ASTROCYTES, A KALEIDOSCOPE OF DIVERSITIES, A PHARMACOLOGICAL HORIZON

EDITED BY: Lorenzo Di Cesare Mannelli, Stefania Ceruti, Juan Andrés Orellana and Laura E. Clarke

PUBLISHED IN: Frontiers in Pharmacology and Frontiers in Neuroscience

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88966-684-3
DOI 10.3389/978-2-88966-684-3

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

ASTROCYTES, A KALEIDOSCOPE OF DIVERSITIES, A PHARMACOLOGICAL HORIZON

Topic Editors:
Lorenzo Di Cesare Mannelli, University of Florence, Italy
Stefania Ceruti, University of Milan, Italy
Juan Andrés Orellana, Pontificia Universidad Católica de chile, Chile
Laura E. Clarke, Stanford University, United States

Citation: Di Cesare Mannelli, L., Ceruti, S., Orellana, J. A., Clarke, L. E., eds. (2021). Astrocytes, a Kaleidoscope of Diversities, a Pharmacological Horizon. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-684-3

Table of Contents

frontiers in Pharmacology

EDITORIAL
published: 08 March 2021
doi: 10.3389/fphar.2021.638239

Editorial: Astrocytes, a Kaleidoscope of Diversities, a Pharmacological Horizon

Lorenzo Di Cesare Mannelli[1]*, *Stefania Ceruti*[2] and *Juan A. Orellana*[3]

[1]Department of Neuroscience, Psychology, Drug Research and Child Health – Neurofarba – Section of Pharmacology and Toxicology, Università degli Studi di Firenze, Florence, Italy, [2]Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy, [3]Departamento de Neurología, Escuela de Medicina and Centro interdisciplinario de Neurociencias, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile

Keywords: glia, neurorestoration, astrocyte population, astrocyte polarization, neuroplasticity, pharmacological targets

Editorial on the Research Topic

Astrocytes, a Kaleidoscope of Diversities, a Pharmacological Horizon

Astrocytes are specialized glia, vital for neural circuit function, and represent a population of complex and functionally diversified cells (Chai et al., 2017). Physiological multiplicity of astrocytes is apparent among different brain circuits and microcircuits, further individual astrocytes display heterogenous signaling properties depending on the subcellular compartments. With respect to injury and disease, astrocytes undergo several phenotypic changes that may be protective or deleterious with regard to pathology in a context-dependent manner (Liddelow and Barres, 2017). Damages to the peripheral and central nervous tissue as well as pathological alterations of complex organs, like the intestine, lead to astrocyte activation, causing neuroanatomical and neurochemical transformations which sustain pathological signals participating in maladaptive plasticity. Nevertheless, also during pathology, astrocytes (as a whole or specific phenotypes or some yet-to-be identified population) maintain their neuroconservative role (Zhou et al., 2020).

Thus research has the challenge to pharmacologically regulate astrocyte functions with special focus on reducing neural aberrant excitation and promoting restorative signals.

The present research topic is intended to be a collection of new physiological and pathological evidence regarding astrocyte features and functions focusing on the concept that astrocytes represent a highly variegated population of cells that mediate neural circuit-specific roles in health and disease.

Spampinato et al. have focused on two important astrocyte functions with pathophysiological relevance: i) regulation of neural stem cell properties within adult neurogenic niches, positive pleiotropic actions of utmost importance under neurodegenerative conditions as an attempt to replace lost cell populations and ii) regulation of the integrity and functions of the blood-brain barrier (BBB) in physiological condition and as a reaction to harmful events contributing to either exacerbate or reduce BBB damage.

Another crucial physiological need satisfied by astrocytes is the cleansing of the cerebral tissue from waste molecules. Aquaporin-4 (AQP-4), a brain water channel, plays a pivotal role in this process. As shown in the review article of Valenza et al., it is mainly expressed on astrocytic endfeet closest to blood vessels participating in several astrocyte signals. The review points out the latest AQP-4 findings related to aging and Alzheimer's disease as well as the available knowledge on pharmacological tools to target AQP-4.

OPEN ACCESS

Edited and reviewed by:
Nicholas M. Barnes, University of Birmingham, United Kingdom

***Correspondence:**
Lorenzo Di Cesare Mannelli
lorenzo.mannelli@unifi.it

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

Received: *05 December 2020*
Accepted: *18 January 2021*
Published: *08 March 2021*

Citation:
Di Cesare Mannelli L, Ceruti S and Orellana JA (2021) Editorial: Astrocytes, a Kaleidoscope of Diversities, a Pharmacological Horizon. Front. Pharmacol. 12:638239. doi: 10.3389/fphar.2021.638239

As regards the cross-talk with the other nervous cells, classic astrocyte-to-neuron communication encompasses the release of messengers via exocytosis, carrier membrane transport and opening of a wide-range of channels (Gundersen et al., 2015). Nevertheless, recent evidence indicates that brain cells may communicate via alternative pathways, including the release of exosomes (Frühbeis et al., 2013). In this context, Venturini et al. have found that astroglial processes could release neuroglobin-containing exosomes as new non-conventional signals.

Among channels implied in the intercellular crosstalk, connexins (Giaume et al., 2021) represents a conserved family of membrane proteins that allow the ionic and molecular exchange between the cytoplasm of adjacent cells (through gap junction channels) or the communication between the extracellular and intracellular space (via hemichannels) (Leybaert et al., 2017). On this subject, Lagos-Cabré et al. have reviewed and discussed evidence suggesting that cell adhesion and cytoskeletal dynamics, both of which are relevant to cell migration, take place by modulation of hemichannels rather than gap junction channels.

Despite the evident astrocyte complexity in terms of phenotype and function, the molecular basis of these differences are unclear. Lozzi and co-workers, by using bioinformatic approaches have demonstrated that cohorts of transcription factors may modulate region-specific molecular signatures in astrocytes. This evidence points out the idea that differential expression of transcription factors governs astrocyte diversity in the brain parenchyma.

Melatonin is produced in the pineal gland and released according to the circadian rhythm (Cipolla-Neto and Amaral, 2018). Recently, this hormone has received attention due to its neuroprotective effect via Nrf2 pathway (Cao et al., 2017). In this issue, Chen and co-workers showed the protective action of melatonin from heme-induced toxicity observed upon intracerebral hemorrhage. They found that this response is mediated by the activation of M2 receptors and the transcription factor Nrf2.

Astrocytes do not express endothelin-1 (ET-1) in healthy conditions, but they prominently express and release this protein in multiple sclerosis demyelinated plaques (D'haeseleer et al., 2013). In this scenario, the work of Hostenbach et al. determined that diversity of pro-inflammatory cytokines causes the production of ET-1, the latter being dramatically prevented by the statin and the natural phenol simvastatin and resveratrol, respectively.

The relevance of astrocytes in pathological conditions was deepened by Siracusa et al. The loss of astrocyte functionality as a result of cellular senescence has been related to neurodegenerative disorders as well as to aging. Astrocytes can drive the inflammatory response and contribute to the altered neuronal activity in several frontal cortex pathologies such as ischemic stroke and epilepsy. For these reasons, the authors discuss the possibilities to target astrocytes as an approach toward pharmacological therapies.

In this view, astroglia is implicated in the pharmacodynamic of already known products. Recent developments have demonstrated that astrocytes can indeed be the cellular targets of neuroprotective agents. As demonstrated in the paper by Zhao et al., vinpocetin, a semi-synthetic alkaloid from the leaves of *Phyllostachys pubescens*, has anti-inflammatory, anti-oxidant and anti-apoptotic actions both *in vitro* following oxygen-glucose deprivation and *in vivo* against ischemia/reperfusion injury by targeting specific astrocytic pathways. Specifically, it promotes Connexin43 phosphorylation through the PI3K/Akt pathway, which in turn promotes BBB integrity, cell-to-cell communication with an overall reduction in brain edema and tissue damage.

The natural compound 2,7,2′-trihydroxy-4,4′7′-trimethoxy-1,1′-biphenanthrene (TTB) isolated from the orchid *Liparis nervosa* (Thunb.) Lindl. has been studied by Liu et al. in an *in vitro* model of oxygen-glucose deprivation/reoxygenation injury (OGD/RI) on astrocytic cultures to mimic the pathological condition named neonatal hypoxic/ischemic. Data demonstrate that TTB is effective against cell death preserving the intracellular antioxidant activity by activating the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and related pathways. Additionally, TTB reverts neurite loss induced by OGD/RI in neuron-astrocyte cocultures.

In conclusion, this Research Topic offers novel information about the role of astrocytes in neurophysiology and in neuropathology as well as possible therapeutic approaches. The pharmacological modulation of astrocytic targets is encouraged as a breakthrough strategy for the relief from several debilitating pathologies.

AUTHOR CONTRIBUTIONS

LDCM, SC, and JAO drafted and revised the manuscript.

REFERENCES

Cao, S., Shrestha, S., Li, J., Yu, X., Chen, J., Yan, F., et al. (2017). Melatonin-mediated mitophagy protects against early brain injury after subarachnoid hemorrhage through inhibition of NLRP3 inflammasome activation. *Sci. Rep.* 7 (1), 2417. doi:10.1038/s41598-017-02679-z

Chai, H., Diaz-Castro, B., Shigetomi, E., Monte, E., Octeau, J. C., Yu, X., et al. (2017). Neural circuit-specialized astrocytes: transcriptomic, proteomic, morphological, and functional evidence. *Neuron* 95 (3), 531–549.e9. doi:10.1016/j.neuron.2017.06.029

Cipolla-Neto, J., and Amaral, F. G. D. (2018). Melatonin as a hormone: new physiological and clinical insights. *Endocr. Rev.* 39 (6), 990–1028. doi:10.1210/er.2018-00084

D'haeseleer, M., Beelen, R., Fierens, Y., Cambron, M., Vanbinst, A. M., Verborgh, C., et al. (2013). Cerebral hypoperfusion in multiple sclerosis is reversible and mediated by endothelin-1. *Proc. Natl. Acad. Sci. U.S.A.* 110 (14), 5654–5658. doi:10.1073/pnas.1222560110

Frühbeis, C., Fröhlich, D., Wen, P. K, and Krämer-Albers, E.-M. (2013). Extracellular vesicles mediators of neuron-glia communication. *Front Cell Neurosci.* 7, 182. doi:10.3389/fncel.2013.00182

Giaume, C., Naus, C. C., Sáez, J. C., and Leybaert, L. (2021). Glial connexins and pannexins in the healthy and diseased brain. *Physiol. Rev.* 101 (1), 93–145. doi:10.1152/physrev.00043.2018

Gundersen, V., Storm-Mathisen, J., and Bergersen, L. H. (2015). Neuroglial transmission. *Physiol. Rev.* 95 (3), 695–726. doi:10.1152/physrev.00024.2014

Leybaert, L., Lampe, P. D., Dhein, S. B., Ferdinandy, P., Beyer, E. C., et al. (2017). Connexins in cardiovascular and neurovascular health and disease: pharmacological implications. *Pharmacol. Rev.* 69 (4), 396–478. doi:10.1124/pr.115.012062

Liddelow, S. A., and Barres, B. A. (2017). Reactive astrocytes: production, function, and therapeutic potential. *Immunity* 46 (6), 957–967. doi:10.1016/j.immuni.2017.06.006

Zhou, Y., Shao, A., Yao, Y., Tu, S., Deng, Y., and Zhang, J. (2020). Dual roles of astrocytes in plasticity and reconstruction after traumatic brain injury. *Cell Commun. Signal.* 18 (1), 62. doi:10.1186/s12964-020-00549-2

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

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 16 July 2019
doi: 10.3389/fphar.2019.00792

TTB Protects Astrocytes Against Oxygen-Glucose Deprivation/Reoxygenation-Induced Injury *via* Activation of Nrf2/HO-1 Signaling Pathway

Liang Liu[1,2,3]*, *Zhichen Zhao*[1], *Qimeng Yin*[1] and *Xiaolu Zhang*[4]*

[1] Institute of Translational Medicine, Medical College, Yangzhou University, Yangzhou, China, [2] Jiangsu Key Laboratory of Experimental & Translational Non-coding RNA Research, Yangzhou University, Yangzhou, China, [3] Jiangsu Key Laboratory of Zoonosis, Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, College of Veterinary Medicine, Yangzhou University, Yangzhou, China, [4] Department of Pharmacy, Clinical Medical College, Yangzhou University, Yangzhou, China

OPEN ACCESS

Edited by:
Stefania Ceruti,
University of Milan, Italy

Reviewed by:
Zhouguang Wang,
Albert Einstein College of Medicine, United States
Marta Boccazzi,
University of Milan, Italy

***Correspondence:**
Xiaolu Zhang
xiaoluz2006@163.com
Liang Liu
enjoyyz@163.com

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

Received: 28 March 2019
Accepted: 18 June 2019
Published: 16 July 2019

Citation:
Liu L, Zhao Z, Yin Q and Zhang X (2019) TTB Protects Astrocytes Against Oxygen-Glucose Deprivation/Reoxygenation-Induced Injury via Activation of Nrf2/HO-1 Signaling Pathway. Front. Pharmacol. 10:792. doi: 10.3389/fphar.2019.00792

Neonatal hypoxic/ischemic encephalopathy (NHIE) is a severe condition that leads to death or neurological disability in newborns. The underlying pathological mechanisms are unclear, and developing the target neuroprotective strategies are urgent. 2,7,2′-trihydroxy-4,4′7′-trimethoxy-1,1′-biphenanthrene (TTB) is a natural product isolated from *Cremastra appendiculata* (D. Don) Makino and *Liparis nervosa* (Thunb.) Lindl. TTB has demonstrated potent cytotoxic activity against stomach (HGC-27) and colon (HT-29) cancer cell lines. However, none of the studies have addressed the effects of TTB in NHIE. In the present study, an oxygen-glucose deprivation/reoxygenation (OGD/R)-induced astrocyte injury model was established to investigate the effect of TTB and its potential mechanisms. Our results showed that TTB alleviated the OGD/R-induced reactive oxygen species increase and the intracellular antioxidant capacity of superoxide dismutase activity decrease. Moreover, TTB potentially prolonged the activation state of the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) pathway and maintained the protection against oxidative stress in OGD/R-induced astrocytes by inducing the nuclear translocation and up-regulation of Nrf2 along with the enhanced expression of the downstream target gene HO-1. Furthermore, TTB treatment diminished the accumulation of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression induced by OGD/R. We also found TTB-treated astrocytes reversed the inhibition of OGD/R on neurite growth of neurons by the astrocyte-neuron coculture system. In conclusion, TTB inhibited the OGD/R-induced astrocyte oxidative stress at least partially through the inhibition of HIF-1α and VEGF *via* the Nrf2/HO-1 signaling pathway.

Keywords: neonatal hypoxia/ischemic encephalopathy, TTB, oxygen-glucose deprivation/reoxygenation, astrocytes, Nrf2/HO-1 signaling pathway, HIF-1α, VEGF

INTRODUCTION

Neonatal hypoxia/ischemic encephalopathy (NHIE), also described as stroke in the neonatal period, is one of the most prevalent causes of a potentially devastating neonatal brain injury with long-term neurological deficits such as mental retardation, cerebral palsy, motor deficits, epilepsy, and learning and behavioral disabilities, which affects 1 to 8 of every 1000 live term births, with the highest rates in developing countries (Dilenge et al., 2001; Kurinczuk et al., 2010). The cases of infantile cerebral palsy are caused by the same factors that cause adult cerebral palsy (Nelson, 2007). At present, therapeutic hypothermia protocols are formally endorsed treatments, which significantly improve outcomes by leading to delayed cell death. However, its effectiveness is limited in severe cases, as 40% to 50% of children with NHIE still die or suffer from long-term neurological disorders (Edwards et al., 2010). There are no effective pharmacological interventions available. To reduce the neurological consequences of NHIE, new and effective neuroprotective strategies are urgently needed.

Astrocytes are the largest population of glial cells in the brain and have been implicated in many functions as key mediators in the central nervous system (CNS). Astrocytes are highly involved in neuronal migration, adaptive plasticity, and synaptogenesis in the developing brain (Ullian et al., 2001; Guizzetti et al., 2014). The developing neonatal brain is particularly vulnerable to oxidative stress based on the immature free radical scavenging systems (Zorec et al., 2018). Several evidences have identified that NHIE causes long-lasting oxidative stress, a process aggravated by mitochondrial dysfunction. Reactive oxygen species (ROS) have been involved in the pathogenesis of NHIE and induce cell death *via* the oxidation of membrane lipids and proteins (Fatemi et al., 2009). Recently, a study demonstrated that astrocytes are a major source of increased brain ROS production during neonatal asphyxia (Parfenova et al., 2018).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a member of the basic region/leucine zipper transcription factor family that regulates several antioxidant pathways (Sandberg et al., 2014). Under unstressed conditions, Nrf2 is binding to the homodimeric protein Kelch-like ECH-associated protein 1 (Keap1), which becomes the Nrf2 Keap1 complex in the cytoplasm. In pathological processes, such as oxidative stress and other insult attacks, Nrf2 is activated by release from the antioxidant response element of Keap1 and translocated to the nucleus from the cytoplasm, which leads to accumulate in the nucleus, regulates genetic activities, and induces cytoprotective action (Cao et al., 2015). Nrf2 activation drives several functions, including antioxidative stress, antiapoptosis, and anti-inflammation, *via* several molecules and pathways (Shu et al., 2016).

2,7,2′-trihydroxy-4,4′7′-trimethoxy-1,1′-biphenanthrene (TTB) is a biphenanthrene isolated from *Cremastra appendiculata* (D. Don) Makino (Xue et al., 2006; Liu et al., 2016a) and *Liparis nervosa* (Thunb.) Lindl. (Liu et al., 2016b), which both belong to the family Orchidaceae. Research concerning bioactivities of TTB was very limited, and it was only reported to have cytotoxic activity against stomach (HGC-27) and colon (HT-29) cancer cell lines (Liu et al., 2016b). Therefore, it is necessary to explore other bioactivities of TTB.

Oxygen-glucose deprivation/reoxygenation (OGD/R) is a widely used cell model to mimic the aspects of cell death observed in a hypoxia brain injury model, including neonate HI and adult ischemic stroke (Cengiz et al., 2014; Tasca et al., 2015). Several recent studies demonstrated that Nrf2 was regulated by special compounds in the rat neonatal HI brain injury model (Cui et al., 2017; Gao et al., 2018). We speculated that TTB may offer neuroprotection in part by regulating Nrf2 in reactive astrocytes. In the current study, we investigated the effect of TTB on the OGD/R-induced astrocyte injury model, which is to mimic NHIE *in vitro*. We observed that Nrf2 activation *via* TTB treatment improved astrocyte function by targeting oxidative stress. Our findings suggested that astrocytic Nrf2 could be a potential therapeutic target for the treatment of NHIE.

MATERIALS AND METHODS

Compound

The ethyl acetate extract of *L. nervosa* (Thunb.) Lindl. was isolated and purified using repeated column chromatography over Sephadex LH-20, RP-C18, silica gel, and semi-preparative high performance liquid chromatography (HPLC) to obtain TTB. The purity of TTB was at least 99% as judged by HPLC analysis. All the extraction, separation, and purification were performed by our group (Liu et al., 2016b).

Cell Culture

Postnatal day 1 Sprague–Dawley rats were purchased from the Comparative Medicine Center of Yangzhou University (Yangzhou, China) and used for culturing astrocytes as described previously (Hertz et al., 1998; Zhang et al., 2014). Briefly, the cerebral cortex was taken in a sterile environment and then dispersed with 0.25% trypsin (Gibco Co., Grand Island, NY, USA) for 10 min at 37°C. The cells were plated in 75 cm^2 flasks precoated with 40 μg/ml poly-D-lysine, grown in high-glucose Dulbecco's modified Eagle medium (DMEM; Gibco Co.) containing 10% fetal bovine serum (FBS; Gibco Co)., 100 units/ml penicillin, and 100 μg/ml streptomycin (Solarbio, Beijing, China), and placed in an incubator at 5% CO_2, 95% air at 37°C. The flasks were gentle shaken about 150 times by hand to remove the layer of nonadherent cells growing on the top of the flat monolayer when changing the medium every 2 to 3 days. More than 95% astrocytes were achieved by

Abbreviations: NHIE, neonatal hypoxic/ischemic encephalopathy; TTB, 2,7,2′-trihydroxy-4,4′7′-trimethoxy-1,1′-biphenanthrene; OGD/R, oxygen-glucose deprivation/reoxygenation; CNS, central nervous system; ROS, reactive oxygen species; SOD, superoxide dismutase; LDH, lactate dehydrogenase; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; GFAP, glial fibrillary acidic protein; Keap1, Kelch-like ECH-associated protein 1; DMEM, high-glucose Dulbecco's modified Eagle medium; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; H/I, hypoxia/ischemia.

the cultures. After 14 days in culture, astrocytes were grown to confluence and then plated in the appropriate vessel. When the cultures reached 70% to 80% confluence, cells were ready for treatment.

Establishment of OGD/R-Induced Injury of Astrocytes

The OGD/R model was established in astrocytes. Briefly, the cells were washed twice with phosphate-buffered saline (PBS) and incubated in glucose- and FBS-free medium and then placed in an anoxic incubator at 94% N_2, 1% O_2, 5% CO_2 at 37°C. After OGD for 6 h, the medium was changed back to high-glucose DMEM containing 10% FBS and returned to the normal oxygen incubator for another 24 h. The blank control group in the experiment was always kept in a normal oxygen incubator and cultured in high-glucose medium containing 10% FBS. The cells were given OGD/R treatment with or without TTB (1.5625, 6.25, and 25 μM). For certain experiments, the astrocytes were preincubated in ML385 (Nrf2-specific inhibitor) for 12 h before OGD/R.

Cell Viability

Primary astrocytes were incubated into 96-well plates at a density of about 1×10^4 cells per well. Cell viability was estimated using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Solarbio) and lactate dehydrogenase (LDH) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The optical absorbance was read on a plate reader at a wavelength of 490 nm for MTT. LDH release from damaged cell membrane was indicated as a percentage of total LDH according to the manufacturer's instruction.

Measurement of Superoxide Dismutase (SOD) Levels

The SOD activity in the astrocytes was measured by a commercially available kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instruction. Briefly, after treatment, cells were washed with cold PBS twice and collected. The homogenates were centrifuged for 10 min at 10,000 rpm at 4°C and supernatants were used for SOD activities. The optical absorbance was read on a plate reader at a wavelength of 450 nm. The protein concentration was determined by BCA assay.

Intracellular ROS Assay

Astrocytes were seeded in 96-well plates at a density of 1×10^4 cells per well. After exposure to OGD/R, the medium with different concentrations of TTB was replaced with 2′,7′-dichlorodihydrofluorescein diacetate (10 μM) in DMEM. The cells were incubated at 37°C for 30 min in the dark and then washed twice with PBS. The fluorescence was tested on a microplate reader using excitation/emission wavelengths (Ex/Em) of 488/525 nm.

Western Blot Analysis

Total proteins were extracted with lysis radioimmunoprecipitation assay buffer (Applygen, Beijing, China) and protease inhibitor cocktail (Applygen). The protein concentrations were determined by BCA assay (Beyotime, Shanghai, China). All steps were carried out on ice. Nuclear and cytosolic proteins were extracted using a commercial kit (KeyGEN BioTECH's, Nanjing, China). The extracts were boiled in a metal bath at 95°C for 5 min. Subsequently, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out to separate the proteins. The proteins were then transferred to a polyvinylidene fluoride (PVDF; Solarbio) membrane for about 1.5 h. After blocking in 5% nonfat milk (Applygen) for 2 h, the PVDF membrane was incubated with the primary antibodies anti-Nrf2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-heme oxygenase-1 (HO-1; 1:500; Wanlei Biotechnology, Shenyang, China), anti-hypoxia-inducible factor-1α (HIF-1α; 1:500; BBI, Shanghai, China), anti-β-actin (1:5,000; abclonal, Wuhan, China), and anti-lamin B (1:500; abclonal) overnight at 4°C then followed by horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The membranes were washed three times for 10 min before obtaining protein bands by enhanced chemiluminescence reagents (Beyotime) and analyzed by ImageJ.

Immunofluorescence Assay

The astrocytes were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 (Solarbio) for 10 min at room temperature. After blocking with 3% bovine serum albumin for 30 min at room temperature, the cells were incubated with the primary antibody anti-Nrf2 (1:200; Santa Cruz Biotechnology) at 4°C overnight followed by Alexa Fluor 488 donkey anti-mouse antibody or Alexa Fluor 594 donkey anti-rabbit antibody (1:500; Invitrogen, Carlsbad, CA, USA). Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml; Beyotime), and images were acquired using a Zeiss fluorescence microscope attached to a digital camera.

Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted using Trizol reagent and dissolved in ultrapure distilled water (Invitrogen). Equal amounts of RNA were reverse transcribed at 25°C for 5 min, 42°C for 60 min, and 70°C for 5 min using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA amplification was carried out in 20 μl PCR buffer using AceQ quantitative PCR (qPCR) SYBR Green Master Mix (Vazyme, Nanjing, China). The primers used for amplification in the experiment were as follows: HIF-1α sense 5′-GTCTCCATTACCTGCCTCTG-3′ and antisense 5′-GATTCTTCGCTTCTGTGTCTTC-3′, vascular endothelial growth factor (VEGF) sense 5′-ACCCCACAAAG AGCTAGATAG-3′ and antisense 5′-CCTCTTCACTAAATGAC AGTCCC-3′, and glial fibrillary acidic protein (GFAP) sense 5′-CCTTGCGCGGCACGAACGAG-3′ and antisense 5′-CCGA GCGAGTGCCTCCTGGT-3′. mRNA levels were normalized

to levels of β-actin measured in the same samples (sense 5′-GCGTCCACCCGCGAGTACAA-3′ and antisense 5′-TCCA TGGCGAACTGGTGGCG-3′).

Astrocyte-Neuron Coculture

The astrocytes were passed in glass coverslips placed into 24-well plate at cell density of 1×10^5 astrocytes per coverslip. When the cultures reached 70% to 80% confluence, the cells were subjected to OGD for 6 h and reoxygenation for 24 h; at the same time, primary neurons were extracted. After OGD/R in astrocytes was completed, primary neurons were seeded at a concentration of 1.2×10^4 cells per well above the astrocytes and cocultured with DMEM for 24 h.

Statistical Analysis

Statistical analyses were performed using Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). Data were expressed as the mean ± standard error (SE) of at least three independent experiments and compared using one-way analysis of variance with Tukey's test. $p < 0.05$ was considered statistically significant difference.

RESULTS

TTB Attenuated OGD/R-Induced Damage in Astrocytes

To examine the cell toxicity of TTB and the protective effect of TTB against cytotoxicity induced by OGD/R, the MTT assay was used to assess the viability of astrocytes. There was no cytotoxicity in the TTB concentration range from 1.5625 to 50 μM (**Figure 1A**). The viability of astrocytes exposed to OGD/R was significantly decreased compared to the blank control group, but this effect was reversed after treatment with TTB at concentrations of 1.5625, 6.25, and 25 μM (**Figure 1B**). These results indicated that TTB treatment was noncytotoxic and TTB attenuated OGD/R-induced astrocyte damage. Exposure to OGD/R significantly increased the release of LDH, whereas treatment with TTB markedly reduced the OGD/R-induced LDH release in astrocytes (**Figure 1C**).

TTB Alleviated OGD/R-Induced Oxidative Stress in Astrocytes

To examine the effect of TTB treatment on OGD/R-induced oxidative stress in astrocytes, we examined the SOD activity and the ROS level. We found that treatment with TTB significantly

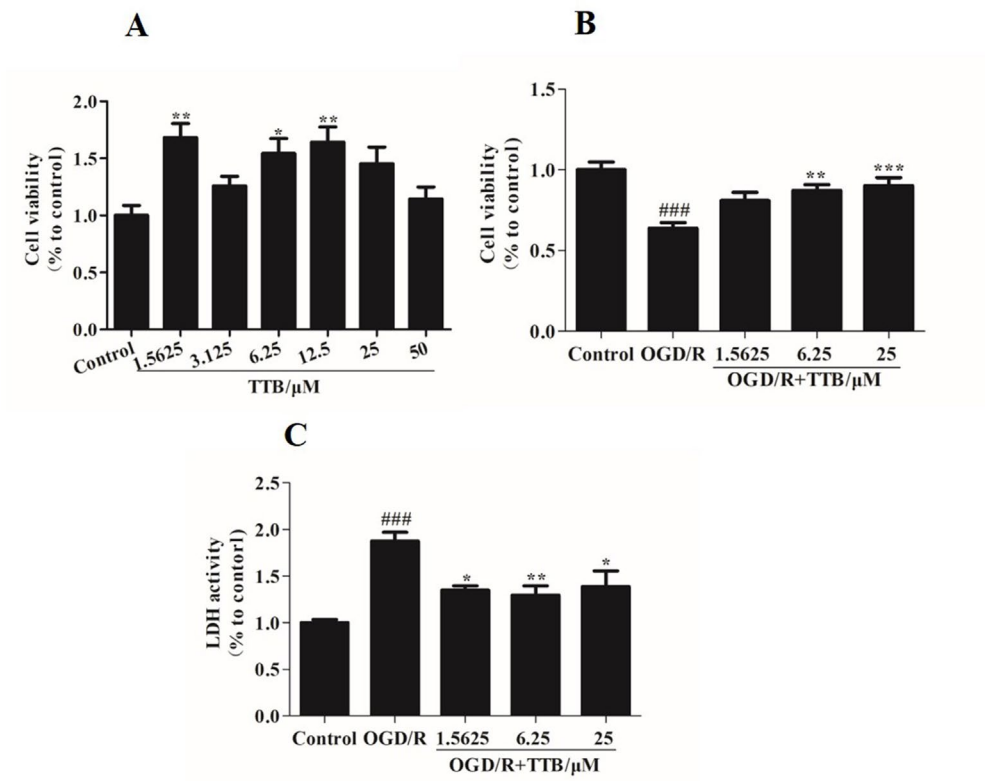

FIGURE 1 | Cell toxicity of TTB in regular astrocytes and effect of TTB on cell viability under oxygen-glucose deprivation/reoxygenation (OGD/R) were evaluated. **(A)** Primary astrocytes were incubated with TTB at the concentration range from 1.5625 to 50 μM for 30 h in the normal incubator. Cell survival was estimated by the MTT assay. **(B** and **C)** Primary astrocytes were incubated with TTB at 1.5625, 6.25, and 25 μM for 6 h OGD and 24 h reoxygenation. Cell survival and cell death were estimated by the MTT and LDH assays, respectively. $^*p < 0.05$, $^{**}p < 0.01$ vs. blank control group; $^{\#\#\#}p < 0.001$ vs. blank control group; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. OGD/R group. Data are mean ± SE of three independent experiments.

reversed the decrease of SOD activity and the increase of intracellular ROS due to OGD/R (**Figures 2A, B**). These results indicated that TTB significantly improved OGD/R-induced oxidative stress in astrocytes.

TTB Induced Nrf2 Up-Regulation and Nuclear Translocation in OGD/R-Injured Astrocytes

When a stress response occurs, intracellular Nrf2 is easily transferred to the nucleus from the cytoplasm, which subsequently initiates the transcriptional activation of various antioxidant enzymes and phase II detoxification enzymes. The effect of TTB on Nrf2 expression and nuclear translocation in OGD/R-induced astrocytes was determined by Western blot and immunofluorescence. Lamin B was used to assess the purity of the nuclear fraction. As shown in **Figure 3A**, compared to the control group, Nrf2 protein expression was dramatically increased by OGD for 6 h and reoxygenation for 24 h but decreased by 12 and 24 h OGD and 24 h reoxygenation. TTB at 6.25 μM up-regulated Nrf2 protein expression in all three time courses under OGD/R. Moreover, OGD for 6 h and reoxygenation for 24 h significantly increased Nrf2 nuclear translocation in astrocytes, and treatment with TTB at 1.5625, 6.25, and 25 μM further facilitated Nrf2 translocation to the nucleus compared to the OGD/R group (**Figures 3B–D**). These results indicated that TTB treatment could up-regulate Nrf2 expression and promote Nrf2 nuclear translocation under OGD/R condition.

TTB Activates the Nrf2/HO-1 Pathway

Nrf2 is activated under stress conditions and translocates to the nucleus to initiate transcriptional activation of HO-1. Therefore, we examined the effect of TTB on the expression of Nrf2 and HO-1 proteins in astrocytes by Western blot. Cells were incubated with TTB at 1.5625, 6.25, and 25 μM under OGD for 6 h and reoxygenation for 24 h. As a result, we found that, compared to the OGD/R group, Nrf2 and HO-1 expression in protein level was significantly up-regulated in the OGD/R+TTB group (**Figures 4A**, **B**), which indicated that TTB might get involved in the Nrf2/HO-1 signal pathway.

TTB Inhibited OGD-Induced HIF-1α Accumulation, VEGF Release, and GFAP Expression in Astrocytes

OGD/R injury could trigger HIF-1α up-regulation. Nrf2 and HIF-1α are two transcription factors that represent oxygen and redox state. We further observed the effect of TTB on HIF-1α expression in both protein and mRNA levels under OGD/R condition. Cells were treated with TTB in different concentrations (1.5625, 6.25, and 25 μM) for 6 h OGD and 24 h reoxygenation in astrocytes. The results showed that TTB treatment inhibited OGD/R-induced up-regulation of HIF-1α (**Figures 5A**, **B**). Moreover, VEGF and GFAP gene expressions were measured by qPCR. TTB inhibited OGD/R-induced increase in GFAP and VEGF gene expressions (**Figures 5C**, **D**).

TTB Prevented OGD/R-Induced Inhibition of Neurite Outgrowth in Neuron-Astrocyte Coculture System

To test the hypothesis that OGD-treated astrocytes inhibit neurite outgrowth and whether TTB could alleviate the inhibition, we plated the neurons on top of the OGD/R-treated astrocytes in the presence of TTB at a concentration of 6.25 μM. We observed that neurons cocultured with OGD/R-induced astrocytes developed shorter major and minor neurites compared to neurons cocultured with the control astrocytes, whereas TTB attenuated the OGD/R-induce inhibition of neurite growth. (**Figure 6A**–**D**). This result indicated that TTB could regulate astrocyte function and promote neuronal growth.

Nrf2 Inhibitor Abolished the Protective Effect of TTB by the Nrf2/HO-1 Pathway in OGD/R-Injured Astrocytes

To investigate whether Nrf2 function contributes to the neuroprotective effects of TTB, astrocytes were incubated with ML385, the Nrf2-specific inhibitor, to inhibit Nrf2 expression. Under OGD/R treatment, the expression of Nrf2 and HO-1 was inhibited by ML385 at a concentration of 5 μM. ML385 also

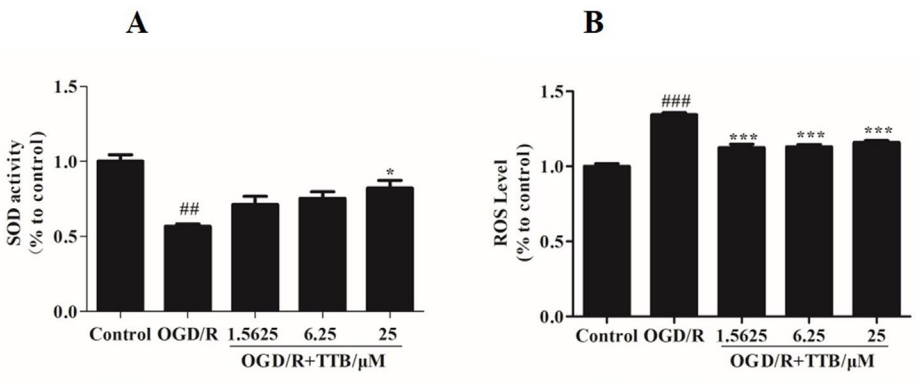

FIGURE 2 | TTB inhibited the oxidative stress in astrocytes under OGD/R exposure. Primary astrocytes were treated with OGD for 6 h and reoxygenation for 24 h in the presence of TTB at 1.5625, 6.25, and 25 μM. **(A)** SOD activity was determined by the SOD assay. **(B)** ROS levels were estimated by the ROS assay. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. blank control group; $^{*}p < 0.05$, $^{***}p < 0.001$ vs. OGD/R group. Data are mean ± SE of three independent experiments.

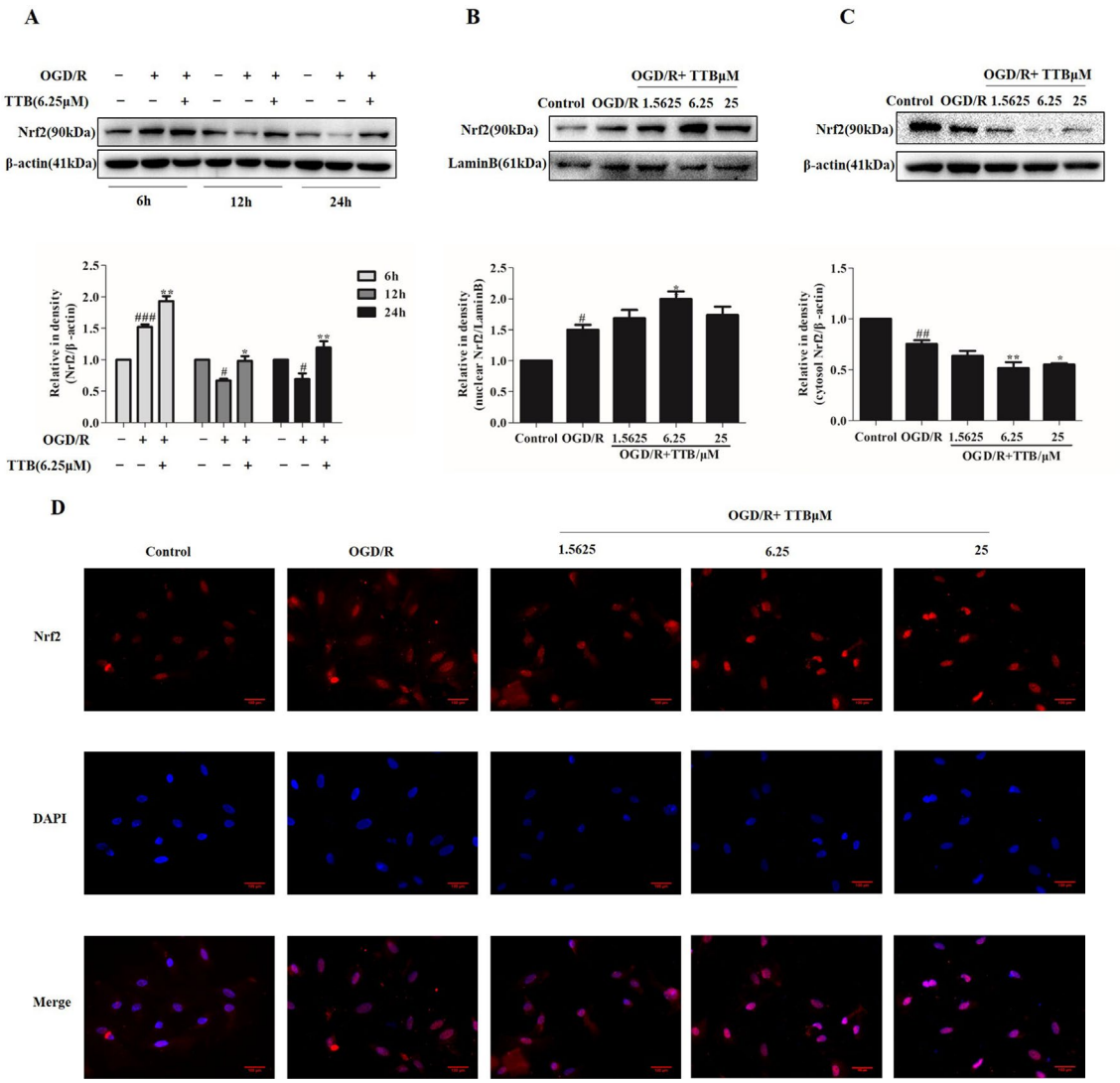

FIGURE 3 | TTB induced Nrf2 activation and nuclear translocation in OGD/R-injured astrocytes. Primary astrocytes were incubated with TTB under OGD/R. Total proteins from treated astrocytes were extracted and used for Western blot. Nuclear and cytosolic proteins were determined by Western blot and immunofluorescence. **(A)** TTB at 6.25 μM increased Nrf2 expression in total protein under OGD for 6, 12, and 24 h after 24 h reoxygenation. **(B** and **C)** TTB at 1.5625, 6.25, and 25 μM decreased Nrf2 expression in the cytosol of astrocytes but increased in the nucleus at 6 h OGD and 24 h rexoygenation. Data are presented as relative density units normalized to β-actin. **(D)** Immunofluorescence staining was performed to detect the effects of TTB on Nrf2 translocation at 6 h OGD followed by 24 h rexoygenation. DAPI was used as a nuclei marker (40× magnification). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. blank control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. OGD/R group. Data are mean ± SE of three independent experiments.

abolished TTB-induced increase in Nrf2 and HO-1 expression (**Figures 7A–C**). Moreover, the effect of TTB on the expression of HIF-1α and VEGF was inhibited by ML385 (**Figures 7A**, **D**, **E**). These data demonstrated that the neuroprotective effect of TTB may be through the activation of the Nrf2/HO-1 pathway.

DISCUSSION

NHIE causes a series of oxidative bursts, cell apoptosis, and cascade of inflammatory responses. The potential therapy strategies have been limited and unsatisfactory (Mulkey et al., 2011; Zalewska et al., 2015). Astrocyte dysfunction is critically involved in oxidative stress, apoptosis, and inflammation in the pathologic process of NHIE. The current studies were undertaken to identify the hypothesis that TTB exposure would initiate the protective response against OGD/R-induced injury in astrocytes, which is an *in vitro* model to mimic NHIE. The transcription factor Nrf2 was identified to play an important role in modulating the neuroprotective effects of TTB.

TTB is a natural biphenanthrene that is a relatively rare secondary metabolite in the plant kingdom. TTB was only reported to have cytotoxicity against HGC-27 and HT-29 cancer cell lines (Liu et al., 2016b). TTB contains phenolic

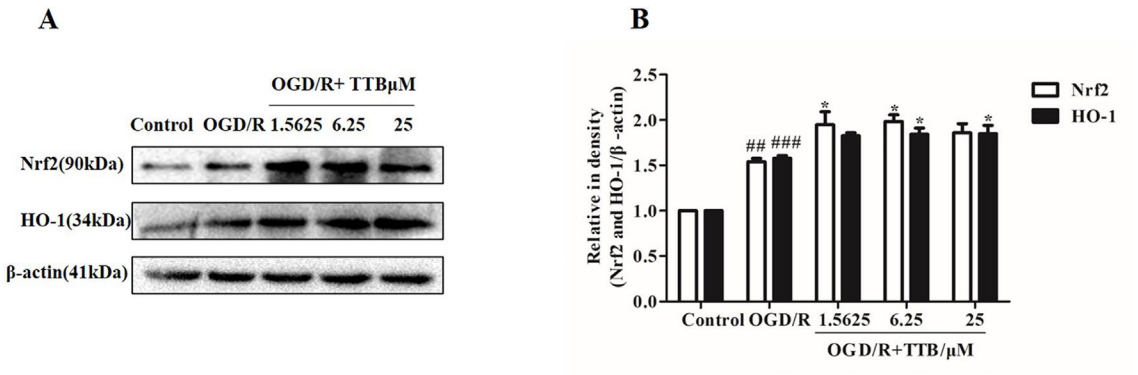

FIGURE 4 | TTB treatment activated Nrf2/HO-1 expression. Primary astrocytes were incubated with TTB at 1.5625, 6.25, and 25 μM for 6 h OGD and 24 h reoxygenation. **(A** and **B)** Western blot was carried out to determine the expression of Nrf2 and HO-1, respectively. Data are presented as relative density units normalized to β-actin $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. blank control group; $^{*}p < 0.05$ vs. OGD/R group. Data are mean ± SE of three independent experiments.

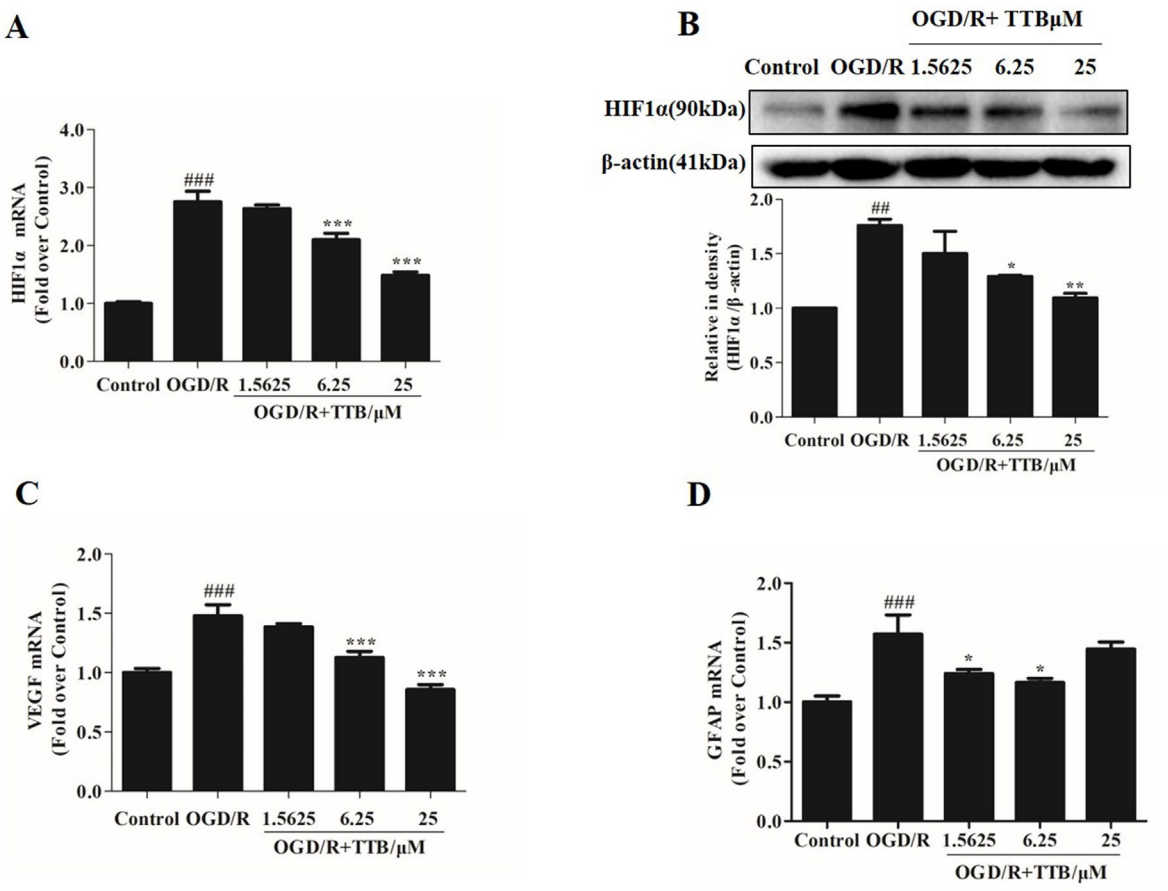

FIGURE 5 | TTB reduced HIF-1α, VEGF, and GFAP expression in OGD/R-injured astrocytes. Primary astrocytes were incubated with TTB at 1.5625, 6.25, and 25 μM for 6 h OGD and 24 h reoxygenation. RNA was extracted and HIF-1α, VEGF, and GFAP mRNA levels were quantified by qPCR. Western blot was carried out for HIF-1α protein determination. Results were normalized to β-actin and expressed as fold over control. **(A** and **B)** HIF-1α mRNA expression was reduced by TTB treatment in OGD/R-injured astrocytes, which was consistent with HIF-1α protein level. **(C** and **D)** TTB inhibited VEGF and GFAP mRNA expression in OGD/R-injured astrocytes. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. blank control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. OGD/R group. Data are mean ± SE of three independent experiments.

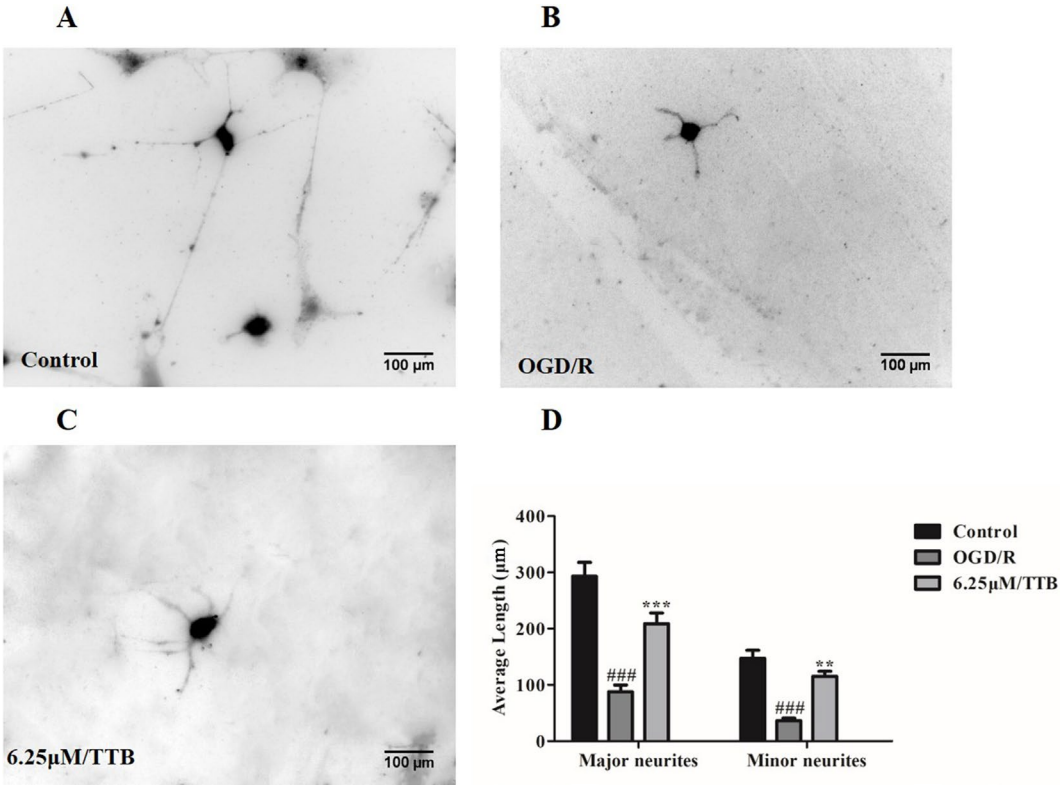

FIGURE 6 | Effects of TTB-treated astrocytes on hippocampal neuron neurite outgrowth. Astrocytes were treated with 6.25 μM TTB for 6 h OGD and 24 h reoxygenation. Hippocampal neurons were plated on top of pretreated astrocytes for an additional 24 h. Then, cultures were fixed and stained with the antibody of neuron-specific β-III-tubulin and a fluorescent secondary antibody. ImageJ was used to measure neurite length. **(A)** Control, **(B)** OGD/R, **(C)** TTB treatment, and **(D)** morphometric quantification of major neurite and minor neurite length. $^{###}p < 0.001$ vs. blank control group; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. OGD/R group. Data are mean ± SE of three independent experiments.

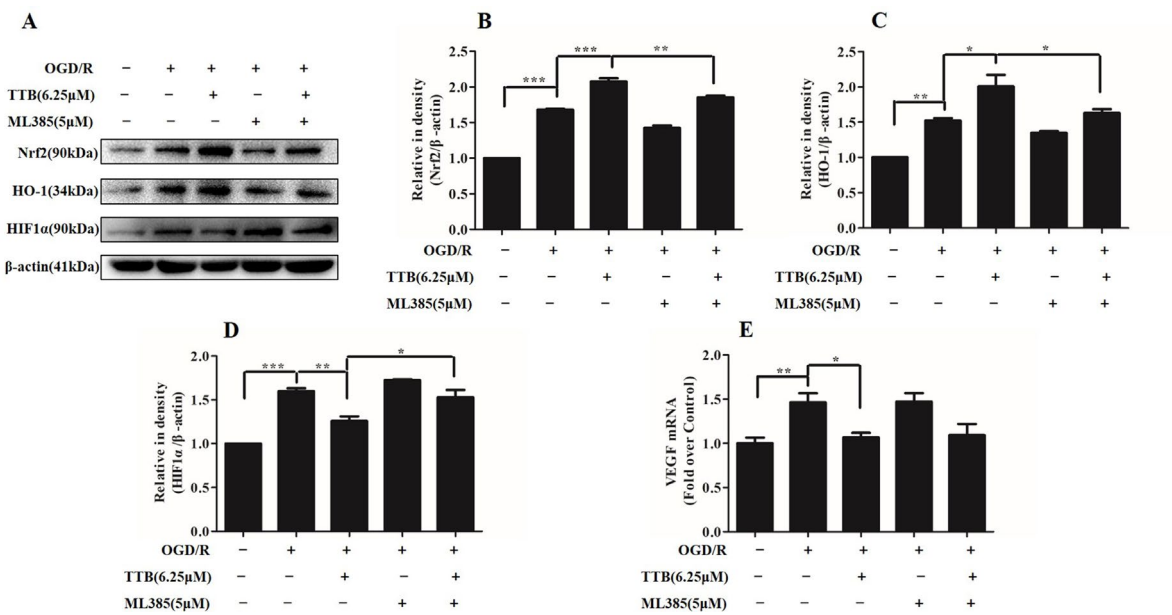

FIGURE 7 | Effects of ML385 (Nrf2-specific inhibitor) on Nrf2, HO-1, HIF-1α, and VEGF levels in OGD/R-treated astrocytes. Western blot and qPCR were carried out. Astrocytes were preincubated with ML385 at a concentration of 5 μM for 12 h and then cells were treated with 6.25 μM TTB for 6 h OGD and 24 h reoxygenation. **(A–D)** Expression of Nrf2, HO-1, and HIF-1α was determined by Western blot, respectively. **(E)** Expression of VEGF in the mRNA level was determined by qPCR. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data are mean ± SE of three independent experiments.

hydroxyl groups, which determines its significant antioxidant activity (Quiniou et al., 2008). Recent studies have identified that antioxidants can protect astrocytes from hypoxia/ischemia (H/I)-induced dysfunction (Bao et al., 2016). In the present study, we exposed primary cultures of astrocytes to OGD for 6 h and reoxygenation for 24 h. Our results demonstrated for the first time that TTB at 6.25 and 25 μM increased cell survival significantly in OGD/R-induced injury. TTB also decreased the LDH release to the culture medium.

H/I exposure causes an oxidative stress and induces a significant damage in brain tissue, which can be described as an increase in the rate of ROS generation and imbalance of antioxidant defense system in the molecular level (Brekke et al., 2017; Parfenova et al., 2018). Excessive ROS initiates pro-inflammatory or growth stimulatory signals that are associated with cell death. Therefore, new pharmacological strategies aimed at the antioxidant system may potentially improve clinical management. The present study demonstrated that OGD/R exposure markedly increased ROS production in astrocytes compared to the control group and this increase was attenuated by TTB treatment. SODs, the antioxidant enzymes, are generally considered as O^{2-} scavengers against tissue and cellular damage caused by ROS (Fridovich, 1995; Li et al., 2008). In our study, OGD/R induced the decrease of SOD activity and TTB prevented the decrease of SOD activity in response to OGD/R. These data illustrated that TTB treatment had protective roles against OGD/R-induced oxidative stress.

Nrf2 is a well-known key regulator of cellular resistance to oxidants and is activated through translocation from the cytoplasm to the nucleus, where it induces HO-1 gene expression as a target antioxidative gene (Kensler et al., 2007; Wu et al., 2015). HO-1, a rate-limiting enzyme in the transition of heme into biliverdin, also has a pivotal function in response to oxidative stress (Cao et al., 2017). When Nrf2 is up-regulated and translocated into the nucleus from the cytoplasm under stress conditions, the process is also essential for the activation of HO-1 expression (Kang et al., 2015). Growing evidence demonstrated that the Nrf2/HO-1 signaling pathway participated in the process of oxidative stress in several brain dysfunctional diseases (Meng et al., 2016; Bellaver et al., 2017; Zhao et al., 2018). Several antioxidant ingredients indicated that they protected cell damage by up-regulating Nrf2 and HO-1 expression in various diseases (Hsu et al., 2012; Yao et al., 2015; Jung et al., 2017). To explore whether TTB-induced cytoprotection was dependent on the presence of Nrf2 by inhibiting oxidative stress, the astrocytes were treated with OGD in different time courses of 6, 12, and 24 h followed by reoxygenation for 24 h. Our results showed that the protein levels of Nrf2 were increased by OGD for 6 h and reoxygenation for 24 h but decreased by OGD for 12 or 24 h and reoxygenation for 24 h compared to the control group. Meanwhile, TTB up-regulated Nrf2 expression in total protein at all time points compared to the OGD/R group. The results demonstrated TTB potentially prolonged the activation state of the Nrf2 pathway and maintained the protection against oxidative stress in OGD/R-induced astrocytes. Furthermore, with OGD for 6 h and reoxygenation for 24 h treatment, TTB facilitated Nrf2 translocation to the nucleus and increased Nrf2 expression in the nucleus, suggesting that TTB promoted the activation of the Nrf2/HO-1 pathway in H/I injury in the early phase.

HIF-1α is an important transcription factor in a wide variety of responses to hypoxia (Chavez et al., 2000). Using the astrocyte-neuron coculture model, the selective loss of HIF-1α function in neuron induced neuronal susceptibility to H/I injury, whereas the loss of HIF-1α function in astrocytes inhibits neuronal death by hypoxia (Vangeison et al., 2008). During hypoxia-induced CNS injury, HIF-1α expression targets multiple genes, including VEGF. The activation of VEGF expression under hypoxic conditions has been investigated in several studies. Notably, astrocytes secrete basal levels of VEGF under physiological conditions and the expression is further up-regulated by hypoxia. VEGF gene expression is transcriptionally regulated by HIF-1α (Marti et al., 2000; Schmid-Brunclik et al., 2008; Wiesner et al., 2013). Some previous studies identified that VEGF protects neurons from ischemic insults and promoted neurogenesis after cerebral ischemic injury (Ma et al., 2012; Liu et al., 2018). However, other studies reported that anti-VEGF treatment blocks vascular leakage in hypoxia (Nordal et al., 2004; Kaur et al., 2006). In the present study, it showed that OGD/R induced HIF-1α and VEGF up-regulation. TTB inverted the effect of OGD/R on HIF-1α/VEGF expression in astrocytes. The results disclosed that the HIF-1α/VEGF pathway might be involved in the astrocyte oxidative stress, providing new insights into TTB protection.

Nrf2 and HIF-1α represent the oxygen and redox state-dependent transcription factors. Their stabilization by redox status decides the cell fate, which means the existence of interplay between Nrf2 and the HIF-1α/VEGF signaling pathway under H/I injury. One study demonstrated that hypoxia induced Nrf2 activation, resulting in the induction of Nrf2-dependent target thioredoxin-1 enhancement of HIF-1α response in A549 cells (Malec et al., 2010). Li et al. indicated that Nrf2 knockdown inhibits venous hypertension-induced activation of the HIF-1α/VEGF pathway (Li et al., 2016). In our study, TTB may act as an Nrf2 activator that up-regulated and maintained Nrf2 expression after OGD/R. To further explore whether the protection of TTB on OGD/R-induced injury in astrocytes was dependent on the activation of the Nrf2 pathway, ML385, a small-molecule Nrf2 inhibitor, was implemented to observe the protective mechanism of TTB. ML385 increases the ubiquitination and inhibits the proteasome degradation of Nrf2 binding to Keap1 and subsequently suppresses Nrf2 expression (Jung et al., 2018). Our results displayed that, with the combination of TTB and MLB385 treatment, MLB385 reversed the TTB-induced up-regulation of Nrf2 and HO-1 expression in OGD/R-induced astrocytes. These results indicated that the activation of the Nrf2/HO-1 signaling pathway after TTB treatment was responsible for the protection of antioxidative stress. Furthermore, we found that, under OGD/R treatment in astrocytes, MLB385 induced the maintenance of the high level of HIF-1α expression. The combination of TTB and MLB385 decreased the HIF-1α protein level compared to MLB385 alone. These results suggested that TTB inhibited OGD/R-induced astrocyte oxidative stress at least partially through the down-regulation of HIF-1α and VEGF *via* the Nrf2/HO-1 signaling pathway.

CNS diseases, such as trauma, H/I injury, neuroinflammation, or neurodegeneration, cause astrocytes to become reactive. Reactive astrocytes were verified to control formation, maintenance, function, and the removal of neuronal synapses (Eroglu and Barres, 2010; Koizumi et al., 2018). Our study verified that OGD/R induced astrocyte reactivation by up-regulating GFAP expression. Meanwhile, TTB inhibited GFAP expression, which revealed that TTB inhibited OGD/R-induced astrocyte reactivation. The mechanism might go through the alteration of factor secretion and gene expression. A previous study showed that proteins released by astrocytes selectively increased neuron axon length, branching, function, and synapse formation (Hughes et al., 2010). Other study demonstrated that astrocytes produced mRNAs that encoded synaptic adhesion proteins, which affected neuronal synapse formation (Cahoy et al., 2008). Astrocyte-neuron interaction might participate in neuronal plasticity. In our study, with neuron-astrocyte coculture, OGD/R-induced astrocytes inhibited neurite growth in neurons compared to the control group. TTB-treated astrocytes reversed the inhibition of OGD/R on neurite growth of neurons in the coculture system. It suggested that TTB regulated astrocyte function and subsequently promoted neuronal plasticity under H/I injury. However, the deep mechanism of which factors were secreted by astrocytes and which genes were regulated remains unknown.

Taken together, TTB displays antioxidant activities in OGD/R-induced astrocytes. Our study provides evidence that TTB effectively suppresses excessive ROS production and increases SOD activity in terms of attenuation of HIF-1α and VEGF expression by activating the Nrf2/HO-1 pathway, which depends on Nrf2 nuclear translocation and up-regulation of HO-1, to protect OGD/R-induced cell oxidative stress. Also, TTB administration in reactive astrocytes by OGD/R might contribute to reverse the inhibition of OGD/R on neurite growth in neurons. These data suggest that TTB could be a novel medication that imparts effective neuroprotection against NHIE to prevent cerebral oxidative stress-induced injury.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because all the data can be found in the manuscript. Requests to access the datasets should be directed to enjoyyz@163.com.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Medical College of Yangzhou University Guide for Care and Use of Laboratory Animals. The protocol was approved by the Committee of Care and Use of Laboratory of Medical College of Yangzhou University.

AUTHOR CONTRIBUTIONS

XZ and LL contributed to the design of the study. LL, ZZ, QY, and XZ performed the experiments. ZZ, LL, and XZ analyzed and interpreted the data. XZ, LL, and ZZ drafted and revised the manuscript. All the authors approved the final version of manuscript.

FUNDING

This study was supported by the National Natural Science Foundation of China (81701211 and 81703812), Traditional Chinese Medicine Bureau of Jiangsu Province Project (YB2015182), Jiangsu Pharmaceutical Association-Aosaikang Clinical Pharmacy Foundation (A201737), Qinglan Project of Yangzhou University (20180210), and "Summit of the Six Top Talents" Program of Jiangsu Province (WSN-277).

REFERENCES

Bao, G., Li, C., Qi, L., Wang, N., and He, B. (2016). Tetrandrine protects against oxygen-glucose-serum deprivation/reoxygenation-induced injury *via* PI3K/AKT/NF-kappaB signaling pathway in rat spinal cord astrocytes. *Biomed. Pharmacother.* 84, 925–930. doi: 10.1016/j.biopha.2016.10.007

Bellaver, B., Souza, D. G., Souza, D. O., and Quincozes-Santos, A. (2017). Hippocampal astrocyte cultures from adult and aged rats reproduce changes in glial functionality observed in the aging brain. *Mol. Neurobiol.* 54, 2969–2985. doi: 10.1007/s12035-016-9880-8

Brekke, E., Berger, H. R., Widerøe, M., Sonnewald, U., and Morken, T. S. (2017). Glucose and intermediary metabolism and astrocyte-neuron interactions following neonatal hypoxia-ischemia in rat. *Neurochem. Res.* 42, 115–132. doi: 10.1007/s11064-016-2149-9

Cahoy, J. D., Emery, B., Kaushal, A., Foo, L. C., Zamanian, J. L., Christopherson, K. S., et al. (2008). A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J. Neurosci.* 28, 264–278. doi: 10.1523/JNEUROSCI.4178-07.2008

Cao, S., Du, J., and Hei, Q. (2017). Lycium barbarum polysaccharide protects against neurotoxicity *via* the Nrf2-HO-1 pathway. *Exp. Ther. Med.* 14, 4919–4927. doi: 10.3892/etm.2017.5127

Cao, S., Chao, D., Zhou, H., Balboni, G., and Xia, Y. (2015). A novel mechanism for cytoprotection against hypoxic injury: delta-opioid receptor-mediated increase in Nrf2 translocation. *Br. J. Pharmacol.* 172, 1869–1881. doi: 10.1111/bph.13031

Cengiz, P., Kintner, D. B., Chanana, V., Yuan, H., Akture, E., Kendigelen, P., et al. (2014). Sustained Na^+/H^+ exchanger activation promotes gliotransmitter release from reactive hippocampal astrocytes following oxygen-glucose deprivation. *PLoS One* 9, e84294. doi: 10.1371/journal.pone.0084294

Chavez, J. C., Agani, F., Pichiule, P., and Lamanna, J. C. (2000). Expression of hypoxia-inducible factor-1alpha in the brain of rats during chronic hypoxia. *J. Appl. Physiol.* 89, 1937–1942. doi: 10.1152/jappl.2000.89.5.1937

Cui, X., Song, H., and Su, J. (2017). Curcumin attenuates hypoxic-ischemic brain injury in neonatal rats through induction of nuclear factor erythroid-2-related factor 2 and heme oxygenase-1. *Exp. Ther. Med.* 14, 1512–1518. doi: 10.3892/etm.2017.4683

Dilenge, M. E., Majnemer, A., and Shevell, M. I. (2001). Long-term developmental outcome of asphyxiated term neonates. *J. Child Neurol.* 16, 781–792. doi: 10.1177/088307380101601102O1

Edwards, A. D., Brocklehurst, P., Gunn, A. J., Halliday, H., Juszczak, E., Levene, M., et al. (2010). Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. *BMJ* 340, c363. doi: 10.1136/bmj.c363

Eroglu, C., and Barres, B. A. (2010). Regulation of synaptic connectivity by glia. *Nature* 468, 223–231. doi: 10.1038/nature09612

Fatemi, A., Wilson, M. A., and Johnston, M. V. (2009). Hypoxic-ischemic encephalopathy in the term infant. *Clin. Perinatol.* 36, 835–858. doi: 10.1016/j.clp.2009.07.011

Fridovich, I. (1995). Superoxide radical and superoxide dismutases. *Annu. Rev. Biochem.* 64, 97–112. doi: 10.1146/annurev.bi.64.070195.000525

Gao, Y., Fu, R., Wang, J., Yang, X., Wen, L., and Feng, J. (2018). Resveratrol mitigates the oxidative stress mediated by hypoxic-ischemic brain injury in neonatal rats *via* Nrf2/HO-1 pathway. *Pharm. Biol.* 56, 440–449. doi: 10.1080/13880209.2018.1502326

Guizzetti, M., Zhang, X., Goeke, C., and Gavin, D. P. (2014). Glia and neurodevelopment: focus on fetal alcohol spectrum disorders. *Front. Pediatr.* 2, 123. doi: 10.3389/fped.2014.00123

Hertz, L., Peng, L., and Lai, J. C. (1998). Functional studies in cultured astrocytes. *Methods* 16, 293–310. doi: 10.1006/meth.1998.0686

Hsu, Y. Y., Chen, C. S., Wu, S. N., Jong, Y. J., and Lo, Y. C. (2012). Berberine activates Nrf2 nuclear translocation and protects against oxidative damage via a phosphatidylinositol 3-kinase/Akt-dependent mechanism in NSC34 motor neuron-like cells. *Eur. J. Pharm. Sci.* 46, 415–425. doi: 10.1016/j.ejps.2012.03.004

Hughes, E. G., Elmariah, S. B., and Balice-Gordon, R. J. (2010). Astrocyte secreted proteins selectively increase hippocampal GABAergic axon length, branching, and synaptogenesis. *Mol. Cell. Neurosci.* 43, 136–145. doi: 10.1016/j.mcn.2009.10.004

Jung, B. J., Yoo, H. S., Shin, S., Park, Y. J., and Jeon, S. M. (2018). Dysregulation of NRF2 in cancer: from molecular mechanisms to therapeutic opportunities. *Biomol. Ther. (Seoul)*. 26, 57–68. doi: 10.4062/biomolther.2017.195

Jung, E., Kim, J. H., Kim, M. O., Lee, S. Y., and Lee, J. (2017). Melanocyte-protective effect of afzelin is mediated by the Nrf2-ARE signalling pathway via GSK-3beta inactivation. *Exp. Dermatol.* 26, 764–770. doi: 10.1111/exd.13277

Kang, J. S., Choi, I. W., Han, M. H., Kim, G. Y., Hong, S. H., Park, C., et al. (2015). The cytoprotective effects of 7,8-dihydroxyflavone against oxidative stress are mediated by the upregulation of Nrf2-dependent HO-1 expression through the activation of the PI3K/Akt and ERK pathways in C2C12 myoblasts. *Int. J. Mol. Med.* 36, 501–510. doi: 10.3892/ijmm.2015.2256

Kaur, C., Sivakumar, V., Zhang, Y., and Ling, E. A. (2006). Hypoxia-induced astrocytic reaction and increased vascular permeability in the rat cerebellum. *Glia* 54, 826–839. doi: 10.1002/glia.20420

Kensler, T. W., Wakabayashi, N., and Biswal, S. (2007). Cell survival responses to environmental stresses *via* the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* 47, 89–116. doi: 10.1146/annurev.pharmtox.46.120604.141046

Koizumi, S., Hirayama, Y., and Morizawa, Y. M. (2018). New roles of reactive astrocytes in the brain; an organizer of cerebral ischemia. *Neurochem. Int.* 119, 107–114. doi: 10.1016/j.neuint.2018.01.007

Kurinczuk, J. J., White-Koning, M., and Badawi, N. (2010). Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. *Early Hum. Dev.* 86, 329–338. doi: 10.1016/j.earlhumdev.2010.05.010

Li, L., Pan, H., Wang, H., Li, X., Bu, X., Wang, Q., et al. (2016). Interplay between VEGF and Nrf2 regulates angiogenesis due to intracranial venous hypertension. *Sci. Rep.* 6, 37338. doi: 10.1038/srep37338

Li, Y., Bao, Y., Jiang, B., Wang, Z., Liu, Y., Zhang, C., et al. (2008). Catalpol protects primary cultured astrocytes from *in vitro* ischemia-induced damage. *Int. Dev. Neurosci.* 26, 309–317. doi: 10.1016/j.ijdevneu.2008.01.006

Liu, L., Li, J., Zeng, K. W., Jiang, Y., and Tu, P. F. (2016a). Five new biphenanthrenes from *Cremastra appendiculata*. *Molecules* 21, 1089–1098. doi: 10.3390/molecules21081089

Liu, L., Yin, Q. M., Zhang, X. W., Wang, W., Dong, X. Y., Yan, X., et al. (2016b). Bioactivity-guided isolation of biphenanthrenes from *Liparis nervosa*. *Fitoterapia* 115, 15–18. doi: 10.1016/j.fitote.2016.09.006

Liu, M., Wu, Y., Liu, Y., Chen, Z., He, S., Zhang, H., et al. (2018). Basic fibroblast growth factor protects astrocytes against ischemia/reperfusion injury by upregulating the caveolin-1/VEGF signaling pathway. *J. Mol. Neurosci.* 64, 211–223. doi: 10.1007/s12031-017-1023-9

Ma, Y., Zechariah, A., Qu, Y., and Hermann, D. M. (2012). Effects of vascular endothelial growth factor in ischemic stroke. *J. Neurosci. Res.* 90, 1873–1882. doi: 10.1002/jnr.23088

Malec, V., Gottschald, O. R., Li, S., Rose, F., Seeger, W., and Hanze, J. (2010). HIF-1alpha signaling is augmented during intermittent hypoxia by induction of the Nrf2 pathway in NOX1-expressing adenocarcinoma A549 cells. *Free Radic. Biol. Med.* 48, 1626–1635. doi: 10.1016/j.freeradbiomed.2010.03.008

Marti, H. J., Bernaudin, M., Bellail, A., Schoch, H., Euler, M., Petit, E., et al. (2000). Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. *Am. J. Pathol.* 156, 965–976. doi: 10.1016/S0002-9440(10)64964-4

Meng, X. E., Zhang, Y., Li, N., Fan, D. F., Yang, C., Li, H., et al. (2016). Effects of hyperbaric oxygen on the Nrf2 signaling pathway in secondary injury following traumatic brain injury. *Genet. Mol. Res.* 15, gmr6933. doi: 10.4238/gmr.15016933

Mulkey, S. B., Fontenot, E. E., Imamura, M., and Yap, V. L. (2011). Therapeutic hypothermia in a neonate with perinatal asphyxia and transposition of the great arteries. *Ther. Hypothermia Temp. Manag.* 1, 205–208. doi: 10.1089/ther.2011.0016

Nelson, K. B. (2007). Perinatal ischemic stroke. *Stroke* 38, 742–745. doi: 10.1161/01.STR.0000247921.97794.5e

Nordal, R. A., Nagy, A., Pintilie, M., and Wong, C. S. (2004). Hypoxia and hypoxia-inducible factor-1 target genes in central nervous system radiation injury: a role for vascular endothelial growth factor. *Clin. Cancer Res.* 10, 3342–3353. doi: 10.1158/1078-0432.CCR-03-0426

Parfenova, H., Pourcyrous, M., Fedinec, A. L., Liu, J., Basuroy, S., and Leffler, C. W. (2018). Astrocyte-produced carbon monoxide and the carbon monoxide donor CORM-A1 protect against cerebrovascular dysfunction caused by prolonged neonatal asphyxia. *Am. J. Physiol. Heart Circ. Physiol.* 315, H978–H988. doi: 10.1152/ajpheart.00140.2018

Quiniou, C., Kooli, E., Joyal, J. S., Sapieha, P., Sennlaub, F., Lahaie, I., et al. (2008). Interleukin-1 and ischemic brain injury in the newborn: development of a small molecule inhibitor of IL-1 receptor. *Semin. Perinatol.* 32, 325–333. doi: 10.1053/j.semperi.2008.07.001

Sandberg, M., Patil, J., D'Angelo, B., Weber, S. G., and Mallard, C. (2014). NRF2-regulation in brain health and disease: implication of cerebral inflammation. *Neuropharmacology* 79, 298–306. doi: 10.1016/j.neuropharm.2013.11.004

Schmid-Brunclik, N., Burgi-Taboada, C., Antoniou, X., Gassmann, M., and Ogunshola, O. O. (2008). Astrocyte responses to injury: VEGF simultaneously modulates cell death and proliferation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 295, R864–873. doi: 10.1152/ajpregu.00536.2007

Shu, L., Wang, C., Wang, J., Zhang, Y., Zhang, X., Yang, Y., et al. (2016). The neuroprotection of hypoxic preconditioning on rat brain against traumatic brain injury by up-regulated transcription factor Nrf2 and HO-1 expression. *Neurosci. Lett.* 611, 74–80. doi: 10.1016/j.neulet.2015.11.012

Tasca, C. I., Dal-Cim, T., and Cimarosti, H. (2015). *In vitro* oxygen-glucose deprivation to study ischemic cell death. *Methods Mol. Biol.* 1254, 197–210. doi: 10.1007/978-1-4939-2152-2_15

Ullian, E. M., Sapperstein, S. K., Christopherson, K. S., and Barres, B. A. (2001). Control of synapse number by glia. *Science* 291, 657–661. doi: 10.1126/science.291.5504.657

Vangeison, G., Carr, D., Federoff, H. J., and Rempe, D. A. (2008). The good, the bad, and the cell type-specific roles of hypoxia inducible factor-1alpha in neurons and astrocytes. *J. Neurosci.* 28, 1988–1993. doi: 10.1523/JNEUROSCI.5323-07.2008

Wiesner, D., Merdian, I., Lewerenz, J., Ludolph, A. C., Dupuis, L., and Witting, A. (2013). Fumaric acid esters stimulate astrocytic VEGF expression through HIF-1alpha and Nrf2. *PLoS One* 8, e76670. doi: 10.1371/journal.pone.0076670

Wu, L., Li, H. H., Wu, Q., Miao, S., Liu, Z. J., Wu, P., et al. (2015). Lipoxin A_4 activates Nrf2 pathway and ameliorates cell damage in cultured cortical astrocytes exposed to oxygen-glucose deprivation/reperfusion insults. *J. Mol. Neurosci.* 56, 848–857. doi: 10.1007/s12031-015-0525-6

Xue, Z., Li, S., Wang, S., Wang, Y., Yang, Y., Shi, J., et al. (2006). Mono-, bi-, and triphenanthrenes from the tubers of *Cremastra appendiculata*. *J. Nat. Prod.* 69, 907–913. doi: 10.1021/np060087n

Yao, J., Zhang, B., Ge, C., Peng, S., and Fang, J. (2015). Xanthohumol, a polyphenol chalcone present in hops, activating Nrf2 enzymes to confer protection against oxidative damage in PC12 cells. *J. Agric. Food Chem.* 63, 1521–1531. doi: 10.1021/jf505075n

Zalewska, T., Jaworska, J., and Ziemka-Nalecz, M. (2015). Current and experimental pharmacological approaches in neonatal hypoxic-ischemic encephalopathy. *Curr. Pharm. Des.* 21, 1433–1439. doi: 10.2174/1381612820999141029162457

Zhang, X., Bhattacharyya, S., Kusumo, H., Goodlett, C. R., Tobacman, J. K., and Guizzetti, M. (2014). Arylsulfatase B modulates neurite outgrowth *via* astrocyte chondroitin-4-sulfate: dysregulation by ethanol. *Glia* 62, 259–271. doi: 10.1002/glia.22604

Zhao, Y. J., Nai, Y., Ma, Q. S., Song, D. J., Ma, Y. B., Zhang, L. H., et al. (2018). Dl-3-n-butylphthalide protects the blood brain barrier of cerebral infarction by activating the Nrf2/HO-1 signaling pathway in mice. *Eur. Rev. Med. Pharmacol. Sci.* 22, 2109–2118. doi: 10.26355/eurrev20180414744

Zorec, R., Parpura, V., and Verkhratsky, A. (2018). Astroglial vesicular network: evolutionary trends, physiology and pathophysiology. *Acta Physiol. (Oxf.)* 222, e12915. doi: 10.1111/apha.12915

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

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 25 July 2019
doi: 10.3389/fnins.2019.00760

Melatonin Prevents Mice Cortical Astrocytes From Hemin-Induced Toxicity Through Activating PKCα/Nrf2/HO-1 Signaling *in vitro*

Xiao Chen[1†], Zhiyu Xi[1†], Huaibin Liang[2], Yuhao Sun[1], Zhihong Zhong[1], Baofeng Wang[1], Liuguan Bian[1] and Qingfang Sun[1,3*]*

[1] Department of Neurosurgery, Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China,
[2] Department of Neurology, Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China,
[3] Department of Neurosurgery, Ruijin Hospital Luwan Branch, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

OPEN ACCESS

Edited by:
Juan Andrés Orellana,
Pontifical Catholic University of Chile, Chile

Reviewed by:
Arturo Ortega,
Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV), Mexico
Fernanda Amaral,
Federal University of São Paulo, Brazil
Hee-Sun Kim,
Ewha Womans University, South Korea

***Correspondence:**
Liuguan Bian
blg11118@rjh.com.cn
Qingfang Sun
rjns123@163.com

[†] These authors have contributed equally to this work

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

Received: 11 April 2019
Accepted: 09 July 2019
Published: 25 July 2019

Citation:
Chen X, Xi Z, Liang H, Sun Y, Zhong Z, Wang B, Bian L and Sun Q (2019) Melatonin Prevents Mice Cortical Astrocytes From Hemin-Induced Toxicity Through Activating PKCα/Nrf2/HO-1 Signaling in vitro. Front. Neurosci. 13:760. doi: 10.3389/fnins.2019.00760

Secondary injuries mediated by oxidative stress lead to deterioration of neurological functions after intracerebral hemorrhage (ICH). Cortical astrocytes are among the most important cells in the central nervous system (CNS), and play key roles in maintaining redox homeostasis by providing oxidative stress defense. Hemin is a product of hemoglobin degradation, which has strong toxicity and can induce reactive oxygen species (ROS). Melatonin (Mel) and its metabolites are well tolerated without toxicity, prevent tissue damage as well as effectively assist in scavenging free radicals. We evaluated the hemin neurotoxicity to astrocytes and the resistance of Mel-treated astrocytes to hemin neurotoxicity. And we found Mel induced PKCα phosphorylation (p-PKC), nuclear translocation of Nrf2 in astrocytes, and upregulation of HO-1, which contributed to the reduction of ROS accumulation and cell apoptosis. Nrf2 and HO1 protein expression upregulated by Mel were decreased after administration of PKC inhibitor, Ro 31-8220 (Ro 31). Luzindole (Luz), a melatonin receptor inhibitor, suppressed p-PKCα, HO-1, and Nrf2 expression upregulated by Mel and increased cell apoptosis rate. The upregulation of HO-1 induced by Mel was depressed by knocking down Nrf2 expression by siRNA, which also decreased the resistance of astrocytes to toxicity of hemin. Mel activates astrocytes through PKCα/Nrf2/HO-1 signaling pathway to acquire resistance to toxicity of hemin and resist from oxidative stress and apoptosis. The positive effect of Mel on PKCα/Nrf2/HO-1 signaling pathway may become a new target for neuroprotection after intracerebral hemorrhage.

Keywords: intracerebral hemorrhage, hemin, melatonin, PKCα, Nrf2, oxidative stress

INTRODUCTION

ICH is a particularly destructive form of stroke with high mortality and morbidity, and survivors typically have severe nervous harm (Qureshi et al., 2009; Keep et al., 2012). Although surgical decompression of hemorrhage is widely believed to be a life-saving method, there is no authenticated medical or surgical treatment for ICH (Adeoye and Broderick, 2010; Keep et al., 2012; Hemphill et al., 2015). Mounting evidence suggests that intracerebral infusion of hemoglobin

(Hb) and its catabolite such as iron, bilirubin and hemin is a major cause of brain injury induced by ICH (Zhao et al., 2011; Keep et al., 2012; Xi et al., 2014). These molecules increase the secretion of inflammatory cytokines including IL-1β and TNF-α, which play a key role in inflammation and enlarge the inflammatory cascade (Wang J. et al., 2018). Oxidative stress is a state in which reactive oxygen species (ROS) production and antioxidant capacity are imbalanced due to the dysfunction of the cellular antioxidant system (Santofimia-Castano et al., 2015). Excessive ROS could lead to oxidative stress, destroying DNA, lipids and protein, and ultimately leading to irreversible damage and apoptosis of cells (Jung et al., 2010; Reczek and Chandel, 2015). The astrocyte, the major gliocyte in CNS, helps to maintain CNS stability and protects neurons against oxidative stress, besides providing neurotrophic factors (Huang et al., 2016; Liu et al., 2017; Wu et al., 2017). Therefore, inhibition of oxidative stress in astrocytes is paramount.

Protein kinase C (PKC) enzymes play a major role in many metabolic and signaling pathways, and participate in the regulation of gene expression, cell growth, migration, proliferation, differentiation and apoptosis. Therefore, lack of PKC and/or its dysregulation may lead to different pathologies, such as diabetes, heart failure, Alzheimer's and Parkinson's diseases, inflammatory diseases, oxidative stress, and even cancer (Isakov, 2018). PKCα is a typical subtype of PKC and plays an important role in antioxidant stress (Chueakula et al., 2018). It has been reported that phosphorylation of Nrf2 by PKC is a key event for Nrf2 nuclear translocation in response to oxidative stress (Huang et al., 2000, 2002). Nrf2 is a member of NF-E2 family of nuclear basic leucine zipper transcription factors, being a key transcription factor that regulates antioxidant reaction against ROS (Itoh et al., 1999; Shih et al., 2003). Nrf2 is usually combined with an actin binding protein Kelch-like ECH associated protein 1 (Keap1) and anchored in the cytoplasm (Jung et al., 2010; Negi et al., 2011; Wang et al., 2012; Deng et al., 2015). Upon cells stimulation, Nrf2 then escapes from Keap1-mediated degradation, transfers from cytosol into nucleus, and subsequently binds to a promoter sequence called antioxidant response (ARE) (Tao et al., 2013; Kleszczynski et al., 2016) to produce a cytoprotective response characterized by high expression of antioxidant enzymes such as hemeoxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), Superoxide Dismutase 2 (SOD2), glutamate cysteine ligase (GCL), and glutathione S-transferase (GST), for example (Kensler et al., 2007; Liu et al., 2015; Kleszczynski et al., 2016). Among them, HO-1 presents a cytoprotective effect on oxidative and inflammatory stress, showing an important metabolic function. It is also the rate-limiting step of oxidative catabolism in heme group (Parada et al., 2014). In different cellular models, the induction of HO-1 is usually related to cell protection, including cerebral ischemia (Parada et al., 2014; Liu et al., 2015).

Melatonin (N-acetyl-5-methoxytryptamine, Mel) is a neurohormone produced in the pineal gland and released in the blood and cerebrospinal fluid (CSF) in a circadian rhythm (Aladag et al., 2009; Cao et al., 2017; Cipolla-Neto and Amaral, 2018). Mel as well as its metabolites are well tolerated without toxicity, prevent tissue damage as well as effectively assist in scavenging hydroxyl radical (HO), nitric oxide (NO), superoxide anion radical (O2 -), peroxynitrite anion (ONOO–), and peroxynitrous acid (ONOOH), and other free radicals (Reiter et al., 2000; Wu et al., 2012). Recently, the effects of Mel on Nrf2 pathway have attracted more attention, specially due to its neuroprotective effect (Negi et al., 2011; Wang et al., 2012; Deng et al., 2015; Kleszczynski et al., 2016; Trivedi et al., 2016; Cao et al., 2017). Mel has already been reported to decrease neuroinflammation and oxidative stress via Nrf2 in experimental diabetic neuropathy (Negi et al., 2011). Wang et al. (2012) have evaluated the protection of Mel on early brain injury from subarachnoid hemorrhage (SAH) via the Nrf2-ARE pathway.

Whether the anti-oxidative effect of Mel in hemin treated astrocytes is related to the PKCα/Nrf2/HO-1 signaling pathway has not been thoroughly studied. Consequently, our team assumed Mel regulated the signaling pathway of PKCα/Nrf2/HO-1 and might be an effective way to combat oxidative damage induced by hemin. In our study, we evaluated cell viability and apoptosis of Mel-treated astrocytes exposed to hemin. ROS, TNNEL staining, immunostaining, and protein expression of PKCα, Nrf2 and HO-1 were evaluated to study the resistance mechanisms of Mel-treated astrocytes to Hemin oxidative stress through PKCα/Nrf2/HO-1 signaling pathway.

MATERIALS AND METHODS

Isolation and Culture of Astrocytes

All experimental schemes were authorized by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University in Shanghai, China. The primary astrocyte cells were prepared from pallium of newborn C57BL/6 mice, within 24 h from birth, obtained from Jester Laboratory Animal Co., Ltd. (Shanghai, China). After removing meninges and blood vessels as much as possible, the remaining cortical tissues were gently ground with 0.25% trypsin and digested at 37°C for 10 min, then plated on a 75 cm^2 flask coated with poly-D-lysine (Corning, United States) at a density of 20,000 cells/cm^2, and kept at 37°C at 95% humidity and 5% carbon dioxide (CO_2). The cells fused in 13–14 days, and half of the media was replaced by fresh media every 4 days. Pure second-to eighth-generation astrocytes were used for the following experiments.

Study Design

The study was performed in three parts. *In vitro* experiments were designed as follows. In the first part, we evaluated the hemin neurotoxicity to astrocytes and the resistance of Mel-treated astrocytes to hemin neurotoxicity. In the second part, we specifically focused on PKC inhibitor, Ro 31-8220 (Ro 31) and the Mel receptor inhibitor, Luzindole (Luz). The regulation of Ro 31 and Luz on hemin resistance in Mel-treated astrocytes was studied in this part. In the third part, we studied whether Mel-treated astrocytes transfected with 50 nM si-Nrf2 could resist the hemin neurotoxicity.

Experiment 1

Astrocytes were seeded on 6, 12, 24, or 96-well plates, treated with 30 μM hemin for 24 h, with or without 30 or 60 μM Mel (Control, Control + 60 μM Mel, Hemin, Hemin + 30 μM Mel, Hemin + 60 μM Mel). Dosages of hemin and Mel were chosen as described previously (Wang Z. et al., 2018). Cells were gathered for cell viability assay, luciferase reporter gene assay, TUNEL staining, intracellular ROS detection, immunostaining, western blotting analysis, and real-time PCR analysis.

Experiment 2

Astrocytes were seeded on 6, 12, or 24-well plates, pre-treated with or without 1 μM Luz/3 μM Ro 31 for 6 h, then exposed to 30 μM hemin, with or without 60 μM Mel for 24 h (Control, Hemin, Hemin + Mel, Hemin + Mel + Luz/ Ro 31). The dosage of Ro 31 and Luz was selected according to previous studies (Juszczak et al., 2014; Santofimia-Castano et al., 2015). Cells were gathered for TUNEL staining, immunostaining and western blotting analysis.

Experiment 3

Fifty nanomolar si-Nrf2 or negative control siRNA (si-NC) were transfected to astrocytes for 48 h, followed by 30 μM hemin incubation, with or without 60 μM Mel for 24 h (Control, Hemin + Mel, Hemin + si-NC + Mel, Hemin + si-Nrf2 + Mel). Cells were gathered and operated according to methods of experiment 2.

Drug Administration and siRNA Transfection

Hemin and Mel (Aladdin, China) were dissolved in absolute ethyl alcohol and diluted with 0.9% normal saline. Ro 31 were purchased from TargetMol, United States. Luz were purchased from Santa Cruz Biotechnology, United States. We transfected astrocytes with Nrf2 specific small interfering RNA (si-Nrf2) (GenePharma, China) by Lipofectamine® 2000 transfection reagent (Invitrogen, United States) according to the manufacturer's instructions. Western blotting was applied to prove the si-Nrf2 knockdown efficiency.

Cell Viability Assay

Cell viability was assessed using Cell Counting Kit-8 (CCK-8) (Beyotime, China) according to the manufacturer's instructions. Cells were seeded into a 96-well plate at a density of 10^4 per well. After 24 h, the cells were treated with 0, 5, 10, 20, 30, 40, and 50 μM hemin with or without 60 μM Mel for 24 h. Then 10 μL CCK-8 working fluid was added to each pore and cultured for 4h at cell culture incubator with 37°C, 95% humidity and 5% CO_2. The results of CCK-8 was tested by the microplate reader (Biotec, United States) at 450 nm and expressed as a percentage of the control group.

Cytotoxicity Assessment by Lactate Dehydrogenase (LDH) Assay

Lactate dehydrogenase cytotoxicity kit (Beyotime Biotechnology, China) was used to detected cytotoxicity according to the manufacturer's instructions. Cells were plated in 24-well plates. Cells were exposed to 30 μM hemin with or without 30 or 60 μM Mel for 24 h. The results of LDH was tested by the microplate reader (Biotec, United States) at 490 nm and expressed as a percentage of the control group.

Detection of ROS

Cells were plated on 6-well plates, handled according to experiment design. ROS assay kit (Beyotime Biotechnology, China) was used to detect ROS accumulation according to the manufacturer's instructions. Astrocytes were incubated with 10 μM DCFH-DA in serum free DMEM for 30 min at cell culture incubator with 37°C under 95% humidity with 5% CO2. Cells were washed with phosphate buffer solution (PBS) three times, and five randomly fields were pictured using a fluorescence microscope (Leica, Germany).

Plasmid Constructs and Luciferase Reporter Gene Assay

Luciferase reporter gene assay was used to analyze whether there was a direct link between Nrf2 and HO-1 in primary astrocytes. A fragment of HO-1 that contains the promotor binding sequence (−500 bp upstream to 100 bp downstream) was cloned into a luciferase reporter construct (GenePharma). Overexpressed Nrf2 plasmid (Nrf2) (GenePharma) was constructed using the empty vector PCDNA 3.1 and transfected into primary astrocytes on 12-well plates. PCDNA 3.1 was used as a negative control (NC). Luciferase activity was measured 24 h after transfection using Dual-Glo Luciferase Reporter Assay kit (Promega) according to the directions of the manufacturer. Experiments were repeated three times independently.

TUNEL Staining

TUNEL staining (*In Situ* Cell Death Detection Kit, Roche, Germany) was used to detected cellular apoptosis. Astrocytes were seeded onto coverslips and handled according to experiment designs. Then the cells were fixed in 4% paraformaldehyde (PFA) for 10 min. After washing with PBS three times, cells were permeated with 0.3% Triton X-100 for 10 min. The coverslips were embedded under the reaction fluid in dark humidified atmosphere for 60 min at 37°C. Then the nuclei were stained with DAPI (1:5000, Beyotime Biotechnology, China) for 5 min at room temperature in the dark. The TUNEL-positive cells displaying red nuclear staining were observed and analyzed by a confocal laser-scanning microscope (Leica, Germany). Five fields were chosen randomly under high power magnification, and the apoptosis ratio was calculated as number of TUNEL-positive cells to total number of cells.

Immunostaining

Cells were plated onto coverslips, handled according to experiment design. The cells were fixed in 4% PFA for 10 min. After washing with PBS three times, fixed cells were permeated with 0.3% Triton X-100 for 10 min, and blocked with 1% bovine serum albumin (BSA) for 1h at room temperature, then incubated overnight at 4°C with primary antibodies: rabbit

anti-Nrf2 polyclonal antibody (1:200, Santa Cruz Biotechnology, United States) and rabbit anti-HO-1 polyclonal antibody (1:300, Abcam, United Kingdom). After washing with PBS three times, the cells were incubated with corresponding secondary fluorescent antibodies (1:300, Invitrogen, United States): Alexa Fluor 488 donkey anti-rabbit IgG for 1 h at room temperature. And then the nuclei were counterstained with DAPI (1:5000, Beyotime Biotechnology, China). A confocal laser-scanning microscope (Leica, Germany) was used to observe and analyze fluorescence images.

Western Blotting Analysis

Cells were plated on 6-well plates, handled according to experiment design and a previous study (Jing et al., 2019). RIPA lysis buffer (Merk Millipore, Germany) with protease inhibitor cocktail (Roche, Swiss) was used to obtain the cell lysate. BCA Protein Assay Kit (Thermo Fisher Scientific, United States) was used to determine the protein concentrations. Each protein sample (30 μg) was loaded for electrophoresis, then transferred onto polyvinylidene difluoride (PVDF) membranes, blocked with 5% non-fat milk and 0.05% Tween-20 at room temperature for 1 h. The membranes were incubated overnight at 4°C with primary antibodies: rabbit anti-Nrf2, mouse anti-β-actin (1:1000, Santa Cruz Biotechnology, United States), rabbit anti-HO-1 (1:2000, Abcam, United Kingdom) and rabbit anti-p-PKCα (1:2000, ABclonal Technology, China). Later, the membranes were washed three times and incubated with proper horseradish peroxidase conjugated secondary antibody for 1 h at room temperature. After washing, the membranes were reacted with enhanced chemiluminescence (ECL) solution (Thermo Fisher Scientific, United States). We used Tanon image system (Shanghai, China) to detect the chemiluminescence signal. The relative intensity of the bands was performed by ImageJ 1.6.0 (NIH, United States).

Total RNA Extraction and Quantitative Real-Time PCR (RT-PCR) Analysis

Cells were plated on 12-well plates and handled according to experiment design. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's protocol. Reverse transcription and amplifying was carried out using reverse transcriptase and Taq DNA polymerase (Yeasen Biotech Co., Ltd., Shanghai, China), respectively. RT-PCR analyses were performed using the SYBR Green Master Mix Kit (Yeasen Biotech Co., Ltd.) and the PCR thermal cycler (Applied Biosystems, CA, United States). Nrf2, HO-1, NQO1, SOD2, IL-6, IL-10, and TNFα mRNA expressions were determined and quantified to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The mRNA relative expressions were normalized to control group. The primer sequences are listed in **Table 1**.

Statistical Analysis

Statistical analysis was performed by SPSS 21.0 (SPSS Inc., Chicago, IL, United States). All data were presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. The average bands density for control groups was set as 1.0, and values of all band density were normalized by the average value of control group to facilitate comparisons. Statistical comparison was performed using Student's *t*-test or one-way analysis of variance (ANOVA) tests. Statistical significance was deemed at $P < 0.05$.

RESULTS

Neurotoxicity Induced by Hemin Led to Primary Astrocytes Apoptosis

In order to observe the neurotoxicity induced by hemin on primary astrocytes, CCK-8 and LDH releasing assays were used to evaluate cell viability and cell death. Our results showed that cell viability decreases with hemin dosage (**Figure 1A**). According to our experimental results, 30 μM hemin was chosen for the subsequent experiments as it could significantly increase cell death ($P < 0.01$).

Mel Treatment Enhances the Resistance of Astrocytes to Neurotoxicity From Hemin, and Regulated Cytokines mRNA Expression

To explore whether Mel-treated astrocytes gain resistance to neurotoxicity from hemin, primary astrocytes were exposed to 30 μM hemin for 24 h, with or without 30 or 60 μM Mel. After Mel administration, astrocytes were resistant to neurotoxicity induced by hemin (**Figure 1A**) and LDH releasing assay (**Figure 1B**) showed Mel-treatment significantly decreased LDH releasing. The cell apoptosis rate decreased significantly in Mel-treated astrocytes compared to non-treated cells ($P < 0.001$) (**Figure 2**). We found that the protective effect of Mel was dose-dependent. Higher Mel doses presented a stronger

TABLE 1 | Primer sequences for qRT-PCR.

Gene	Forward primer/ Reverse primer (5′-3′)
HO-1	CAAGGAGGTACACATCCAAGCC/ TACAAGGAAGCCATCACCAGCT
NQO1	TGGTGACATAATCCGACAAGAT/ TTACCCACCTGAATGCCATAAT
SOD2	ACGCCACCGAGGAGAAGTACC/ CGCTTGATAGCCTCCAGCAACTC
IL-6	TGGGACTGATGCTGGTGACA/ ACAGGTCTGTTGGGAGTGGT
IL-10	CTGCTATGCTGCCTGCTCTTACTG/ ATGTGGCTCTGGCCGACTGG
TNFα	TGATCGGTCCCAACAAGGA/ TGCTTGGTGGTTTGCTACGA
GAPDH	GATGGTGAAGGTCGGTGTGA/ TGAACTTGCCGTGGGTAGAG

HO-1, heme oxygenase 1; NQO1, NAD(P)H quinone oxidoreductase 1; SOD2, superoxide dismutase 2; IL-6, interleukin 6; IL-10, interleukin 10; TNFα, tumor necrosis factor α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

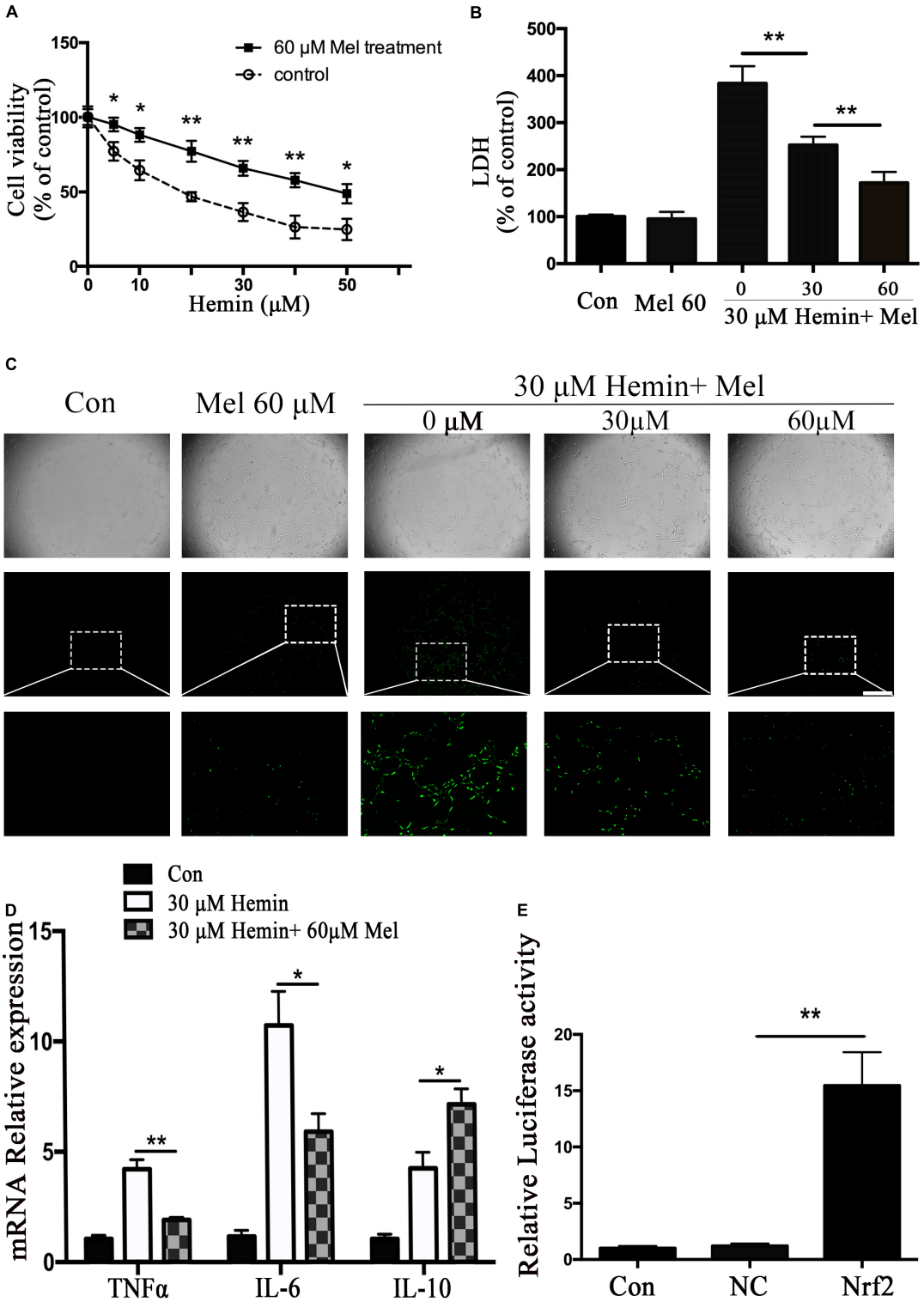

FIGURE 1 | Mel-treatment protected astrocytes from neurotoxicity induced by hemin. **(A)** Astrocytes were exposed to 0, 5, 10, 20, 30, 40, and 50 μM hemin for 24 h with or without 60 μM Mel, then the cell viability was evaluated by CCK-8. **(B)** Astrocytes were exposed to 30 μM hemin with or without 30 or 60 μM Mel for 24 h, and the cell death was evaluated by LDH releasing assay; **(C)** DCFH-DA probes were loaded, the intracellular ROS were observed using fluorescent microscope, bar = 400 μm; **(D)** mRNA expression of TNFα, IL-6, and IL-10 was checked. The relative expression of the mRNA was normalized to control. **(E)** Luciferase activity analysis was examined and normalized to control. The results of densitometric analysis of the bands were plotted into histogram. Difference between groups was analyzed using One-way ANOVA analysis or Student's *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$.

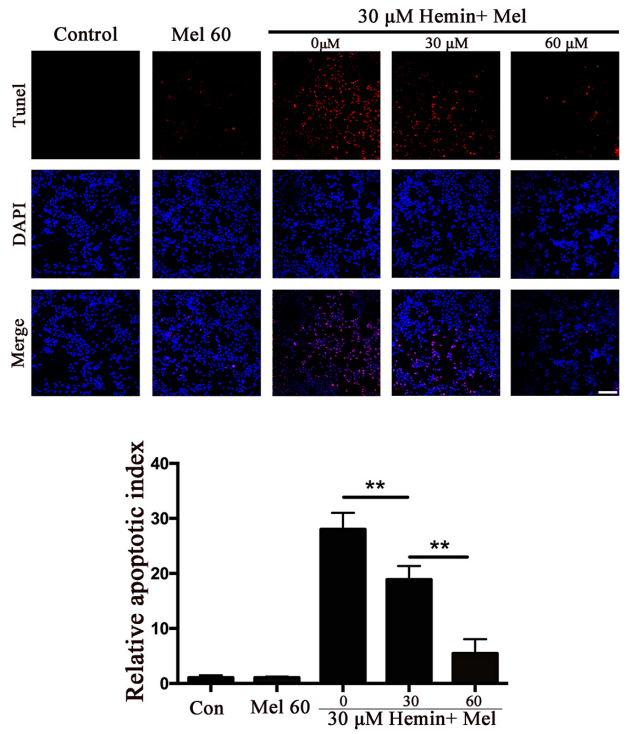

FIGURE 2 | The effect of Mel on astrocytes apoptotic ratio in five groups was indicated. TUNEL staining (red) was used to mark apoptotic cells, bar = 200 μm. The apoptosis rate was plotted into histogram and the relative expression of the proteins was normalized to control. Difference between groups was analyzed using One-way ANOVA analysis or Student's *t*-test. $^{**}P < 0.01$.

antitoxic effect than lower doses (**Figures 1A,B**). Then we checked the mRNA expression of cytokines. TNFα and IL-6 decreased significantly after Mel treatment ($P < 0.001$ and $P < 0.05$, respectively), while IL-10 increased significantly ($P < 0.05$) (**Figure 1D**).

Mel Down-Regulated ROS Accumulation Induced by Hemin

The neurotoxicity of hemin is mainly due to the production of ROS. To explore whether Mel-treatment could protect astrocytes against neurotoxicity from hemin by blocking intracellular ROS accumulation, ROS probe DCFH-DA was loaded into the cells. The results (**Figure 1C**) showed that hemin could significantly increase ROS accumulation while Mel-treatment significantly reduced intracellular ROS accumulation induced by hemin ($P < 0.05$).

Mel Up-Regulated Astrocytes HO-1 Expression After Hemin Exposure, and Up-Regulated NQO1, SOD2 mRNA Expression Simultaneously

HO-1 catalyzes hemin oxidative catabolism. In order to explore whether the protective effect of Mel-treatment is related to HO-1 induction, the expression of HO-1 was detected by immunostaining and western blotting. The immunostaining revealed that the HO-1 staining in astrocytes after hemin exposure increased after Mel-treatment (**Figure 3A**). Then we further investigated HO-1 protein expression by western blotting analysis. The results revealed that the HO-1 protein expression after Mel treatment was increased in parallel with HO-1 immunostaining ($P < 0.01$) (**Figure 3B**). The up-regulation capability of Mel was dose-dependent.

Subsequently, we further examined the effect of Mel on the mRNA expression of phase II antioxidant enzymes besides HO-1. The results were consistent with the protein expression of HO-1 ($P < 0.01$, $P < 0.01$, and $P < 0.01$, respectively) (**Figure 3C**).

Mel Increased Astrocytes Nrf2 Expression and Promoted Nrf2 Nuclear Translocation

Nrf2 is the major endogenous regulator of antioxidant reaction. Luciferase gene reporter showed that the fluorescence activity of astrocytes with overexpressed Nrf2 plasmid increased significantly, compared with the NC plasmid ($P < 0.01$) (**Figure 1E**). By immunostaining and western blotting, we observed whether the HO-1 expression was regulated by Nrf2 signaling pathway. The immunostaining (**Figure 4A**) showed that activated Nrf2 transferred into the nuclei of astrocytes under the stimulation of Mel. The protein expression of Nrf2 after 30 and 60 μM Mel stimulation (**Figure 4B**) was higher than those of no-treatment groups ($P < 0.01$). Western blotting of nuclear and cytoplasmic samples (**Figure 4C**) revealed that the Nrf2 ratio of nucleus to cytoplasm of astrocytes treated with Mel was significantly higher than that of no-treatment group ($P < 0.01$) and Nrf2 nuclear augmentation also showed a dose dependence (**Figure 4C**).

Mel Increased Astrocytes p-PKCα Expression, and Nrf2 and HO1 Expression Decreased After PKC Inhibition

Protein kinase C activation is a key event of Nrf2 nuclear translocation during oxidative stress. We observed that p-PKCα protein expression was higher in the Mel treated group than in untreated ones ($P < 0.01$) (**Figure 5A**), with a simultaneous increase in Nrf2 and HO1 expression. When we administrated the PKC inhibitor (Ro 31) in addition to Mel, both p-PKCα ($P < 0.05$) and HO-1 and Nrf2 ($P < 0.01$ and $P < 0.01$, respectively) upregulations by Mel were suppressed (**Figure 5B**).

Luz Inhibits the Protective Effect of Mel and Down-Regulated HO-1, Nrf2, and p-PKC Expression of Mel-Treated Astrocytes After Hemin Exposure

To explore the potential mechanisms of Mel, we administrated Mel receptor inhibitor (Luz) in addition to Mel treatment.

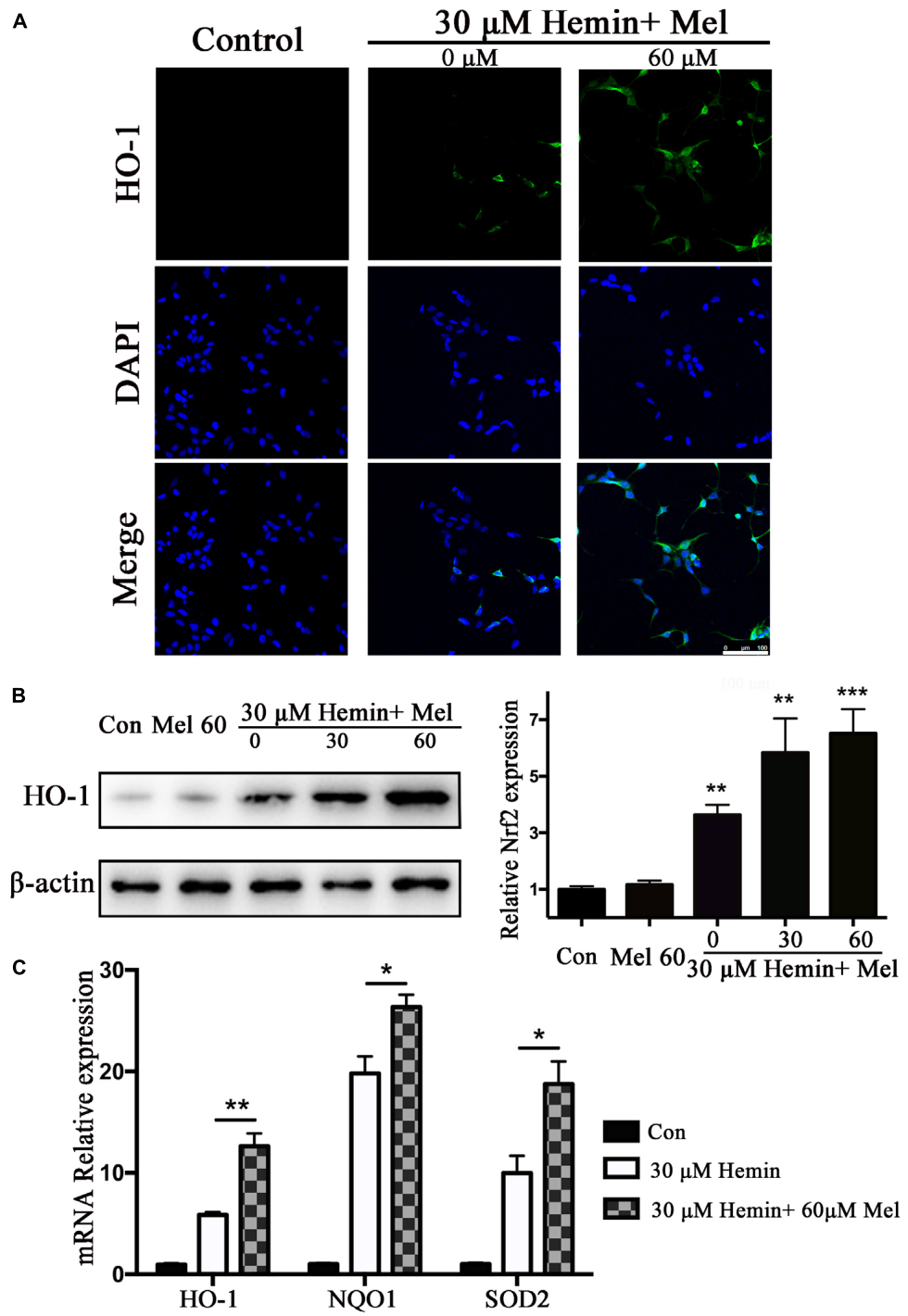

FIGURE 3 | Mel-treatment induced HO-1 expression in astrocytes. **(A)** Immunostaining showed the expression of HO-1 in astrocytes treated with or without 60 μM Mel for 24 h, the nuclei were counterstained with DAPI, bar = 100 μm. **(B)** Western blotting analysis of HO-1 expression in astrocytes treated with Mel of indicated dose. **(C)** mRNA expression of HO-1, NQO1, and SOD2 in astrocytes treated with Mel was further examined. The relative expression of the proteins and mRNA was normalized to control. The results of densitometric analysis of the bands were plotted into histogram. Difference between groups was analyzed using One-way ANOVA analysis or Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. control group.

TUNEL staining (**Figure 6A**) showed that after Luz administration, the numbers of TUNEL-positive cells were significantly increased than in the Mel group ($P < 0.001$). Luz also strongly suppressed the protein expression of HO-1, Nrf2, and p-PKCα up-regulated by Mel compared to Mel group ($P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively) (**Figure 6B**).

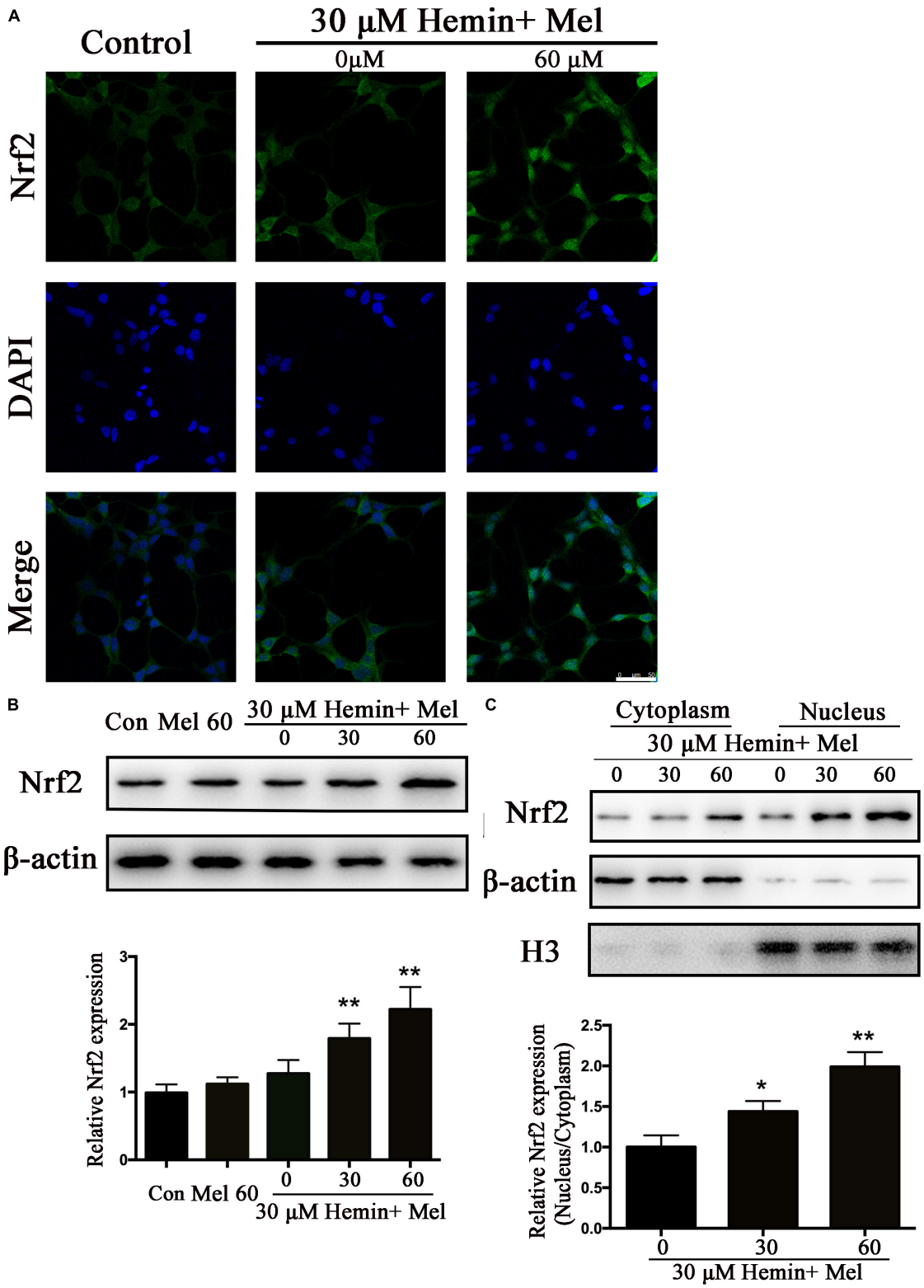

FIGURE 4 | Mel-treatment induced Nrf2 expression and promoted its nuclear translocation. **(A)** Immunostaining showing the subcellular expression of Nrf2 in astrocytes treated with or without 60 μM Mel for 24 h. The nuclei were counterstained with DAPI, bar = 50 μm. **(B)** Western bloting analysis of Nrf2 expression in astrocytes treated with Mel of indicated dose. **(C)** Nrf2 protein expression of cytoplasmic and nuclear from 0, 30, 60 μM Mel treated was analyzed. β-actin and H3 were, respectively, used as loading control for cytoplasmic and nuclear protein expression. The relative expression of the proteins was normalized to control. The results of densitometric analysis of the bands were plotted into histogram. Difference between groups was analyzed using One-way ANOVA analysis or Student's *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group or 0 μM group.

Nrf2 Knockdown Offset the Protection Effect of Mel on Neurotoxicity From Hemin

To explore whether the protection effect of Mel treatment relied on Nrf2, we transfected astrocytes with si-Nrf2 and si-NC. We used western blotting to check the efficiency of knockout, and about 82.14% of Nrf2 expression was restrained by si-Nrf2 ($P < 0.001$) (**Figure 7A**). After astrocytes infected with si-Nrf2 and si-NC were treated with 30 μM hemin for 24 h, with or without 60 μM Mel, TUNEL staining (**Figure 7B**) revealed that si-Nrf2 significantly increased the number of TUNEL-positive cells compared to si-NC ($P < 0.001$). Western blotting showed that si-Nrf2 significantly depressed HO-1 up-regulation induced by Mel, compared to si-NC ($P < 0.01$), but there was no significant difference in p-PKC (**Figure 7C**).

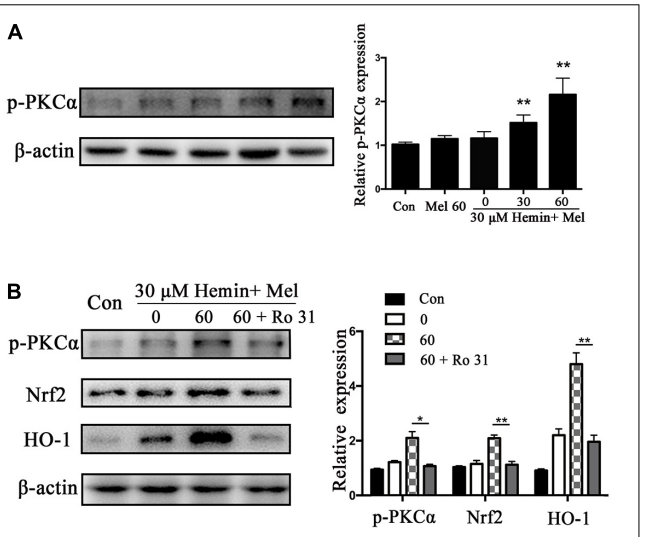

FIGURE 5 | P-PKCα expression in astrocytes exposed to hemin with Mel treatment, with or without PKC inhibitors. Western blotting analysis of p-PKCα expression in astrocytes treated with Mel of indicated dose **(A)**. **(B)** After administration of PKC inhibitors (RO 31), p-PKCα and its downstream expression were significantly inhibited. The relative expression of the proteins was normalized to control. The results of densitometric analysis of the bands were plotted into histogram. Difference between groups was analyzed using One-way ANOVA analysis or Student's *t*-test. $^*P < 0.05$ and $^{**}P < 0.01$ vs. control group.

DISCUSSION

The major findings of this study are as below: (1) the cell viability of astrocytes was decreased after hemin exposure, in a dose-dependent manner; (2) astrocytes are extensively damaged by neurotoxicity induced by hemin without Mel treatment, but after treated with Mel, Mel helped astrocytes resist the neurotoxicity and reduce the degree of damage; (3) Mel administration induced PKCα phosphorylation, Nrf2 upregulation and nuclear translocation in astrocytes, and led to phase II enzyme HO-1 upregulation; (4) Nrf2 and HO1 protein expression upregulated by Mel were blocked after administration of PKC inhibitor, Ro 31; (5) Mel-induced activation of PKCα/Nrf2/HO1 pathway could be partly abolished by Mel receptor inhibitor, Luz; (6) the *in vitro* protective effect of Mel on astrocytes was PKCα/Nrf2 dependent.

Mel, secreted by the pineal gland, possesses multiple pharmacological properties (Cipolla-Neto and Amaral, 2018). Mel as well as its metabolites are highly effective endogenous antioxidants. They are often used as a protective factor and antioxidant in many experiments and studies (Reiter et al., 2000; Cipolla-Neto and Amaral, 2018). Several recent studies propose that Mel prevents kidney injury (Sener et al., 2002), pancreatitis injury (Jung et al., 2010), and liver injury (Jung et al., 2009; Kang and Lee, 2012) by decreasing oxidative stress. In terms of neuroprotection, Mel has been reported to play an active role in several neurological disease, such as epilepsy (Brigo et al., 2016), Parkinson's disease (Mendivil-Perez et al., 2017), cerebral ischemia (Yang et al., 2015), intracerebral Hemorrhage (Wang Z. et al., 2018), and SAH (Dong et al., 2016; Zhao et al., 2017).

These beneficial properties impel us to think over the mechanism of Mel on astrocytes to protect ICH from oxidative stress. Both Mel and Nrf2 pathways play a vital role in oxidative stress. To date, although several studies have reported about the role of Mel on the Nrf2/ARE pathway (Jung et al., 2009, 2010; Negi et al., 2011; Wang et al., 2012; Deng et al., 2015; Kleszczynski et al., 2016; Trivedi et al., 2016; Cao et al., 2017), the mechanism is still not definitely clear. Our results were in favor of neuroprotection of Mel on astrocytes as Mel treatment not only reduced ROS accumulation but also enhances the resistance of astrocytes to neurotoxicity from hemin *in vitro*. Furthermore, we found that Mel treatment increased PKCα phosphorylation and Nrf2 and its phase II enzyme HO1 expression when compared to the untreated group, being those effects dose-dependent, which impelled us to consider Mel as a potential neuroprotection drug against ICH through PKCα/Nrf2/HO1 pathway.

Protein kinase C is one of several protein kinases able to modify Nrf2 to activate its release from keap1 (Huang et al., 2002). PKC phosphorylation of Nrf2 serine 40 results in the escape or release of Nrf2 from Keap1, translocate to the nucleus, and bind to the ARE that leads to coordinated activation of gene expression (Huang et al., 2002; Niture et al., 2010). It was reported that direct phosphorylation of Nrf2 by PKC is a key event of Nrf2 nuclear translocation in oxidative stress (Huang et al., 2000). In addition, PKCα inhibitors could reduce the expression of Nrf2, leading to the down-regulation of HO-1 (Yun et al., 2010). Nrf2 is an important transcription factor regulating antioxidant defense (Deng et al., 2015; Kleszczynski et al., 2016). Once stimulated by oxidative stress, Nrf2 is released by Keap1 and would be translocated to the nucleus binding to ARE and promoting the transcription of HO-1, phase II detoxification enzyme genes (Wang et al., 2012; Chen et al., 2015; Deng et al., 2015; Liu et al., 2015; Santofimia-Castano et al., 2015; Kleszczynski et al., 2016). Nrf2-ARE pathway is considered as a multi-organ protective agent and has been reported to play a key role in several CNS diseases, such as SAH (Chen et al., 2011), cerebral ischemia (Shih et al., 2005), traumatic brain injury (Wang J. et al., 2018), and cerebral hemorrhage (Chen et al., 2015). In addition, Nrf2 signaling pathway would be activated in

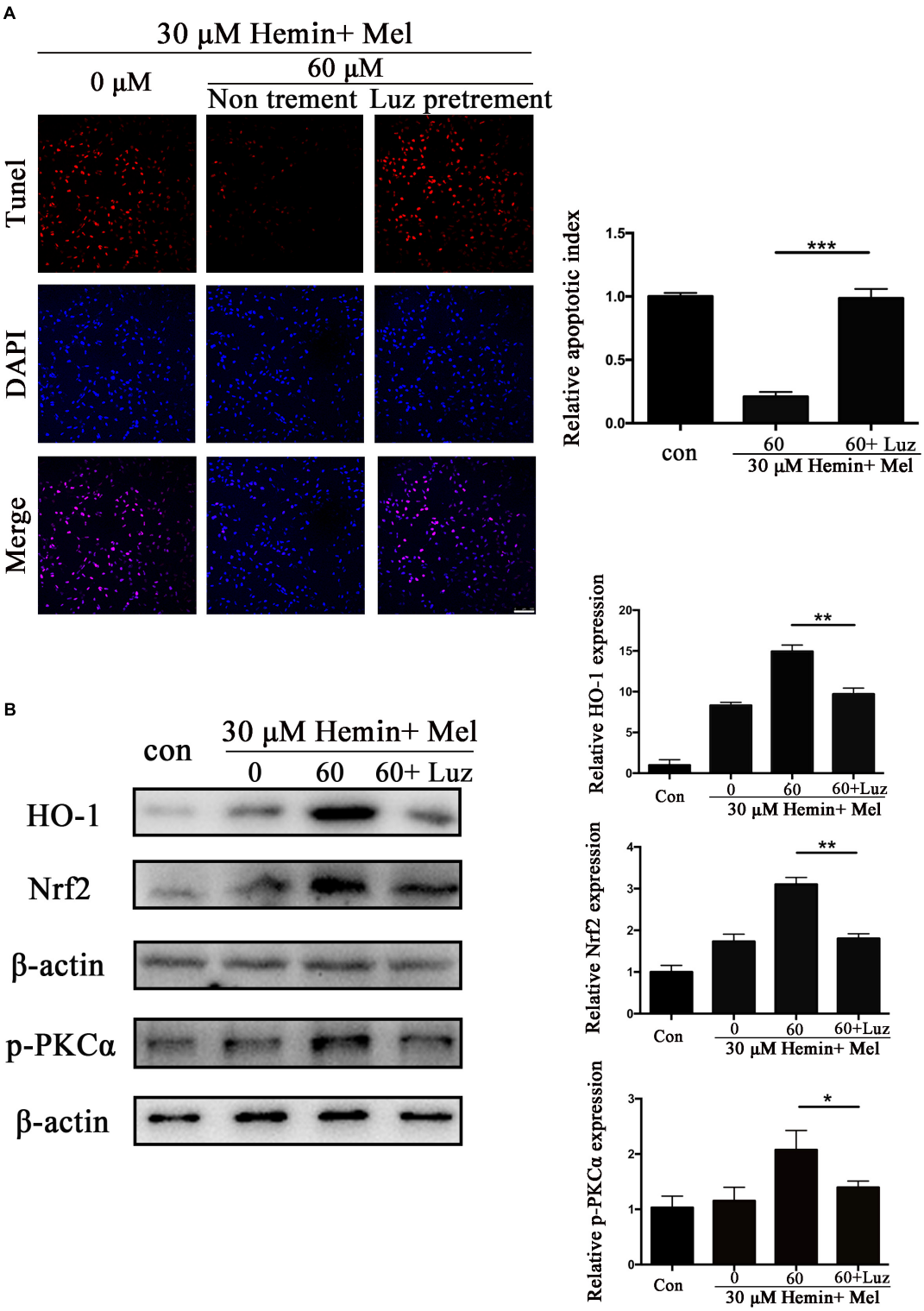

FIGURE 6 | Luz inhibited the protection effect of Mel and suppressed HO-1 and Nrf2 expression after hemin exposure. Astrocytes were transfected with or without Luz for 6 h, then followed by 30 μM hemin incubation, with or without 60 μM Mel for 24 h, **(A)** TUNEL staining (red) was used to mark apoptotic cells, bar = 100 μm. **(B)** Western bloting analysis of p-PKCα, Nrf2, and HO-1 protein expression was examined according to the experimental design. The relative expression was normalized to control. The results of apoptosis rate and densitometric analysis of the bands were plotted into histogram. Difference between groups was analyzed using One-way ANOVA analysis or Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.01$.

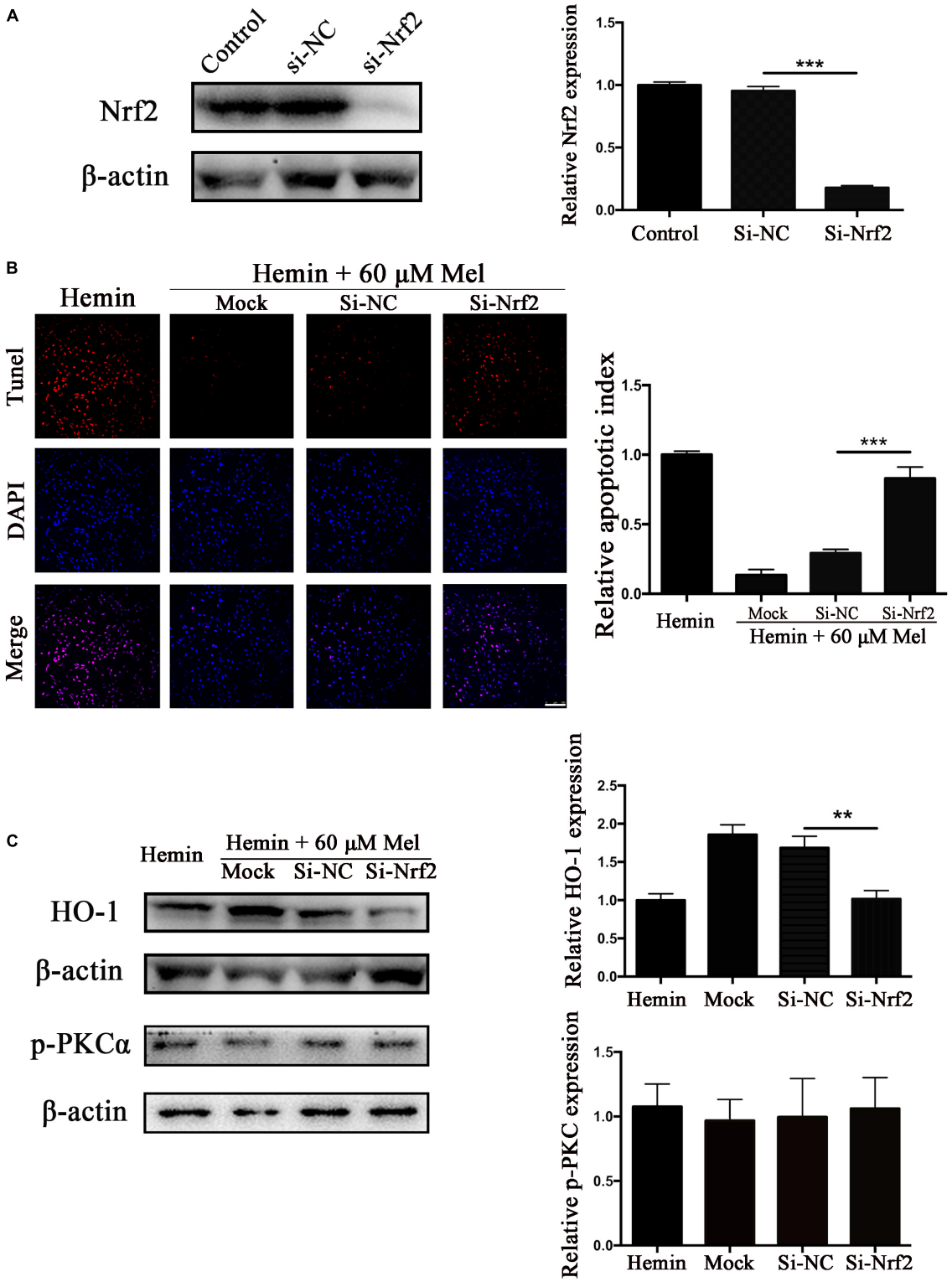

FIGURE 7 | Nrf2 knockdown suppressed Mel-induced upregulation of HO-1 expression and increased the numbers of TUNEL-positive cells. **(A)** Western blotting analysis of Nrf2 expression in control, si-NC, si-Nrf2 transfected astrocytes. Astrocytes were transfected with or without si-NC or si-Nrf2 for 48 h, then followed by 30 μM hemin incubation, with or without 60 μM Mel for 24 h; **(B)** TUNEL staining (red) was used to mark apoptotic cells, bar = 100 μm; **(C)** The HO-1 and p-PKCα expression was analyzed by western blotting. The relative expression was normalized to control. The results of apoptosis rate and densitometric analysis of the bands were plotted into histogram. Difference between groups was analyzed using One-way ANOVA analysis or Student's *t*-test. $^{**}P < 0.01$ and $^{***}P < 0.01$.

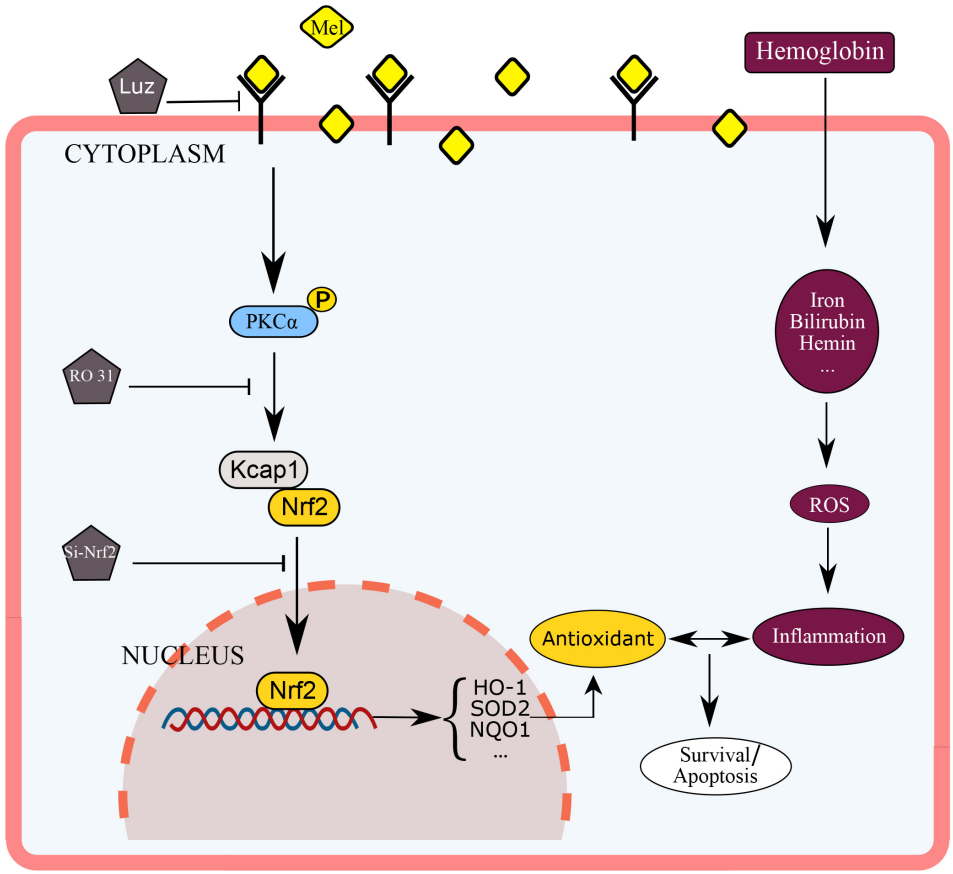

FIGURE 8 | Diagram outlining the mechanism of Mel on PKCα/Nrf2/HO-1 signaling pathways. Mel induced PKCα phosphorylation (p-PKC), nuclear translocation of Nrf2 in astrocytes, and upregulation of HO-1, then restraining ROS accumulation and cell apoptosis.

astrocytes to protect astrocytes as well as their adjacent neurons from oxidative damage (Kraft et al., 2004; Wang J. et al., 2018). Wang et al. (2007) also pointed out that when the Nrf2 gene was knocked out, neurological function might be impaired after the ICH. The mechanism may relate to ROS-induced DNA damage and neuronal cell death by apoptosis (Ito et al., 2001; Wang and Tsirka, 2005; Wang et al., 2007). Our results were parallel with those reports and indicated that phosphorylation of PKCα increased after MEL treatment, followed by up-regulation of Nrf2 and HO-1, which subsequently led to a decrease in ROS accumulation and apoptosis after hemin exposure.

Subcellular distribution of Nrf2 was further studied. The results indicated that Nrf2 expression was upregulated and Nrf2 translocated into the nucleus after Mel treatment. It was reported by Negi et al. (2011) that Mel stabilizes and activates Nrf2 in both cytoplasm and nucleus. In our study, Mel did increase Nrf2 expression both in nucleus and cytoplasm, but the growth increase in nucleus was more significant than that in cytoplasm. When the cellular protective mechanism was activated by stress impacts, Nrf2 will be translocated into the nucleus, which may be an effective way to maintain cell survival (Kleszczynski et al., 2016). This may explain our results that Nrf2 expression in nucleus was higher than that in cytoplasm. We also found that the Nrf2 upregulation and nuclear translocation depended on the phosphorylation of PKCα, and this phenomenon was terminated by PKC inhibitors. When we used Mel receptor inhibitors, Luz, these positive results were blocked, phosphorylation of PKCα was inhibited, upregulation of Nrf2 and HO1 were reversed, and correspondingly, nuclear translocation was suppressed, which confirmed that Mel protects astrocytes against apoptosis through PKCα/Nrf2-HO1 signaling pathway.

To further confirm that the protective effect of Mel treatment is Nrf2 dependent, we knocked down the expression of Nrf2 with Nrf2 specific siRNA. Our results indicated that Mel-induced HO-1upregulation was significantly suppressed by si-Nrf2, and the ROS accumulation and cell apoptosis were significantly increased, compared to si-NC group. Rodriguez et al. (2007) have shown that disruption of Nrf2 enhanced the upregulation of NF-κB activity and pro-inflammatory cytokines in brain injury, and vice versa, a low level of TNFα (2–5 ng/ml) could evoke significant nuclear translocation of Nrf2 with increased DNA/promoter binding and transactivation of Nrf2 targets (Shanmugam et al., 2016). Such phenomenon indicated that there might be an interaction between the pro-inflammatory signaling pathway and the Nrf2/HO-1 signaling pathway (Kleszczynski et al., 2016). Yin et al. (2015) have shown that the HO-1 inhibitor,

ZPP-IX, not only decreased the HO-1 expression and inhibited the Nrf2 entering nucleus, but also triggered the NF-κB entering nucleus, resulted in the over-expression of NF-κB and TNF-α. This result corresponded to the research of Poss and Tonegawa (1997) that animals developed a chronic inflammatory disease in a HO-1 knockout mice model. Our study also showed that MEL therapy downregulated TNFα and IL-6 expression, and upregulated IL-10 expression in astrocytes after hemin exposure. This was probably due to the antioxidant defense mechanism induced by elevated Nrf2 and nuclear translocation, or additional activation of inflammatory pathways, which need to be further explored in our future research.

This experiment used mice astrocytes to simulate oxidative stress of cerebral hemorrhage *in vitro* in order to study the protective effect of Mel on astrocyte through PKCα/Nrf2/HO-1 pathway. As far as we know, this may be the first report that shows that melatonin attenuates hemin induced oxidative damage in primary astrocytes via PKCα/Nrf2/HO1 signaling pathway *in vitro*. The potential mechanism of Mel on PKCα/Nrf2/HO-1 signaling pathways is shown in **Figure 8**. Unfortunately, there were still several limits in our studies. We only used *in vitro* Nrf2 knockout model and PKC inhibition to verify the effect of Mel on PKCα/Nrf2/HO-1 pathway. We lack Nrf2 knockout and PKC inhibition experiments *in vivo* to verify our theory, there may be other possibilities that Mel affects PKCα/Nrf2/HO1 pathways through other independent effects, such as cross-transmission between signaling pathways, microenvironment effects and cell-to-cell connections. It is important to note that Luz is just a Mel receptor inhibitor. The inhibition of the membrane receptors influence many of Mel actions, but does not inhibit all of them, as Mel presents direct actions, that are non-receptor dependent, and nuclear receptor-dependent actions. This study deals with the inhibition of the membrane receptor of Mel, so we set up blank group, control group and so on to minimize the error. In further research, we will focus on the protective effect of Mel on ICH through PKCα/Nrf2/HO1 signaling pathway *in vivo*.

Our results suggest that Mel activated PKCα/Nrf2/HO1 signaling pathway, inducing PKCα phosphorylation, upregulation as well as nuclear translocation of Nrf2, to protect astrocytes against neurotoxicity, and apoptosis from hemin. The protective effect of Mel on astrocyte depends on PKCα phosphorylation and the activation of Nrf2. The mechanisms by which Mel is coupled to PKCα and Nrf2 deserve future study. It is still worthwhile to take PKCα/Nrf2/HO1 signaling pathway combined with Mel as a target for neuroprotection after ICH.

DATA AVAILABILITY

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

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "Evidence of the Animal Experimental Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine." The protocol was approved by the "Laboratory Animal Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine."

AUTHOR CONTRIBUTIONS

XC, QS, and LB designed the research and wrote the manuscript. XC and ZX analyzed the results. HL prepared and completed the astrocytes isolation and culture. HL and ZZ collected the data and carried out the statistical analysis. YS, BW, and LB provided useful suggestions on the experiment design and reviewed the manuscript. YS and LB provided funds collection. QS and LB assisted with reviewing, editing the manuscript, and provided expertize and feedback.

FUNDING

This study was financially supported by the National Natural Science Foundation of China (Grant Nos. 816705021 and 8177050156).

REFERENCES

Adeoye, O., and Broderick, J. P. (2010). Advances in the management of intracerebral hemorrhage. *Nat. Rev. Neurol.* 6, 593–601. doi: 10.1038/nrneurol.2010.146

Aladag, M. A., Turkoz, Y., Parlakpinar, H., Ozen, H., Egri, M., and Unal, S. C. (2009). Melatonin ameliorates cerebral vasospasm after experimental subarachnoidal haemorrhage correcting imbalance of nitric oxide levels in rats. *Neurochem. Res.* 34, 1935–1944. doi: 10.1007/s11064-009-9979-7

Brigo, F., Igwe, S. C., and Del Felice, A. (2016). Melatonin as add-on treatment for epilepsy. *Cochrane Database Syst. Rev.* 8:Cd006967. doi: 10.1002/14651858.CD006967.pub4

Cao, S., Shrestha, S., Li, J., Yu, X., Chen, J., Yan, F., et al. (2017). Melatonin-mediated mitophagy protects against early brain injury after subarachnoid hemorrhage through inhibition of NLRP3 inflammasome activation. *Sci. Rep.* 7:2417. doi: 10.1038/s41598-017-02679-z

Chen, G., Fang, Q., Zhang, J., Zhou, D., and Wang, Z. (2011). Role of the Nrf2-ARE pathway in early brain injury after experimental subarachnoid hemorrhage. *J. Neurosci. Res.* 89, 515–523. doi: 10.1002/jnr.22577

Chen, Z.-Y., Yin, X.-P., Zhou, J., Wu, D., and Bao, B. (2015). Mechanisms underlying the perifocal neuroprotective effect of the Nrf2–ARE signaling pathway after intracranial hemorrhage. *Drug Design Dev. Ther.* 9, 5973–5986. doi: 10.2147/dddt.S79399

Chueakula, N., Jaikumkao, K., Arjinajarn, P., Pongchaidecha, A., Chatsudthipong, V., Chattipakorn, N., et al. (2018). Diacerein alleviates kidney injury through attenuating inflammation and oxidative stress in obese insulin-resistant rats. *Free Radic. Biol. Med.* 115, 146–155. doi: 10.1016/j.freeradbiomed.2017.11.021

Cipolla-Neto, J., and Amaral, F. G. D. (2018). Melatonin as a hormone: new physiological and clinical insights. *Endocr. Rev.* 39, 990–1028. doi: 10.1210/er.2018-00084

Deng, Y., Zhu, J., Mi, C., Xu, B., Jiao, C., Li, Y., et al. (2015). Melatonin antagonizes Mn-induced oxidative injury through the activation of keap1-Nrf2-ARE signaling pathway in the striatum of mice. *Neurotox Res.* 27, 156–171. doi: 10.1007/s12640-014-9489-5

Dong, Y., Fan, C., Hu, W., Jiang, S., Ma, Z., Yan, X., et al. (2016). Melatonin attenuated early brain injury induced by subarachnoid hemorrhage via regulating NLRP3 inflammasome and apoptosis signaling. *J. Pineal Res.* 60, 253–262. doi: 10.1111/jpi.12300

Hemphill, J. C. III, Greenberg, S. M., Anderson, C. S., Becker, K., Bendok, B. R., Cushman, M., et al. (2015). Guidelines for the management of spontaneous intracerebral hemorrhage: a guideline for healthcare professionals from the american heart Association/American stroke association. *Stroke* 46, 2032–2060. doi: 10.1161/str.0000000000000069

Huang, H. C., Nguyen, T., and Pickett, C. B. (2000). Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. *Proc. Natl. Acad. Sci. U.S.A.* 97, 12475–12480. doi: 10.1073/pnas.220418997

Huang, H. C., Nguyen, T., and Pickett, C. B. (2002). Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J. Biol. Chem.* 277, 42769–42774. doi: 10.1074/jbc.M206911200

Huang, Z., Wang, Y., Hu, G., Zhou, J., Mei, L., and Xiong, W. C. (2016). YAP is a critical inducer of SOCS3, preventing reactive astrogliosis. *Cereb. Cortex* 26, 2299–2310. doi: 10.1093/cercor/bhv292

Isakov, N. (2018). Protein kinase C (PKC) isoforms in cancer, tumor promotion and tumor suppression. *Semin. Cancer Biol.* 48, 36–52. doi: 10.1016/j.semcancer.2017.04.012

Ito, D., Tanaka, K., Suzuki, S., Dembo, T., and Fukuuchi, Y. (2001). Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. *Stroke* 32, 1208–1215. doi: 10.1161/01.str.32.5.1208

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., et al. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 13, 76–86. doi: 10.1101/gad.13.1.76

Jing, Y., Zhang, L., Xu, Z., Chen, H., Ju, S., Ding, J., et al. (2019). Phosphatase actin Regulator-1 (PHACTR-1) knockdown suppresses cell proliferation and migration and promotes cell apoptosis in the bEnd.3 mouse brain capillary endothelial cell line. *Med. Sci. Monit.* 25, 1291–1300. doi: 10.12659/msm.912586

Jung, K. H., Hong, S.-W., Zheng, H.-M., Lee, D.-H., and Hong, S.-S. (2009). Melatonin downregulates nuclear erythroid 2-related factor 2 and nuclear factor-kappaB during prevention of oxidative liver injury in a dimethylnitrosamine model. *J. Pineal Res.* 47, 173–183. doi: 10.1111/j.1600-079X.2009.00698.x

Jung, K. H., Hong, S. W., Zheng, H. M., Lee, H. S., Lee, H., Lee, D. H., et al. (2010). Melatonin ameliorates cerulein-induced pancreatitis by the modulation of nuclear erythroid 2-related factor 2 and nuclear factor-kappaB in rats. *J. Pineal Res.* 48, 239–250. doi: 10.1111/j.1600-079X.2010.00748.x

Juszczak, M., Roszczyk, M., Kowalczyk, E., and Stempniak, B. (2014). The influence od melatonin receptors antagonists, luzindole and 4-phenyl-2-propionamidotetralin (4-P-PDOT), on melatonin-dependent vasopressin and adrenocorticotropic hormone (ACTH) release from the rat hypothalamo-hypophysial system. In vitro and in vivo studies. *J. Physiol. Pharmacol.* 65, 777–784.

Kang, J.-W., and Lee, S.-M. (2012). Melatonin inhibits type 1 interferon signaling of toll-like receptor 4 via heme oxygenase-1 induction in hepatic ischemia/reperfusion. *J. Pineal Res.* 53, 67–76. doi: 10.1111/j.1600-079X.2012.00972.x

Keep, R. F., Hua, Y., and Xi, G. (2012). Intracerebral haemorrhage: mechanisms of injury and therapeutic targets. *Lancet Neurol.* 11, 720–731. doi: 10.1016/s1474-4422(12)70104-7

Kensler, T. W., Wakabayashi, N., and Biswal, S. (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* 47, 89–116. doi: 10.1146/annurev.pharmtox.46.120604.141046

Kleszczynski, K., Zillikens, D., and Fischer, T. W. (2016). Melatonin enhances mitochondrial ATP synthesis, reduces reactive oxygen species formation, and mediates translocation of the nuclear erythroid 2-related factor 2 resulting in activation of phase-2 antioxidant enzymes (gamma-GCS, HO-1, NQO1) in ultraviolet radiation-treated normal human epidermal keratinocytes (NHEK). *J. Pineal Res.* 61, 187–197. doi: 10.1111/jpi.12338

Kraft, A. D., Johnson, D. A., and Johnson, J. A. (2004). Nuclear factor E2-related factor 2-dependent antioxidant response element activation by tert-butylhydroquinone and sulforaphane occurring preferentially in astrocytes conditions neurons against oxidative insult. *J. Neurosci.* 24, 1101–1112. doi: 10.1523/jneurosci.3817-03.2004

Liu, B., Teschemacher, A. G., and Kasparov, S. (2017). Astroglia as a cellular target for neuroprotection and treatment of neuro-psychiatric disorders. *Glia* 65, 1205–1226. doi: 10.1002/glia.23136

Liu, Y., Qiu, J., Wang, Z., You, W., Wu, L., Ji, C., et al. (2015). Dimethylfumarate alleviates early brain injury and secondary cognitive deficits after experimental subarachnoid hemorrhage via activation of Keap1-Nrf2-ARE system. *J. Neurosurg.* 123, 915–923. doi: 10.3171/2014.11.Jns132348

Mendivil-Perez, M., Soto-Mercado, V., Guerra-Librero, A., Fernandez-Gil, B. I., Florido, J., Shen, Y. Q., et al. (2017). Melatonin enhances neural stem cell differentiation and engraftment by increasing mitochondrial function. *J. Pineal Res.* 63:e12415. doi: 10.1111/jpi.12415

Negi, G., Kumar, A., and Sharma, S. S. (2011). Melatonin modulates neuroinflammation and oxidative stress in experimental diabetic neuropathy: effects on NF-kappaB and Nrf2 cascades. *J. Pineal Res.* 50, 124–131. doi: 10.1111/j.1600-079X.2010.00821.x

Niture, S. K., Kaspar, J. W., Shen, J., and Jaiswal, A. K. (2010). Nrf2 signaling and cell survival. *Toxicol. Appl. Pharmacol.* 244, 37–42. doi: 10.1016/j.taap.2009.06.009

Parada, E., Buendia, I., Leon, R., Negredo, P., Romero, A., Cuadrado, A., et al. (2014). Neuroprotective effect of melatonin against ischemia is partially mediated by alpha-7 nicotinic receptor modulation and HO-1 overexpression. *J. Pineal Res.* 56, 204–212. doi: 10.1111/jpi.12113

Poss, K. D., and Tonegawa, S. (1997). Heme oxygenase 1 is required for mammalian iron reutilization. *Proc. Natl. Acad. Sci. U.S.A.* 94, 10919–10924. doi: 10.1073/pnas.94.20.10919

Qureshi, A. I., Mendelow, A. D., and Hanley, D. F. (2009). Intracerebral haemorrhage. *Lancet* 373, 1632–1644. doi: 10.1016/s0140-6736(09)60371-8

Reczek, C. R., and Chandel, N. S. (2015). ROS-dependent signal transduction. *Curr. Opin. Cell Biol.* 33, 8–13. doi: 10.1016/j.ceb.2014.09.010

Reiter, R. J., Tan, D. X., Osuna, C., and Gitto, E. (2000). Actions of melatonin in the reduction of oxidative stress. A review. *J. Biomed. Sci.* 7, 444–458. doi: 10.1159/000025480

Rodriguez, M. I., Escames, G., Lopez, L. C., Lopez, A., Garcia, J. A., Ortiz, F., et al. (2007). Chronic melatonin treatment reduces the age-dependent inflammatory process in senescence-accelerated mice. *J. Pineal Res.* 42, 272–279. doi: 10.1111/j.1600-079X.2006.00416.x

Santofimia-Castano, P., Clea Ruy, D., Garcia-Sanchez, L., Jimenez-Blasco, D., Fernandez-Bermejo, M., Bolanos, J. P., et al. (2015). Melatonin induces the expression of Nrf2-regulated antioxidant enzymes via PKC and Ca2+ influx activation in mouse pancreatic acinar cells. *Free Radic. Biol. Med.* 87, 226–236. doi: 10.1016/j.freeradbiomed.2015.06.033

Sener, G., Sehirli, A. O., Keyer-Uysal, M., Arbak, S., Ersoy, Y., and Yegen, B. C. (2002). The protective effect of melatonin on renal ischemia-reperfusion injury in the rat. *J. Pineal Res.* 32, 120–126.

Shanmugam, G., Narasimhan, M., Sakthivel, R., Kumar, R. R., Davidson, C., Palaniappan, S., et al. (2016). A biphasic effect of TNF-alpha in regulation of the Keap1/Nrf2 pathway in cardiomyocytes. *Redox Biol.* 9, 77–89. doi: 10.1016/j.redox.2016.06.004

Shih, A. Y., Johnson, D. A., Wong, G., Kraft, A. D., Jiang, L., Erb, H., et al. (2003). Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. *J. Neurosci.* 23, 3394–3406. doi: 10.1523/jneurosci.23-08-03394.2003

Shih, A. Y., Li, P., and Murphy, T. H. (2005). A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. *J. Neurosci.* 25, 10321–10335. doi: 10.1523/jneurosci.4014-05.2005

Tao, R.-R., Huang, J.-Y., Shao, X.-J., Ye, W.-F., Tian, Y., Liao, M.-H., et al. (2013). Ischemic injury promotes Keap1 nitration and disturbance of antioxidative responses in endothelial cells: a potential vasoprotective effect of melatonin. *J. Pineal Res.* 54, 271–281. doi: 10.1111/jpi.12009

Trivedi, P. P., Jena, G. B., Tikoo, K. B., and Kumar, V. (2016). Melatonin modulated autophagy and Nrf2 signaling pathways in mice with colitis-associated colon carcinogenesis. *Mol. Carcinogenesis* 55, 255–267. doi: 10.1002/mc.22274

Wang, J., Fields, J., Zhao, C., Langer, J., Thimmulappa, R. K., Kensler, T. W., et al. (2007). Role of Nrf2 in protection against intracerebral hemorrhage injury in mice. *Free Radic. Biol. Med.* 43, 408–414. doi: 10.1016/j.freeradbiomed.2007.04.020

Wang, J., Jiang, C., Zhang, K., Lan, X., Chen, X., Zang, W., et al. (2018). Melatonin receptor activation provides cerebral protection after traumatic brain injury by mitigating oxidative stress and inflammation via the Nrf2 signaling pathway. *Free Radic Biol. Med.* 131, 345–355. doi: 10.1016/j.freeradbiomed.2018.12.014

Wang, Z., Zhou, F., Dou, Y., Tian, X., Liu, C., Li, H., et al. (2018). Melatonin alleviates intracerebral hemorrhage-induced secondary brain injury in rats via suppressing apoptosis, inflammation, oxidative stress, DNA damage, and mitochondria injury. *Transl. Stroke Res.* 9, 74–91. doi: 10.1007/s12975-017-0559-x

Wang, J., and Tsirka, S. E. (2005). Neuroprotection by inhibition of matrix metalloproteinases in a mouse model of intracerebral haemorrhage. *Brain* 128(Pt 7), 1622–1633. doi: 10.1093/brain/awh489

Wang, Z., Ma, C., Meng, C. J., Zhu, G. Q., Sun, X. B., Huo, L., et al. (2012). Melatonin activates the Nrf2-ARE pathway when it protects against early brain injury in a subarachnoid hemorrhage model. *J. Pineal Res.* 53, 129–137. doi: 10.1111/j.1600-079X.2012.00978.x

Wu, C.-C., Lu, K.-C., Lin, G.-J., Hsieh, H.-Y., Chu, P., Lin, S.-H., et al. (2012). Melatonin enhances endogenous heme oxygenase-1 and represses immune responses to ameliorate experimental murine membranous nephropathy. *J. Pineal Res.* 52, 460–469. doi: 10.1111/j.1600-079X.2011.00960.x

Wu, X. M., Qian, C., Zhou, Y. F., Yan, Y. C., Luo, Q. Q., Yung, W. H., et al. (2017). Bi-directionally protective communication between neurons and astrocytes under ischemia. *Redox Biol.* 13, 20–31. doi: 10.1016/j.redox.2017.05.010

Xi, G., Strahle, J., Hua, Y., and Keep, R. F. (2014). Progress in translational research on intracerebral hemorrhage: is there an end in sight? *Prog. Neurobiol.* 115, 45–63. doi: 10.1016/j.pneurobio.2013.09.007

Yang, Y., Jiang, S., Dong, Y., Fan, C., Zhao, L., Yang, X., et al. (2015). Melatonin prevents cell death and mitochondrial dysfunction via a SIRT1-dependent mechanism during ischemic-stroke in mice. *J. Pineal Res.* 58, 61–70. doi: 10.1111/jpi.12193

Yin, X. P., Wu, D., Zhou, J., Chen, Z. Y., Bao, B., and Xie, L. (2015). Heme oxygenase 1 plays role of neuron-protection by regulating Nrf2-ARE signaling post intracerebral hemorrhage. *Int. J. Clin. Exp. Pathol.* 8, 10156–10163.

Yun, B. R., Lee, M. J., Kim, J. H., Kim, I. H., Yu, G. R., and Kim, D. G. (2010). Enhancement of parthenolide-induced apoptosis by a PKC-alpha inhibition through heme oxygenase-1 blockage in cholangiocarcinoma cells. *Exp. Mol. Med.* 42, 787–797. doi: 10.3858/emm.2010.42.11.082

Zhao, F., Hua, Y., He, Y., Keep, R. F., and Xi, G. (2011). Minocycline-induced attenuation of iron overload and brain injury after experimental intracerebral hemorrhage. *Stroke* 42, 3587–3593. doi: 10.1161/strokeaha.111.623926

Zhao, L., Liu, H., Yue, L., Zhang, J., Li, X., Wang, B., et al. (2017). Melatonin attenuates early brain injury via the melatonin Receptor/Sirt1/NF-kappaB signaling pathway following subarachnoid hemorrhage in mice. *Mol. Neurobiol.* 54, 1612–1621. doi: 10.1007/s12035-016-9776-7

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

frontiers in Pharmacology

MINI REVIEW
published: 27 September 2019
doi: 10.3389/fphar.2019.01114

Astrocytes: Role and Functions in Brain Pathologies

Rosalba Siracusa[1†], *Roberta Fusco*[1†] *and Salvatore Cuzzocrea*[1,2*]

[1] Department of Chemical, Biological, Pharmaceutical and Environmental Science, University of Messina, Messina, Italy, [2] Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, Saint Louis, MO, United States

Astrocytes are a population of cells with distinctive morphological and functional characteristics that differ within specific areas of the brain. Postnatally, astrocyte progenitors migrate to reach their brain area and related properties. They have a regulatory role of brain functions that are implicated in neurogenesis and synaptogenesis, controlling blood–brain barrier permeability and maintaining extracellular homeostasis. Mature astrocytes also express some genes enriched in cell progenitors, suggesting they can retain proliferative potential. Considering heterogeneity of cell population, it is not surprising that their disorders are related to a wide range of different neuro-pathologies. Brain diseases are characterized by the active inflammatory state of the astrocytes, which is usually described as up-regulation of glial fibrillary acidic protein (GFAP). In particular, the loss of astrocytes function as a result of cellular senescence could have implications for the neurodegenerative disorders, such as Alzheimer disease and Huntington disease, and for the aging brain. Astrocytes can also drive the induction and the progression of the inflammatory state due to their Ca^{2+} signals and that it is strongly related to the disease severity/state. Moreover, they contribute to the altered neuronal activity in several frontal cortex pathologies such as ischemic stroke and epilepsy. There, we describe the current knowledge pertaining to astrocytes' role in brain pathologies and discuss the possibilities to target them as approach toward pharmacological therapies for neuro-pathologies.

Keywords: astrocytes, Alzheimer disease, Huntington disease, epilepsy, ischemic stroke, drug

OPEN ACCESS

Edited by:
Lorenzo Di Cesare Mannelli, University of Florence, Italy

Reviewed by:
Livio Luongo, Second University of Naples, Italy
Caterina Scuderi, Sapienza University of Rome, Italy
Abbas Mirshafiey, Tehran University of Medical Sciences, Iran

***Correspondence:**
Salvatore Cuzzocrea
salvator@unime.it

[†]*These authors have contributed equally to this work*

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

Received: *17 June 2019*
Accepted: *30 August 2019*
Published: *27 September 2019*

Citation:
Siracusa R, Fusco R and Cuzzocrea S (2019) Astrocytes: Role and Functions in Brain Pathologies. Front. Pharmacol. 10:1114. doi: 10.3389/fphar.2019.01114

INTRODUCTION

During development, radial glial cells are the primary neural stem cells developing all neurons such as astrocytes, microglia cells, ependymal cells, and oligodendrocytes (Taverna et al., 2014). Mature astrocytes are categorized for functional and morphology proprieties. In the frontal cortex, these cells can be morphologically distinguished in four types: fibrous astroglia, protoplasmic, varicose, and interlaminar projections placed in the white matter and I, II, III, IV, V, and VI layers (Vasile et al., 2017). Other functional and morphological distinct astrocytes are unipolar Bergmann glia with radial ascending processes and elongated radial glia-like tanycytes. In the cerebellum, Bergmann glia control the synapsis of Purkinje cells (De Zeeuw and Hoogland, 2015), while in the hypothalamus, tanycytes are specialized in the modulation of neuroendocrine functions (Prevot et al., 2018). One of the most important astrocytes function is to deliver energy to neurons by the astrocyte-neuron lactate shuttle (Bass et al., 1971; Sherwood et al., 2006). Astrocytes modulate Ca^{2+} variations that influence neuronal activity releasing gliotransmitters (Peteri et al., 2019).

The modulation of the neurotransmitter uptake involves the excitatory transporters 1 and 2 (EAAT1 and 2) (Roberts et al., 2014). In response to inflammation and injury, astrocytes become reactive. They can be divided in two main categories: scar-forming astrocytes and hypertrophic astrocytes (Khakh et al., 2017). Several studies underline that reactive astrocytes alter them homeostatic functions such as potassium ion uptake, ion buffering, Ca^{2+} signaling, and excitatory neurotransmitter uptake (Rossi and Volterra, 2009). Regulation of astrocytes functions affected several brain pathologies such as Alzheimer disease, Huntington disease, Ischemic stroke, and epilepsy.

ALZHEIMER DISEASE

AD is a neurodegenerative disease with motor abnormalities, cognitive changes, and behavioral impairment. It is characterized by the aggregation of amyloid-β plaques in vessel walls and accumulation of the protein tau in neural cells. Astrocytes in this pathology contribute to the loss of neuroprotection and to the gaining of pathological characteristics. At the beginning, astrocytes have a protective role up-taking and degrading amyloid-β. The progression of disease leads to reduced astrocyte clearance of amyloid-β that contribute to gain of function (Garwood et al., 2017). Furthermore, amyloid-β accumulation stimulates astrocytes to produce pro-inflammatory mediators inducing a positive feedback of activation (González-Reyes et al., 2017).

It has been shown that amyloid-β co-operates with several receptors located on astrocytes such as scavenger receptors, TLRs, lipoprotein, glycoprotein and acetylcholine receptors, chemokine, and complement receptors (Farfara et al., 2008). Scavenger receptors are a group of evolutionally conserved membrane receptors expressed on the surface of microglia, macrophages, and dendritic cells (Wilkinson and El Khoury, 2012). To date, they have been classified into six classes (scavenger receptor A, B, C, D, E, and F) even if some members of this family remain unclassified (RAGE, CD163, and SR-PSOX). Of particular interest during AD are CD36, RAGE (receptor for advanced glycation end products), SCARA-1 (scavenger receptor A-1), and MARCO (macrophage scavenger receptor with collagenous structure). SCARA-1 is involved in clearance of Aβ, while MARCO forms a complex with formyl peptide–receptor-like 1 (FPR1) upon encountering Aβ. MARCO may decrease the inflammatory response in microglia through the FPR-1 *via* the ERK 1/2 intracellular signaling and the inhibition of cAMP (Brandenburg et al., 2010). CD36 and RAGE are implicated in activation of microglia by Aβ. CD36 cooperates with the other innate immune pattern recognition receptor like the TLRs to outline pathogen-specific responses. Once engaged by Aβ, CD36 forms a complex with TLR-6 and TLR-4 causing ROS production and inflammasome activation (Stewart et al., 2010). RAGE receptor is one of the most characterized unclassified scavenger receptor and has been reported to produce proinflammatory modifications in astrocytes when binds amyloid-β (González-Reyes et al., 2017). RAGE in turn activates the NF-κB (Yan et al., 1994) and its downstream pathway including p21, Cdc42-Rac, ras, MAPK (Taguchi et al., 2000), ERK (Wilkinson and El Khoury, 2012), and JNK (González-Reyes et al., 2017). RAGE is highly expressed vasculature and neurons in AD brains compared with the un-diseased (Arancio et al., 2004). RAGE located on endothelial cells in implicated in transporting Aβ into the brain (Mackic et al., 1998), and also increasing the diapedesis of monocytes across the blood–brain barrier (Giri et al., 2000). Once bound to soluble Aβ, RAGE induces microglial activation and chemotaxis following a concentration gradient, leading to a microglial accumulation around Aβ plaques (Wilkinson and El Khoury, 2012). RAGE mediates also the phagocytic profile of astrocytes and the interaction with other ligands, including S100β, involved in Alzheimer disease neuroinflammation (Cirillo et al., 2015). S100β produced by astrocytes is a common feature of Alzheimer disease (Bosch et al., 2015). It is associated with depressive behavior and cognitive flexibility and regulates neuronal oscillations (Stroth and Svenningsson, 2015; Brockett et al., 2018).

Moreover, morphological modifications of astrocytes in Alzheimer disease involve alterations in K^+ neurovascular regulation, by downregulation of Kir4.1 and BK_{Ca}, causing irregular cerebral blood flow (González-Reyes et al., 2017). Also, Ca^{2+} signaling is altered by amyloid-β accumulation (Haughey and Mattson, 2003). In astrocytes, this accumulation modifies the expression of the nicotinic acetylcholine receptors (nAchRs) and metabotropic glutamate receptor 5 (mGluR5), changing Ca^{2+} homeostasis (Xiu et al., 2005; Lim et al., 2013). Through this pathway, astrocytes increase glutamate signaling and led to the downregulation of its transporters (Masliah et al., 1996). Glutamate aberrant trafficking is linked to the modified cholesterol synthesis (Tian et al., 2010; Merlini et al., 2011; Talantova et al., 2013). A prodromal symptom to Alzheimer's disease can be the glucose hypometabolism (Mosconi et al., 2006). Carriers of apolipoprotein Eε4 (APOEε4) allele display lower glucose metabolism in different brain area with an augmented risk for AD (Reiman et al., 2004). Astrocytes signaling is a useful target to prevent and control the development of the AD.

HUNTINGTON DISEASE

Huntington disease is a genetic neurodegenerative disease with neuropsychiatric and motor dysfunctions. It is caused by a trinucleotide repeat (CAG) in the gene for Htt. This expansion caused a different form of Htt (mHtt) which aggregates (Bunner and Rebec, 2016). Astrocytes are more efficient than neurons in clearance of aggregates, so they are more resistant to mHtt accumulation (Zhao et al., 2016; Jansen et al., 2017; Zhao et al., 2017). However, when mHtt aggregates into astrocytes modifies glutamate signaling, causing neuronal excitotoxicity (Shin et al., 2005; Bradford et al., 2009). This condition is a typical feature of Huntington disease but has also been described several cases without alteration in glutamate release (Parsons et al., 2016). Astrocytes in Huntington disease are characterized by a decreased expression of Kir4.1 (Tong et al., 2014; Zhang et al., 2018). It influences GLT1-mediated homeostasis and Ca^{2+} signaling (Tong et al., 2014; Jiang et al., 2016). These dysfunctions

head the reactive state of astrocytes bringing about the possibility neurotoxicity can induce inflammation as secondary effect of Huntington disease (Tong et al., 2014).

During the inflammatory state, microglia trigger the activation of astrocytes releasing factors such as TNF-α, C1q, and IL-1α (Khakh and Sofroniew, 2015; Liddelow et al., 2017). They decreased synaptic maintenance and phagocytic activity (Bradford et al., 2009) and increase degeneration neurons and oligodendrocytes (Liddelow et al., 2017).

mHtt accumulation modifies astrocytes exosome (Hong et al., 2017) and BDNF (Hong et al., 2016) release. Restoration of BDNF expression from astrocytes displays neuroprotective effects (Giralt et al., 2010; Hong et al., 2016; Reick et al., 2016). It has been displayed that astrocytes are intricated in a wide range of pathological features of Huntington disease, so they can be used as a novel therapeutic target.

EPILEPSY

Epilepsy is a group of brain disorders characterized by unpredictable and periodic occurrence of seizures. The cause of most cases of epilepsy is unknown. Some cases occur as the result of brain injury, stroke, brain tumors, infections of the brain, and birth defects through a process known as epileptogenesis (Goldberg and Coulter, 2013). Known genetic mutations are directly linked to a small proportion of cases (Pandolfo, 2011). Although the symptoms of a seizure may affect any part of the body, the electrical events that produce the symptoms occur in the brain. Epileptic seizures are the result of excessive and abnormal neuronal activity in the cortex of the brain (Fisher et al., 2005).

The most common of these pathologies is the hippocampal sclerosis or mesial temporal sclerosis. It is characterized by gliosis, neuronal cell loss in the hippocampal areas, synaptic reorganization, and microvascular proliferation. A study published in PloS Biology shows how the interaction between neurons and astrocytes is one of the mechanisms that contributes to the generation of epileptic discharges. Believed in the past to be simple "helpers" of neurons, astrocytes have revealed over time cells that play a much more active role in the brain (Gomez-Gonzalo et al., 2010). Astrocytes express ion channels, transmitter receptors, and transporters and, thus, are endowed with the machinery to sense and respond to neuronal activity. Glutamate transporters are located on several neuronal cell types, but astrocytes are mainly involved in the glutamate uptake (Steinhauser et al., 2016). GLT-1, the glutamate transporter located on astrocytes, is involved in the bulk of extracellular glutamate clearance and is responsible of the increased levels in epileptogenic foci. Moreover, glutamine synthetase is reduced in the hippocampus of temporal lobe epilepsy patients compared to the healthy one. This downregulation leads to a slow glutamate–glutamine cycling and an accumulation of the transmitter in the extracellular space and astrocytes, providing a metabolic mechanism for astrocyte-dependent hyperexcitability. A few studies have highlighted the contribution of ionotropic glutamate receptors in convulsion generation. AMPA receptors, in particular the subtype composed by subunits GluR1 to GluR4, are abundantly expressed on astrocytes. Epilepsy patients show an enhanced expression of GluR1 flip variants accounts for the prolonged receptor in hippocampal astrocytes. Prolonged receptor opening increases influx of Na^+ and Ca^{2+} ions, blocking astroglial Kir channels which increase depolarization reducing the K^+ buffering capacity of astrocytes (Steinhauser et al., 2012). All this process contributes to hyperexcitability. In this condition, extracellular [K^+] could increase from ~ 3 mM to 10–12 mM; and glial cells take the most K^+ released by active neurons. As already mentioned, the primary mechanism for spatial K^+ buffering and K^+ reuptake is *via* glial inwardly rectifying K^+ channels (Kir channels). Kir channel subtypes (Kir1–Kir7) differ in functional properties and tissue distribution; Kir4.1 is the most abundantly in brain astrocytes. Astrocytes are also joined by gap junctions, which allow these cells to redistribute through the glial network the K^+ ions excessively accumulated at sites of intense neuronal activity. Accordingly, increasing evidence indicates that dysfunctional astrocytes are crucially involved in processes leading to epilepsy (Steinhauser and Seifert, 2012).

ISCHEMIC STROKE

Ischemic stroke is a brain damage which can lead to death or disabilities. It results from a vasculature dysfunction with occlusion of blood vessels by embolus or thrombus. The reduced or blocked blood flow causes loss of oxygen and glucose and in turn synthesis of ATP *via* glycolysis and oxidative phosphorylation. These conditions produce excitotoxicity and malfunction of astrocytes glutamate transporters, fundamental in the synaptic cleft in clearing glutamate release (Yi and Hazell, 2006; Zou et al., 2010). Increased glutamate release in the extracellular area induces the overexpression of rNMDARs and caused overloading of intracellular Ca^{2+} (Tanaka et al., 1997; Medvedeva et al., 2009). This energy depletion influences membrane potential depolarization and ionic gradients in neurons and astrocytes. In particular, astrocytes, comparing neurons, are less susceptible to glutamate cytotoxicity induced by brain stroke, but they display proliferation and up-regulation of GFAP levels producing reactive astrogliosis (Sofroniew, 2000). Reactive astrocytes are usually found in the focal lesions with tissues reorganization and formation of glial scars (Sofroniew, 2000). White matter astrocytes are especially sensible to ischemic stroke (Chen et al., 2016). The ischemic core shows a predominant presence of hypertrophic astrocytes with a larger Ca^{2+} signal compared to the penumbra region, the area surrounding the ischemic locus (Ding et al., 2009). Transcriptome analysis of activated astrocytes from inflamed brain after middle cerebral artery occlusion shows expression of genes encoding neuroprotective mediators and included cytokines (IL-6, IL-1, IL-1β, IL-10), transforming growth factor-β (TGFβ), interferon-γ (IFN-γ), thrombospondins, and neurotrophic factors (Zamanian et al., 2012). High levels of cytokines induce increasing levels of nitric oxide (NO) (Stoll et al., 1998) and apoptosis of neuronal cells (Clark and Lutsep, 2001) and inhibit neurogenesis (Monje et al., 2003). Reactive astrocytes also release chemokines after ischemia (Kim, 1996). In vascular endothelial cells, chemokines increased

adhesion molecules levels, attracting immune cells (Sofroniew, 2000). Astrocytes are the first cells of the nervous system where the class II major histocompatibility complex (MHC) (Dong and Benveniste, 2001) was shown. MHC II presents antigens to CD41 T-helper cells and is expressed on antigen presenting cells (APCs). Moreover, astrocytes express pattern recognition receptors (PRRs) as scavenger receptor, TLRs, and complement proteins playing a role in immune response regulation (Bsibsi et al., 2006).

These features let us to consider astrocyte a possible regulator of the ischemic context, considering that chronic of inflammation is influenced by the degree of tissue injury and exacerbation of the damage.

DISCUSSION

To date, only five drugs are accepted by the Food and Drug Administration (FDA) for the cure of AD: donepezil, galantamine and rivastigmine, memantine, and a drug composed of donezil and memantine (**Table 1**). Unfortunately, the use of these drugs is aimed at improving the excellence of life of patients, and they are not capable to stop the progression of the disorder (Caselli et al., 2017). So, it is important to find innovative treatments that improve therapeutic results. Aβ plaques increase the proinflammatory cytokines (Patel et al., 2005; Colombo and Farina, 2016) and the production of free radicals (Carson et al., 2006; Wyss-Coray and Rogers, 2012) with consequent activation of the astrocytes. In a late study conducted on APP/PS1 transgenic mice and on mixed neuronal/glial cultures, it was shown that curcumin improves spatial memory, stimulates cholinergic neuronal function, and, through PPAR-γ, reduces the activation of the inflammatory process in microglia and astrocytes (González-Reyes et al., 2017). Additional natural phytochemicals have demonstrated an anti-inflammatory and immunosuppressive capacities in AD models (**Table 1**), e.g., the triptolide extract inhibits astrocyte activation in the APP/PS1 transgenic mouse model of AD (Li et al., 2016). Punicalagin, a pomegranate derivative, reduces neuroinflammation (lowering TNF-α and IL-β) and also prevents oxidative stress by reducing iNOS, COX-2, and ROS production (Kim et al., 2017). Other mixtures that may have a probable role against dementia (Libro et al., 2016) are cannabinoid agonists such as WIN, 2-AG, and methanandamide (**Table 1**) that have shown anti-inflammatory activities in primary astrocytes grown later exposure to $A\beta_{1-42}$ or $A\beta_{25-35}$ (Aguirre-Rueda et al., 2015; Gajardo-Gomez et al., 2017). Other approaches to diminish oxidative stress in AD models involve stimulants of endogenous antioxidant factors (**Table 1**) such as pelargonidine (Sohanaki et al., 2016), Bambusae concretio Silicea (Jeong et al., 2005), and the new compound Monascin (Shi et al., 2016). In *in vivo* and in *in vitro* analyses, it has been shown that exogenous antioxidant compounds (**Table 1**) also have beneficial effects. Among these, we have resveratrol (Wang et al., 2016), tocotrienol (vitamin E) (Ibrahim et al., 2017), anthocyanins (Rehman et al., 2017), epicatechin (Cuevas et al., 2009), and 3H-1,2-dithiole-3-thione (a powerful free radical scavenger) (Wang et al., 2017). Aβ accumulation from astrocytes can also be decreased using IL-1β or TNF-α/TNF-α, PPAR-γ receptor agonists, minocycline or nicergoline, and tyrosine kinase inhibitors (Von Bernhardi et al., 2010; Kitazawa et al., 2011; Mandrekar-Colucci et al., 2012; Tweedie et al., 2012). NSAIDs are drugs that bind to and activate the PPAR-γ receptor (Jaradat et al., 2001; Wick et al., 2002) leading to reduced activation of glial cells (Combs et al., 2000; Bernardo and Minghetti, 2006) and cytokine-mediated inflammation (Sastre and Gentleman, 2010; De Nuccio et al., 2015).

The astrocyte carries most of the extracellular glutamate. Therefore, damage to astrocytes affects their capability to perceive or respond to an increase in glutamate levels which leads to the

TABLE 1 | Neurologically active drugs.

Disease	Drug category	References
AD	FDA accepted	Donepezil, galantamine, rivastigmine, memantine, and donezil + memantine (Caselli et al., 2017)
	Natural phytochemicals	Triptolide extract (Li et al., 2016) and punicalagin (Kim et al., 2017)
	Cannabinoid agonists	WIN, 2-AG, and methanandamide (Aguirre-Rueda et al., 2015; Gajardo-Gomez et al., 2017)
	Endogenous antioxidant factors	Pelargonidine (Sohanaki et al., 2016), Bambusae concretio Salicea (Jeong et al., 2005), monascin (Shi et al., 2016)
	Exogenous antioxidant compounds	Resveratrol (Wang et al., 2016), tocotrienol (Ibrahim et al., 2017), anthocyanins (Rehman et al., 2017), epicatechin (Cuevas et al., 2009), and 3H-1,2-dithiole-3-thione (Wang et al., 2017)
	Stimulators of the GLT1 expression	Penicillin, cephalosporin, ampicillin, estrogen, riluzole, and insulin (Frizzo et al., 2004; Brann et al., 2007; Ji et al., 2011)
	Activators of the GLT1 translation	Pyridazine and LDN/OSU-0212320 (Colton et al., 2010; Xing et al., 2011)
	GABA receptor antagonists	(Yuan and Shan, 2014)
Epilepsy	AED	Valproic acid, lamotrigine, phenobarbital, gabapentin, felbamate, and topiramate (French and Gazzola, 2011)
	Anticancer drug	Rapamycin (Huang et al., 2010; Kim and Lee, 2019)
	Allosteric potentiators of glutamine synthetase, regulators of AQP4 trafficking, interleukin 1 antagonists, and agonists or allosteric potentiators of TNFR2	(Wetherington et al., 2008) (Crunelli et al., 2015)
Ischemic stroke	Stimulators of the GLT1 expression	Ceftriaxone (Ouyang et al., 2007; Verma et al., 2010), carnosine (Shen et al., 2010), and tamoxifen (Lee et al., 2009)
	Inhibitors of p53 activity	MicroRNA-29a (Ouyang et al., 2013; Ouyang et al., 2014)
	Stimulators of angiogenesis	Ecdysterone (Luo et al., 2011) and omega-3 polyunsaturated fatty acids (Wang et al., 2014)

destruction of the microenvironment near neurons causing an over-stimulation of NMDA receptors, responsible for changes in cognitive functions in the frontal cortex (Finsterwald et al., 2015). Current studies have shown that the damage to astrocytes induced by Aβ is responsible for the reduced expression of GLT1 in AD. Therefore, drugs that target astrocytic glutamate transporters to ameliorate their expression and role represent a possible target for neurodegenerative syndromes. In this regard, there are two pharmacological approaches to increase GLT expression: either by increasing GLT1 promoter activation or by activating GLT1 translation (Rothstein et al., 2005; Kong et al., 2014). Among the compounds able to stimulate the expression of GLT1 already 48h after drug treatment, there are β-lactam antibiotics comprising penicillin and its derivatives, as well as cephalosporin antibiotics. Other mixtures such as ampicillin, estrogen, riluzole, and insulin have also been found to increase GLT1 expression (Frizzo et al., 2004; Brann et al., 2007; Ji et al., 2011) (**Table 1**). Instead, among compounds that have been found to activate the GLT1 translation (**Table 1**), we have a series of compounds based on pyridazine and LDN/OSU-0212320 (Colton et al., 2010; Xing et al., 2011). Finally, recent studies have correlated GABAergic neurotransmission with the pathological changes of AD (Li et al., 2011). Damaged astrocytes produce a copious amount of GABA that is released to inhibit excitatory neurotransmission in the dentate gyrus. In addition to GABA, monoamine oxidase-B (MAO-B) has been reported to be altered on reactive astrocytes (Jo et al., 2014), and the enzyme is upregulated in the *post mortem* brain of individuals with AD (Saura et al., 1994). In an animal model of Alzheimer, it has been shown that the administration of GABA receptor antagonists (**Table 1**) improve long-term memory in the hippocampus (Yuan and Shan, 2014).

HD is a disease that progressively destroys neurons in the brain and leads to severe motor and cognitive deficits. To date, no cure is available, but researchers have made progress that can lead to effective therapies. Numerous studies suggest that astrocytes may be intricated in HD. In particular, it has been observed that mHTT accumulations in striatal astrocytes are present in the brains of HD patients and in HD mouse models (Bradford et al., 2009). Several HD mouse models have been used to evaluate the contribution of astrocytes to HD pathophysiology. In one of these studies, astrogliosis was evaluated as it frequently accompanies brain disorders. In conjunction with the start of symptoms, a high number of astrocytes showed mHTT inclusions and an important reduction in fundamental functional proteins. One of these proteins was Kir4.1 (Tong et al., 2014). These results propose that mHTT is correlated with early termination of the expression of essential functional astrocyte proteins (e.g., Kir4.1), which modifies the function of astrocytes without triggering astrogliosis. Furthermore, striatal astrocytes of HD mice show depolarized membrane potentials and lower membrane conductances when mice are symptomatic. This is owing to the function and lower expression of the Kir4.1 channels. Deficiencies in latent membrane potential were recovered by selective release of Kir4.1 from adeno-associated viruses (AAV) and a specific astrocyte promoter. Furthermore, it has been observed that the loss of Kir4.1 currents in striatal astrocytes leads to reduced K^+ spatial buffering, which leads to higher environmental K^+ levels in HD mouse models. Therefore, the astrocytic channels Kir.4.1, and other astrocytic molecular mechanisms can represent appreciated targets for therapeutic development (Khakh and Sofroniew, 2014).

Other approaches currently being studied for HD therapy point to both to obtain information on the mechanisms of disease progression and to silence the expression of mHTT using antisense oligonucleotides. A new approach is to detect novel factors that increase neurogenesis and/or stimulate the reprogramming of endogenous neuroblasts and parenchymal astrocytes to produce new healthy neurons to substitute the lost ones and/or strengthen the neuroprotection of preexisting striatal and cortical neurons (Sassone et al., 2018).

Regarding epilepsy, to date, more than 20 antiepileptic drugs (AEDs) (**Table1**) have been developed, including valproic acid, lamotrigine, phenobarbital, gabapentin, felbamate, and topiramate (French and Gazzola, 2011). Despite this, ~30% of patients respond poorly to treatment (Kwan and Brodie, 2000). In contrast, 70% of patients can attain long-term remission under AED treatment. However, many AEDs are associated with adverse side effects that are experienced by a substantial number of patients. Thus, significant unmet medical needs still must be overcome for the real and safe treatment of epilepsy. Many studies have suggested that inequities between excitatory and inhibitory signals may cause epilepsy (White et al., 2007; Bialer and White, 2010). AEDs currently used to stop epileptic seizures act mostly by blocking ion channels and inhibiting neuronal excitability. Rapamycin, which was approved by the FDA as an anticancer drug (**Table 1**), has been demonstrated as another potential antiepileptic agent with broader clinical relevance (Huang et al., 2010; Kim and Lee, 2019). Unfortunately, rapamycin can inhibit cell proliferation and motility; thus, the safety of long-term rapamycin treatments must be assessed in advance. However, the role of the mTOR inhibition strategy for the treatment of epilepsy remains viable (Russo et al., 2014). Today, it is clear that astrocytes play prominent roles in information processing in the epileptic brain. Insights gleaned from careful studies of the properties of reactive astrocytes suggest several novel targets for drug development (**Table 1**), including allosteric potentiators of glutamine synthetase, regulators of AQP4 trafficking, interleukin 1 antagonists, and agonists or allosteric potentiators of TNFR2 (Wetherington et al., 2008) (Crunelli et al., 2015).

To date, pharmacological treatments for ischemia/reperfusion have palliative effects and require almost immediate administration after damage (Van Der Worp and Van Gijn, 2007). To overcome this problem, it is indispensable to find new treatments focused mainly on long-term neuroprotection. Strategies targeting astrocytes may be an option as the increase in astrocyte survival during ischemic stress is connected with increased neuronal survival. It has been observed that induction of glial-specific purinergic receptor activation (P2Y1R) leads to greater consumption of mitochondrial O_2 and stimulation of ATP production by astrocytes thus reducing neuronal damage to astrocytes and cell death and therefore brain damage (Zheng et al., 2013; Liu and Chopp, 2016). Furthermore, infarct area improved even after administration of TGF-α (Sharif et al., 2007). This treatment also led to a significant functional recovery

in rats after MCAO (Justicia et al., 2001). Other experiments indicate that another therapeutic potential involves the increase in astrocytic glutamate transport after stroke. Thus, the increased expression of the glutamate transporter GLT-1 in astrocytes with ceftriaxone (**Table 1**) (Ouyang et al., 2007; Verma et al., 2010) protects neurons from ischemia (Chu et al., 2007). Other compounds that improve neurological function and reduce the infarct area are carnosine (Shen et al., 2010) and tamoxifen (Lee et al., 2009) (**Table 1**). Both substances preserve the expression of GLT-1 on astrocytes by reducing glutamate levels and attenuating the consequent excitotoxicity. Another target for stroke therapy is p53 (**Table 1**) since inhibition of p53 activity has been shown to hinder astrocyte activation and glutamate intake (Ahn et al., 2015). Even microRNAs, approximately of which are expressed in astrocytes as microRNA-29a, appear to be intricate in the control of cerebral ischemia and may represent targets for improving stroke outcome (Ouyang et al., 2013; Ouyang et al., 2014). More recently, reference is made to cell therapy which aims at finding cells that can induce regeneration. Astrocyte transplantation conducts to recovery of axonal myelination, variation of the immune response, and issue of neurotrophic factors that prevent oxidative stress and excitotoxic injury (Choudhury and Ding, 2016). Other studies have suggested to astrocytes a therapeutic target based on their control by genetic change of proteins associated to the immune response and exacerbation of reactivity and cytotoxicity (Merienne et al., 2015). Finally, it was observed that post-stroke angiogenesis not only ameliorate blood perfusion in the ischemic area but also supports cerebral parenchymal cells, comprising astrocytes, the issue of neurotrophic factors, to stimulate neurogenesis, which therefore improves remodeling cerebral and long-term neurological function after stroke (Zhang and Chopp, 2009). Consequently, angiogenesis represents a valid reparative machinery that has been verified in numerous studies (**Table 1**). For example, treatment with ecdysterone ameliorates neurological function by improving astrocyte stimulation and angiogenesis after focal cerebral ischemia in rats (Luo et al., 2011). Transgenic overproduction of omega-3 polyunsaturated fatty acids in mice recovers post-stroke revascularization and increases endogenous angiogenesis by inducing angiopoietin 2 production in astrocytes, which consequently stimulated endothelial cell proliferation and BBB formation, proposing that the integration of omega-3 polyunsaturated fatty acids is a possible angiogenic treatment able to increase brain repair and improve long-term functional recovery after ischemic stroke (Wang et al., 2014).

AUTHOR CONTRIBUTIONS

RS and RF made literature search and wrote the first draft of the manuscript. SC, RS and RF designed the aim of the review. All authors contributed to reading and approving the final version of the manuscript.

REFERENCES

Aguirre-Rueda, D., Guerra-Ojeda, S., Aldasoro, M., Iradi, A., Obrador, E., Mauricio, M. D., et al. (2015). WIN 55,212-2, agonist of cannabinoid receptors, prevents amyloid beta1-42 effects on astrocytes in primary culture. *PLoS One* 10, e0122843. doi: 10.1371/journal.pone.0122843

Ahn, K. C., Mackenzie, E. M., Learman, C. R., Hall, T. C., Weaver, C. L., Dunbar, G. L., et al. (2015). Inhibition of p53 attenuates ischemic stress-induced activation of astrocytes. *Neuroreport* 26, 862–869. doi: 10.1097/WNR.0000000000000439

Arancio, O., Zhang, H. P., Chen, X., Lin, C., Trinchese, F., Puzzo, D., et al. (2004). RAGE potentiates Aβ-induced perturbation of neuronal function in transgenic mice. *EMBO J.* 23, 4096–4105. doi: 10.1038/sj.emboj.7600415

Bass, N. H., Hess, H. H., Pope, A., and Thalheimer, C. (1971). Quantitative cytoarchitectonic distribution of neurons, glia, and DNa in rat cerebral cortex. *J. Comp. Neurol.* 143, 481–490. doi: 10.1002/cne.901430405

Bernardo, A., and Minghetti, L. (2006). PPAR-gamma agonists as regulators of microglial activation and brain inflammation. *Curr. Pharm. Des.* 12, 93–109. doi: 10.2174/138161206780574579

Bialer, M., and White, H. S. (2010). Key factors in the discovery and development of new antiepileptic drugs. *Nat. Rev. Drug Discov.* 9, 68–82. doi: 10.1038/nrd2997

Bosch, M. N., Pugliese, M., Andrade, C., Gimeno-Bayón, J., Mahy, N., and Rodriguez, M. J. (2015). Amyloid-β immunotherapy reduces amyloid plaques and astroglial reaction in aged domestic dogs. *Neurodegener. Dis.* 15, 24–37. doi: 10.1159/000368672

Bradford, J., Shin, J.-Y., Roberts, M., Wang, C.-E., Li, X.-J., and Li, S. (2009). Expression of mutant huntingtin in mouse brain astrocytes causes age-dependent neurological symptoms. *Proc. Natl. Acad. Sci.* 106, 22480–22485. doi: 10.1073/pnas.0911503106

Brandenburg, L. O., Konrad, M., Wruck, C. J., Koch, T., Lucius, R., and Pufe, T. (2010). Functional and physical interactions between formyl-peptide-receptors and scavenger receptor MARCO and their involvement in amyloid beta 1-42-induced signal transduction in glial cells. *J. Neurochem.* 113, 749–760. doi: 10.1111/j.1471-4159.2010.06637.x

Brann, D. W., Dhandapani, K., Wakade, C., Mahesh, V. B., and Khan, M. M. (2007). Neurotrophic and neuroprotective actions of estrogen: basic mechanisms and clinical implications. *Steroids* 72, 381–405. doi: 10.1016/j.steroids.2007.02.003

Brockett, A. T., Kane, G. A., Monari, P. K., Briones, B. A., Vigneron, P.-A., Barber, G. A., et al. (2018). Evidence supporting a role for astrocytes in the regulation of cognitive flexibility and neuronal oscillations through the Ca2+ binding protein S100β. *PloS One* 13, e0195726. doi: 10.1371/journal.pone.0195726

Bsibsi, M., Persoon-Deen, C., Verwer, R. W. H., Meeuwsen, S., Ravid, R., and Van Noort, J. M. (2006). Toll-like receptor 3 on adult human astrocytes triggers production of neuroprotective mediators. *Glia* 53, 688–695. doi: 10.1002/glia.20328

Bunner, K. D., and Rebec, G. V. (2016). Corticostriatal dysfunction in Huntington's disease: the basics. *Front. Hum. Neurosci.* 10, 317. doi: 10.3389/fnhum.2016.00317

Carson, M. J., Thrash, J. C., and Walter, B. (2006). The cellular response in neuroinflammation: The role of leukocytes, microglia and astrocytes in neuronal death and survival. *Clin. Neurosci. Res.* 6, 237–245. doi: 10.1016/j.cnr.2006.09.004

Caselli, R. J., Beach, T. G., Knopman, D. S., and Graff-Radford, N. R. (2017). Alzheimer disease: scientific breakthroughs and translational challenges. *Mayo. Clin. Proc.* 92, 978–994. doi: 10.1016/j.mayocp.2017.02.011

Chen, A., Akinyemi, R. O., Hase, Y., Firbank, M. J., Ndung'u, M. N., Foster, V., et al. (2016). Frontal white matter hyperintensities, clasmatodendrosis and gliovascular abnormalities in ageing and post-stroke dementia. *Brain* 139, 242–258. doi: 10.1093/brain/awv328

Choudhury, G. R., and Ding, S. (2016). Reactive astrocytes and therapeutic potential in focal ischemic stroke. *Neurobiol. Dis* 85, 234–244. doi: 10.1016/j.nbd.2015.05.003

Chu, K., Lee, S. T., Sinn, D. I., Ko, S. Y., Kim, E. H., Kim, J. M., et al. (2007). Pharmacological induction of ischemic tolerance by glutamate transporter-1 (EAAT2) upregulation. *Stroke* 38, 177–182. doi: 10.1161/01.STR.0000252091.36912.65

Cirillo, C., Capoccia, E., Iuvone, T., Cuomo, R., Sarnelli, G., Steardo, L., et al. (2015). S100B inhibitor pentamidine attenuates reactive gliosis and reduces neuronal loss in a mouse model of Alzheimer's disease. *Biomed. Res. Int.* 2015, 11. doi: 10.1155/2015/508342

Clark, W. M., and Lutsep, H. L. (2001). Potential of anticytokine therapies in central nervous system ischaemia. *Expert. Opin. Biol. Ther.* 1, 227–237. doi: 10.1517/14712598.1.2.227

Colombo, E., and Farina, C. (2016). Astrocytes: key regulators of neuroinflammation. *Trends Immunol.* 37, 608–620. doi: 10.1016/j.it.2016.06.006

Colton, C. K., Kong, Q., Lai, L., Zhu, M. X., Seyb, K. I., Cuny, G. D., et al. (2010). Identification of translational activators of glial glutamate transporter EAAT2 through cell-based high-throughput screening: an approach to prevent excitotoxicity. *J. Biomol. Screen.* 15, 653–662. doi: 10.1177/1087057110370998

Combs, C. K., Johnson, D. E., Karlo, J. C., Cannady, S. B., and Landreth, G. E. (2000). Inflammatory mechanisms in Alzheimer's disease: inhibition of beta-amyloid-stimulated proinflammatory responses and neurotoxicity by PPARgamma agonists. *J. Neurosci.* 20, 558–567. doi: 10.1523/JNEUROSCI.20-02-00558.2000

Crunelli, V., Carmignoto, G., and Steinhauser, C. (2015). Novel astrocyte targets: new avenues for the therapeutic treatment of epilepsy. *Neuroscientist* 21, 62–83. doi: 10.1177/1073858414523320

Cuevas, E., Limon, D., Perez-Severiano, F., Diaz, A., Ortega, L., Zenteno, E., et al. (2009). Antioxidant effects of epicatechin on the hippocampal toxicity caused by amyloid-beta 25-35 in rats. *Eur. J. Pharmacol.* 616, 122–127. doi: 10.1016/j.ejphar.2009.06.013

De Nuccio, C., Bernardo, A., Cruciani, C., De Simone, R., Visentin, S., and Minghetti, L. (2015). Peroxisome proliferator activated receptor-gamma agonists protect oligodendrocyte progenitors against tumor necrosis factor-alpha-induced damage: effects on mitochondrial functions and differentiation. *Exp. Neurol.* 271, 506–514. doi: 10.1016/j.expneurol.2015.07.014

De Zeeuw, C. I., and Hoogland, T. M. (2015). Reappraisal of Bergmann glial cells as modulators of cerebellar circuit function. *Front. Cell Neurosci.* 9, 246. doi: 10.3389/fncel.2015.00246

Ding, S. H., Wang, T. N., Cui, W. J., and Haydon, P. G. (2009). Photothrombosis ischemia stimulates a sustained astrocytic Ca2+ signaling *in vivo. Glia* 57, 767–776. doi: 10.1002/glia.20804

Dong, Y. S., and Benveniste, E. N. (2001). Immune function of astrocytes. *Glia* 36, 180–190. doi: 10.1002/glia.1107

Farfara, D., Lifshitz, V., and Frenkel, D. (2008). Neuroprotective and neurotoxic properties of glial cells in the pathogenesis of Alzheimer's disease. *J. Cell Mol. Med.* 12, 762–780. doi: 10.1111/j.1582-4934.2008.00314.x

Finsterwald, C., Magistretti, P. J., and Lengacher, S. (2015). Astrocytes: new targets for the treatment of neurodegenerative diseases. *Curr. Pharm. Des.* 21, 3570–3581. doi: 10.2174/1381612821666150710144502

Fisher, R. S., Van Emde Boas, W., Blume, W., Elger, C., Genton, P., Lee, P., et al. (2005). Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia* 46, 470–472. doi: 10.1111/j.0013-9580.2005.66104.x

French, J. A., and Gazzola, D. M. (2011). New generation antiepileptic drugs: what do they offer in terms of improved tolerability and safety? *Ther. Adv. Drug Saf.* 2, 141–158. doi: 10.1177/2042098611411127

Frizzo, M. E., Dall'onder, L. P., Dalcin, K. B., and Souza, D. O. (2004). Riluzole enhances glutamate uptake in rat astrocyte cultures. *Cell Mol. Neurobiol.* 24, 123–128. doi: 10.1023/B:CEMN.0000012717.37839.07

Gajardo-Gomez, R., Labra, V. C., Maturana, C. J., Shoji, K. F., Santibanez, C. A., Saez, J. C., et al. (2017). Cannabinoids prevent the amyloid beta-induced activation of astroglial hemichannels: a neuroprotective mechanism. *Glia* 65, 122–137. doi: 10.1002/glia.23080

Garwood, C., Ratcliffe, L., Simpson, J., Heath, P., Ince, P., and Wharton, S. (2017). Astrocytes in Alzheimer's disease and other age-associated dementias: a supporting player with a central role. *Neuropathol. Appl. Neurobiol.* 43, 281–298. doi: 10.1111/nan.12338

Giralt, A., Friedman, H., Caneda-Ferron, B., Urban, N., Moreno, E., Rubio, N., et al. (2010). BDNF regulation under GFAP promoter provides engineered astrocytes as a new approach for long-term protection in Huntington's disease. *Gene Ther.* 17, 1294. doi: 10.1038/gt.2010.71

Giri, R., Shen, Y., Stins, M., Du Yan, S., Schmidt, A. M., Stern, D., et al. (2000). β-Amyloid-induced migration of monocytes across human brain endothelial cells involves RAGE and PECAM-1. *Am. J. Physiol. Cell Physiol.* 279, C1772–C1781. doi: 10.1152/ajpcell.2000.279.6.C1772

Goldberg, E. M., and Coulter, D. A. (2013). Mechanisms of epileptogenesis: a convergence on neural circuit dysfunction. *Nat. Rev. Neurosci.* 14, 337–349. doi: 10.1038/nrn3482

Gomez-Gonzalo, M., Losi, G., Chiavegato, A., Zonta, M., Cammarota, M., Brondi, M., et al. (2010). An excitatory loop with astrocytes contributes to drive neurons to seizure threshold. *PLoS Biol.* 8, e1000352. doi: 10.1371/journal.pbio.1000352

González-Reyes, R. E., Nava-Mesa, M. O., Vargas-Sánchez, K., Ariza-Salamanca, D., and Mora-Muñoz, L. (2017). Involvement of astrocytes in Alzheimer's disease from a neuroinflammatory and oxidative stress perspective. *Front. Mol. Neurosci.* 10, 427. doi: 10.3389/fnmol.2017.00427

Haughey, N. J., and Mattson, M. P. (2003). Alzheimer's amyloid β-peptide enhances ATP/gap junction-mediated calcium-wave propagation in astrocytes. *Neuromolecular Med.* 3, 173–180. doi: 10.1385/NMM:3:3:173

Hong, Y., Zhao, T., Li, X.-J., and Li, S. (2016). Mutant huntingtin impairs BDNF release from astrocytes by disrupting conversion of Rab3a-GTP into Rab3a-GDP. *J. Neurosci.* 36, 8790–8801. doi: 10.1523/JNEUROSCI.0168-16.2016

Hong, Y., Zhao, T., Li, X.-J., and Li, S. (2017). Mutant huntingtin inhibits αB-crystallin expression and impairs exosome secretion from astrocytes. *J. Neurosci.* 37, 9550–9563. doi: 10.1523/JNEUROSCI.1418-17.2017

Huang, X., Zhang, H., Yang, J., Wu, J., Mcmahon, J., Lin, Y., et al. (2010). Pharmacological inhibition of the mammalian target of rapamycin pathway suppresses acquired epilepsy. *Neurobiol. Dis* 40, 193–199. doi: 10.1016/j.nbd.2010.05.024

Ibrahim, N. F., Yanagisawa, D., Durani, L. W., Hamezah, H. S., Damanhuri, H. A., Wan Ngah, W. Z., et al. (2017). Tocotrienol-Rich fraction modulates amyloid pathology and improves cognitive function in AbetaPP/PS1 mice. *J. Alzheimers Dis.* 55, 597–612. doi: 10.3233/JAD-160685

Jansen, A. H., Van Hal, M., Op Den Kelder, I. C., Meier, R. T., De Ruiter, A. A., Schut, M. H., et al. (2017). Frequency of nuclear mutant huntingtin inclusion formation in neurons and glia is cell-type-specific. *Glia* 65, 50–61. doi: 10.1002/glia.23050

Jaradat, M. S., Wongsud, B., Phornchirasilp, S., Rangwala, S. M., Shams, G., Sutton, M., et al. (2001). Activation of peroxisome proliferator-activated receptor isoforms and inhibition of prostaglandin H(2) synthases by ibuprofen, naproxen, and indomethacin. *Biochem. Pharmacol.* 62, 1587–1595. doi: 10.1016/S0006-2952(01)00822-X

Jeong, J. C., Yoon, C. H., Lee, W. H., Park, K. K., Chang, Y. C., Choi, Y. H., et al. (2005). Effects of Bambusae concretio Salicea (Chunchukhwang) on amyloid beta-induced cell toxicity and antioxidative enzymes in cultured rat neuronal astrocytes. *J. Ethnopharmacol.* 98, 259–266. doi: 10.1016/j.jep.2004.12.034

Ji, Y. F., Xu, S. M., Zhu, J., Wang, X. X., and Shen, Y. (2011). Insulin increases glutamate transporter GLT1 in cultured astrocytes. *Biochem. Biophys. Res. Commun.* 405, 691–696. doi: 10.1016/j.bbrc.2011.01.105

Jiang, R., Diaz-Castro, B., Looger, L. L., and Khakh, B. S. (2016). Dysfunctional calcium and glutamate signaling in striatal astrocytes from Huntington's disease model mice. *J. Neurosci.* 36, 3453–3470. doi: 10.1523/JNEUROSCI.3693-15.2016

Jo, S., Yarishkin, O., Hwang, Y. J., Chun, Y. E., Park, M., Woo, D. H., et al. (2014). GABA from reactive astrocytes impairs memory in mouse models of Alzheimer's disease. *Nat. Med.* 20, 886–896. doi: 10.1038/nm.3639

Justicia, C., Perez-Asensio, F. J., Burguete, M. C., Salom, J. B., and Planas, A. M. (2001). Administration of transforming growth factor-alpha reduces infarct volume after transient focal cerebral ischemia in the rat. *J. Cereb. Blood Flow Metab.* 21, 1097–1104. doi: 10.1097/00004647-200109000-00007

Khakh, B. S., Beaumont, V., Cachope, R., Munoz-Sanjuan, I., Goldman, S. A., and Grantyn, R. (2017). Unravelling and exploiting astrocyte dysfunction in Huntington's disease. *Trends Neurosci.* 40, 422–437. doi: 10.1016/j.tins.2017.05.002

Khakh, B. S., and Sofroniew, M. V. (2014). Astrocytes and Huntington's disease. *ACS Chem. Neurosci.* 5, 494–496. doi: 10.1021/cn500100r

Khakh, B. S., and Sofroniew, M. V. (2015). Diversity of astrocyte functions and phenotypes in neural circuits. *Nat. Neurosci.* 18, 942. doi: 10.1038/nn.4043

Kim, J. K., and Lee, J. H. (2019). Mechanistic target of rapamycin pathway in epileptic disorders. *J. Korean Neurosurg. Soc.* 62, 272–287. doi: 10.3340/jkns.2019.0027

Kim, J. S. (1996). Cytokines and adhesion molecules in stroke and related diseases. *J. Neurol. Sci.* 137, 69–78. doi: 10.1016/0022-510X(95)00338-3

Kim, Y. E., Hwang, C. J., Lee, H. P., Kim, C. S., Son, D. J., Ham, Y. W., et al. (2017). Inhibitory effect of punicalagin on lipopolysaccharide-induced neuroinflammation, oxidative stress and memory impairment *via* inhibition of nuclear factor-kappaB. *Neuropharmacology* 117, 21–32. doi: 10.1016/j.neuropharm.2017.01.025

Kitazawa, M., Cheng, D., Tsukamoto, M. R., Koike, M. A., Wes, P. D., Vasilevko, V., et al. (2011). Blocking IL-1 signaling rescues cognition, attenuates tau pathology, and restores neuronal beta-catenin pathway function in an Alzheimer's disease model. *J. Immunol.* 187, 6539–6549. doi: 10.4049/jimmunol.1100620

Kong, Q., Chang, L. C., Takahashi, K., Liu, Q., Schulte, D. A., Lai, L., et al. (2014). Small-molecule activator of glutamate transporter EAAT2 translation provides neuroprotection. *J. Clin. Invest.* 124, 1255–1267. doi: 10.1172/JCI66163

Kwan, P., and Brodie, M. J. (2000). Early identification of refractory epilepsy. *N. Engl. J. Med.* 342, 314–319. doi: 10.1056/NEJM200002033420503

Lee, E. S., Sidoryk, M., Jiang, H., Yin, Z., and Aschner, M. (2009). Estrogen and tamoxifen reverse manganese-induced glutamate transporter impairment in astrocytes. *J. Neurochem.* 110, 530–544. doi: 10.1111/j.1471-4159.2009.06105.x

Li, J. M., Zhang, Y., Tang, L., Chen, Y. H., Gao, Q., Bao, M. H., et al. (2016). Effects of triptolide on hippocampal microglial cells and astrocytes in the APP/PS1 double transgenic mouse model of Alzheimer's disease. *Neural Regen. Res.* 11, 1492–1498. doi: 10.4103/1673-5374.191224

Li, L., Zhang, H., Varrin-Doyer, M., Zamvil, S. S., and Verkman, A. S. (2011). Proinflammatory role of aquaporin-4 in autoimmune neuroinflammation. *FASEB J.* 25, 1556–1566. doi: 10.1096/fj.10-177279

Libro, R., Giacoppo, S., Soundara Rajan, T., Bramanti, P., and Mazzon, E. (2016). Natural Phytochemicals in the treatment and prevention of dementia: an overview. *Molecules* 21, 518. doi: 10.3390/molecules21040518

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., Schirmer, L., et al. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* 541, 481. doi: 10.1038/nature21029

Lim, D., Iyer, A., Ronco, V., Grolla, A. A., Canonico, P. L., Aronica, E., et al. (2013). Amyloid beta deregulates astroglial mGluR5-mediated calcium signaling *via* calcineurin and Nf-kB. *Glia* 61, 1134–1145. doi: 10.1002/glia.22502

Liu, Z., and Chopp, M. (2016). Astrocytes, therapeutic targets for neuroprotection and neurorestoration in ischemic stroke. *Prog. Neurobiol.* 144, 103–120. doi: 10.1016/j.pneurobio.2015.09.008

Luo, C., Yi, B., Fan, W., Chen, K., Gui, L., Chen, Z., et al. (2011). Enhanced angiogenesis and astrocyte activation by ecdysterone treatment in a focal cerebral ischemia rat model. *Acta Neurochir. Suppl.* 110, 151–155. doi: 10.1007/978-3-7091-0353-1_26

Mackic, J. B., Stins, M., Mccomb, J. G., Calero, M., Ghiso, J., Kim, K. S., et al. (1998). Human blood-brain barrier receptors for Alzheimer's amyloid-beta 1-40. Asymmetrical binding, endocytosis, and transcytosis at the apical side of brain microvascular endothelial cell monolayer. *J. Clin. Invest.* 102, 734–743. doi: 10.1172/JCI2029

Mandrekar-Colucci, S., Karlo, J. C., and Landreth, G. E. (2012). Mechanisms underlying the rapid peroxisome proliferator-activated receptor-gamma-mediated amyloid clearance and reversal of cognitive deficits in a murine model of Alzheimer's disease. *J. Neurosci.* 32, 10117–10128. doi: 10.1523/JNEUROSCI.5268-11.2012

Masliah, E., Hansen, L., Alford, M., Deteresa, R., and Mallory, M. (1996). Deficient glutamate tranport is associated with neurodegeneration in Alzheimer's disease. *Ann. Neurol.* 40, 759–766. doi: 10.1002/ana.410400512

Medvedeva, Y. V., Lin, B., Shuttleworth, C. W., and Weiss, J. H. (2009). Intracellular Zn2+ accumulation contributes to synaptic failure, mitochondrial depolarization, and cell death in an acute slice oxygen–glucose deprivation model of ischemia. *J. Neurosci.* 29, 1105–1114. doi: 10.1523/JNEUROSCI.4604-08.2009

Merienne, N., Delzor, A., Viret, A., Dufour, N., Rey, M., Hantraye, P., et al. (2015). Gene transfer engineering for astrocyte-specific silencing in the CNS. *Gene Ther.* 22, 830–839. doi: 10.1038/gt.2015.54

Merlini, M., Meyer, E. P., Ulmann-Schuler, A., and Nitsch, R. M. (2011). Vascular β-amyloid and early astrocyte alterations impair cerebrovascular function and cerebral metabolism in transgenic arcAβ mice. *Acta Neuropathol.* 122, 293–311. doi: 10.1007/s00401-011-0834-y

Monje, M. L., Toda, H., and Palmer, T. D. (2003). Inflammatory blockade restores adult hippocampal neurogenesis. *Science* 302, 1760–1765. doi: 10.1126/science.1088417

Mosconi, L., Sorbi, S., De Leon, M. J., Li, Y., Nacmias, B., Myoung, P. S., et al. (2006). Hypometabolism exceeds atrophy in presymptomatic early-onset familial Alzheimer's disease. *J. Nucl. Med.* 47, 1778–1786.

Ouyang, Y. B., Volobouéva, L. A., Xu, L. J., and Giffard, R. G. (2007). Selective dysfunction of hippocampal CA1 astrocytes contributes to delayed neuronal damage after transient forebrain ischemia. *J. Neurosci.* 27, 4253–4260. doi: 10.1523/JNEUROSCI.0211-07.2007

Ouyang, Y. B., Xu, L., Lu, Y., Sun, X., Yue, S., Xiong, X. X., et al. (2013). Astrocyte-enriched miR-29a targets PUMA and reduces neuronal vulnerability to forebrain ischemia. *Glia* 61, 1784–1794. doi: 10.1002/glia.22556

Ouyang, Y. B., Xu, L., Yue, S., Liu, S., and Giffard, R. G. (2014). Neuroprotection by astrocytes in brain ischemia: importance of microRNAs. *Neurosci. Lett.* 565, 53–58. doi: 10.1016/j.neulet.2013.11.015

Pandolfo, M. (2011). Genetics of epilepsy. *Semin. Neurol.* 31, 506–518. doi: 10.1055/s-0031-1299789

Parsons, M. P., Vanni, M. P., Woodard, C. L., Kang, R., Murphy, T. H., and Raymond, L. A. (2016). Real-time imaging of glutamate clearance reveals normal striatal uptake in Huntington disease mouse models. *Nat. Commun.* 7, 11251. doi: 10.1038/ncomms11251

Patel, N. S., Paris, D., Mathura, V., Quadros, A. N., Crawford, F. C., and Mullan, M. J. (2005). Inflammatory cytokine levels correlate with amyloid load in transgenic mouse models of Alzheimer's disease. *J. Neuroinflammation* 2, 9. doi: 10.1186/1742-2094-2-9

Peteri, U.-K., Niukkanen, M., and Castrén, M. L. (2019). Astrocytes in neuropathologies affecting the frontal cortex. *Front. Cell Neurosci.* 13, 44. doi: 10.3389/fncel.2019.00044

Prevot, V., Dehouck, B., Sharif, A., Ciofi, P., Giacobini, P., and Clasadonte, J. (2018). The versatile tanycyte: a hypothalamic integrator of reproduction and energy metabolism. *Endocr. Rev.* 39, 333–368. doi: 10.1210/er.2017-00235

Rehman, S. U., Shah, S. A., Ali, T., Chung, J. I., and Kim, M. O. (2017). Anthocyanins reversed D-galactose-induced oxidative stress and neuroinflammation mediated cognitive impairment in adult rats. *Mol. Neurobiol.* 54, 255–271. doi: 10.1007/s12035-015-9604-5

Reick, C., Ellrichmann, G., Tsai, T., Lee, D.-H., Wiese, S., Gold, R., et al. (2016). Expression of brain-derived neurotrophic factor in astrocytes-beneficial effects of glatiramer acetate in the R6/2 and YAC128 mouse models of Huntington's disease. *Exp. Neurol.* 285, 12–23. doi: 10.1016/j.expneurol.2016.08.012

Reiman, E. M., Chen, K., Alexander, G. E., Caselli, R. J., Bandy, D., Osborne, D., et al. (2004). Functional brain abnormalities in young adults at genetic risk for late-onset Alzheimer's dementia. *Proc. Natl. Acad. Sci.* 101, 284–289. doi: 10.1073/pnas.2635903100

Roberts, R. C., Roche, J. K., and Mccullumsmith, R. E. (2014). Localization of excitatory amino acid transporters EAAT1 and EAAT2 in human postmortem cortex: a light and electron microscopic study. *Neuroscience* 277, 522–540. doi: 10.1016/j.neuroscience.2014.07.019

Rossi, D., and Volterra, A. (2009). Astrocytic dysfunction: insights on the role in neurodegeneration. *Brain Res. Bull.* 80, 224–232. doi: 10.1016/j.brainresbull.2009.07.012

Rothstein, J. D., Patel, S., Regan, M. R., Haenggeli, C., Huang, Y. H., Bergles, D. E., et al. (2005). Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature* 433, 73–77. doi: 10.1038/nature03180

Russo, E., Follesa, P., Citraro, R., Camastra, C., Donato, A., Isola, D., et al. (2014). The mTOR signaling pathway and neuronal stem/progenitor cell proliferation in the hippocampus are altered during the development of absence epilepsy in a genetic animal model. *Neurol. Sci.* 35, 1793–1799. doi: 10.1007/s10072-014-1842-1

Sassone, J., Papadimitriou, E., and Thomaidou, D. (2018). Regenerative Approaches in huntington's disease: from mechanistic insights to therapeutic protocols. *Front. Neurosci.* 12, 800. doi: 10.3389/fnins.2018.00800

Sastre, M., and Gentleman, S. M. (2010). NSAIDs: how they work and their prospects as therapeutics in Alzheimer's disease. *Front. Aging Neurosci.* 2. doi: 10.3389/fnagi.2010.00020

Saura, J., Luque, J. M., Cesura, A. M., Da Prada, M., Chan-Palay, V., Huber, G., et al. (1994). Increased monoamine oxidase B activity in plaque-associated astrocytes of Alzheimer brains revealed by quantitative enzyme radioautography. *Neuroscience* 62, 15–30. doi: 10.1016/0306-4522(94)90311-5

Sharif, A., Legendre, P., Prevot, V., Allet, C., Romao, L., Studler, J. M., et al. (2007). Transforming growth factor alpha promotes sequential conversion of mature astrocytes into neural progenitors and stem cells. *Oncogene* 26, 2695–2706. doi: 10.1038/sj.onc.1210071

Shen, Y., He, P., Fan, Y. Y., Zhang, J. X., Yan, H. J., Hu, W. W., et al. (2010). Carnosine protects against permanent cerebral ischemia in histidine decarboxylase knockout mice by reducing glutamate excitotoxicity. *Free Radic. Biol. Med.* 48, 727–735. doi: 10.1016/j.freeradbiomed.2009.12.021

Sherwood, C. C., Stimpson, C. D., Raghanti, M. A., Wildman, D. E., Uddin, M., Grossman, L. I., et al. (2006). Evolution of increased glia–neuron ratios in the human frontal cortex. *Proc. Natl. Acad. Sci.* 103, 13606–13611. doi: 10.1073/pnas.0605843103

Shi, Y. C., Pan, T. M., and Liao, V. H. (2016). Monascin from Monascus-fermented products reduces oxidative stress and amyloid-beta toxicity *via* DAF-16/FOXO in Caenorhabditis elegans. *J. Agric. Food Chem.* 64, 7114–7120. doi: 10.1021/acs.jafc.6b02779

Shin, J.-Y., Fang, Z.-H., Yu, Z.-X., Wang, C.-E., Li, S.-H., and Li, X.-J. (2005). Expression of mutant huntingtin in glial cells contributes to neuronal excitotoxicity. *J. Cell Biol.* 171, 1001–1012. doi: 10.1083/jcb.200508072

Sofroniew, M. (2000). Astrocyte failure as a cause of CNS dysfunction. *Mol. Psyciatry* 5 (3), 230–232. doi: 10.1038/sj.mp.4000753

Sohanaki, H., Baluchnejadmojarad, T., Nikbakht, F., and Roghani, M. (2016). Pelargonidin improves memory deficit in amyloid beta25-35 rat model of Alzheimer's disease by inhibition of glial activation, cholinesterase, and oxidative stress. *Biomed. Pharmacother.* 83, 85–91. doi: 10.1016/j.biopha.2016.06.021

Steinhauser, C., and Seifert, G. (2012). "Astrocyte dysfunction in epilepsy," in *Jasper's Basic Mechanisms of the Epilepsies*. Eds. Th, J.L. Noebels, M. Avoli, M. A. Rogawski, R. W. Olsen, and A. V. Delgado-Escueta(Bethesda (MD): National Center for Biotechnology Information (US)).

Steinhauser, C., Grunnet, M., and Carmignoto, G. (2016). Crucial role of astrocytes in temporal lobe epilepsy. *Neuroscience* 323, 157–169. doi: 10.1016/j.neuroscience.2014.12.047

Steinhauser, C., Seifert, G., and Bedner, P. (2012). Astrocyte dysfunction in temporal lobe epilepsy: K+ channels and gap junction coupling. *Glia* 60, 1192–1202. doi: 10.1002/glia.22313

Stewart, C. R., Stuart, L. M., Wilkinson, K., Van Gils, J. M., Deng, J., Halle, A., et al. (2010). CD36 ligands promote sterile inflammation through assembly of a toll-like receptor 4 and 6 heterodimer. *Nat. Immunol.* 11, 155–161. doi: 10.1038/ni.1836

Stoll G., Jander S., and Schroeter M. (1998). Inflammation and glial responses ischemic brain lessions. *Prog. Neurobiol.* 56 (2), 149–171. doi: 10.1016/S0301-0082(98)00034-3

Stroth, N., and Svenningsson, P. (2015). S100B interacts with the serotonin 5-HT7 receptor to regulate a depressive-like behavior. *Eur. Neuropsychopharmacol.* 25, 2372–2380. doi: 10.1016/j.euroneuro.2015.10.003

Taguchi, A., Blood, D. C., Del Toro, G., Canet, A., Lee, D. C., Qu, W., et al. (2000). Blockade of RAGE–amphoterin signalling suppresses tumour growth and metastases. *Nature* 405, 354. doi: 10.1038/35012626

Talantova, M., Sanz-Blasco, S., Zhang, X., Xia, P., Akhtar, M. W., Okamoto, S.-I., et al. (2013). Aβ induces astrocytic glutamate release, extrasynaptic NMDA receptor activation, and synaptic loss. *Proc. Natl. Acad. Sci.* 110, E2518–E2527. doi: 10.1073/pnas.1306832110

Tanaka, E., Yamamoto, S., Kudo, Y., Mihara, S., and Higashi, H. (1997). Mechanisms underlying the rapid depolarization produced by deprivation of oxygen and glucose in rat hippocampal CA1 neurons *in vitro*. *J. Neurophysiol.* 78, 891–902. doi: 10.1152/jn.1997.78.2.891

Taverna, E., Götz, M., and Huttner, W. B. (2014). The cell biology of neurogenesis: toward an understanding of the development and evolution of the neocortex. *Annu. Rev. Cell Dev. Biol.* 30, 465–502. doi: 10.1146/annurev-cellbio-101011-155801

Tian, G., Kong, Q., Lai, L., Ray-Chaudhury, A., and Lin, C. L. G. (2010). Increased expression of cholesterol 24S-hydroxylase results in disruption of glial glutamate transporter EAAT2 association with lipid rafts: a potential role in Alzheimer's disease. *J. Neurochem.* 113, 978–989. doi: 10.1111/j.1471-4159.2010.06661.x

Tong, X., Ao, Y., Faas, G. C., Nwaobi, S. E., Xu, J., Haustein, M. D., et al. (2014). Astrocyte Kir4. 1 ion channel deficits contribute to neuronal dysfunction in Huntington's disease model mice. *Nat. Neurosci.* 17, 694. doi: 10.1038/nn.3691

Tweedie, D., Ferguson, R. A., Fishman, K., Frankola, K. A., Van Praag, H., Holloway, H. W., et al. (2012). Tumor necrosis factor-alpha synthesis inhibitor 3,6'-dithiothalidomide attenuates markers of inflammation, Alzheimer pathology and behavioral deficits in animal models of neuroinflammation and Alzheimer's disease. *J. Neuroinflammation* 9, 106. doi: 10.1186/1742-2094-9-106

Van Der Worp, H. B., and Van Gijn, J. (2007). Clinical practice. Acute ischemic stroke. *N. Engl. J. Med.* 357, 572–579. doi: 10.1056/NEJMcp072057

Vasile, F., Dossi, E., and Rouach, N. (2017). Human astrocytes: structure and functions in the healthy brain. *Brain Struct. Funct.* 222, 2017–2029. doi: 10.1007/s00429-017-1383-5

Verma, R., Mishra, V., Sasmal, D., and Raghubir, R. (2010). Pharmacological evaluation of glutamate transporter 1 (GLT-1) mediated neuroprotection following cerebral ischemia/reperfusion injury. *Eur. J. Pharmacol.* 638, 65–71. doi: 10.1016/j.ejphar.2010.04.021

Von Bernhardi, R., Tichauer, J. E., and Eugenin, J. (2010). Aging-dependent changes of microglial cells and their relevance for neurodegenerative disorders. *J. Neurochem.* 112, 1099–1114. doi: 10.1111/j.1471-4159.2009.06537.x

Wang, G., Chen, L., Pan, X., Chen, J., Wang, L., Wang, W., et al. (2016). The effect of resveratrol on beta amyloid-induced memory impairment involves inhibition of phosphodiesterase-4 related signaling. *Oncotarget* 7, 17380–17392. doi: 10.18632/oncotarget.8041

Wang, J., Shi, Y., Zhang, L., Zhang, F., Hu, X., Zhang, W., et al. (2014). Omega-3 polyunsaturated fatty acids enhance cerebral angiogenesis and provide long-term protection after stroke. *Neurobiol. Dis* 68, 91–103. doi: 10.1016/j.nbd.2014.04.014

Wang, L., Wang, M., Hu, J., Shen, W., Hu, J., Yao, Y., et al. (2017). Protective effect of 3H-1, 2-dithiole-3-thione on cellular model of Alzheimer's disease involves Nrf2/ARE signaling pathway. *Eur. J. Pharmacol.* 795, 115–123. doi: 10.1016/j.ejphar.2016.12.013

Wetherington, J., Serrano, G., and Dingledine, R. (2008). Astrocytes in the epileptic brain. *Neuron* 58, 168–178. doi: 10.1016/j.neuron.2008.04.002

White, H. S., Smith, M. D., and Wilcox, K. S. (2007). Mechanisms of action of antiepileptic drugs. *Int. Rev. Neurobiol.* 81, 85–110. doi: 10.1016/S0074-7742(06)81006-8

Wick, M., Hurteau, G., Dessev, C., Chan, D., Geraci, M. W., Winn, R. A., et al. (2002). Peroxisome proliferator-activated receptor-gamma is a target of nonsteroidal anti-inflammatory drugs mediating cyclooxygenase-independent inhibition of lung cancer cell growth. *Mol. Pharmacol.* 62, 1207–1214. doi: 10.1124/mol.62.5.1207

Wilkinson, K., and El Khoury, J. (2012). Microglial scavenger receptors and their roles in the pathogenesis of Alzheimer's disease. *Int. J. Alzheimers Dis.* 2012, 10 doi: 10.1155/2012/489456

Wyss-Coray, T., and Rogers, J. (2012). Inflammation in Alzheimer disease-a brief review of the basic science and clinical literature. *Cold Spring Harb. Perspect. Med.* 2, a006346. doi: 10.1101/cshperspect.a006346

Xing, X., Chang, L. C., Kong, Q., Colton, C. K., Lai, L., Glicksman, M. A., et al. (2011). Structure-activity relationship study of pyridazine derivatives as glutamate transporter EAAT2 activators. *Bioorg. Med. Chem. Lett.* 21, 5774–5777. doi: 10.1016/j.bmcl.2011.08.009

Xiu, J., Nordberg, A., Zhang, J.-T., and Guan, Z.-Z. (2005). Expression of nicotinic receptors on primary cultures of rat astrocytes and up-regulation of the α7, α4 and β2 subunits in response to nanomolar concentrations of the β-amyloid peptide1–42. *Neurochem. Int.* 47, 281–290. doi: 10.1016/j.neuint.2005.04.023

Yan, S. D., Schmidt, A. M., Anderson, G. M., Zhang, J., Brett, J., Zou, Y. S., et al. (1994). Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J. Biol. Chem.* 269, 9889–9897.

Yi, J.-H., and Hazell, A. S. (2006). Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury. *Neurochem. Int.* 48, 394–403. doi: 10.1016/j.neuint.2005.12.001

Yuan, T. F., and Shan, C. (2014). "Glial inhibition" of memory in Alzheimer's disease. *Sci. China Life Sci.* 57, 1238–1240. doi: 10.1007/s11427-014-4780-5

Zamanian, J. L., Xu, L. J., Foo, L. C., Nouri, N., Zhou, L., Giffard, R. G., et al. (2012). Genomic analysis of reactive astrogliosis. *J. Neurosci.* 32, 6391–6410. doi: 10.1523/JNEUROSCI.6221-11.2012

Zhang, X., Wan, J. Q., and Tong, X. P. (2018). Potassium channel dysfunction in neurons and astrocytes in Huntington's disease. *CNS Neurosci. Ther.* 24, 311–318. doi: 10.1111/cns.12804

Zhang, Z. G., and Chopp, M. (2009). Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic. *Lancet Neurol.* 8, 491–500. doi: 10.1016/S1474-4422(09)70061-4

Zhao, T., Hong, Y., Li, S., and Li, X.-J. (2016). Compartment-dependent degradation of mutant huntingtin accounts for its preferential accumulation in neuronal processes. *J. Neurosci.* 36, 8317–8328. doi: 10.1523/JNEUROSCI.0806-16.2016

Zhao, T., Hong, Y., Yin, P., Li, S., and Li, X.-J. (2017). Differential HspBP1 expression accounts for the greater vulnerability of neurons than astrocytes to misfolded proteins. *Proc. Natl. Acad. Sci.* 114, E7803–E7811. doi: 10.1073/pnas.1710549114

Zheng, W., Talley Watts, L., Holstein, D. M., Wewer, J., and Lechleiter, J. D. (2013). P2Y1R-initiated, IP3R-dependent stimulation of astrocyte mitochondrial metabolism reduces and partially reverses ischemic neuronal damage in mouse. *J. Cereb. Blood Flow Metab.* 33, 600–611. doi: 10.1038/jcbfm.2012.214

Zou, J., Wang, Y.-X., Dou, F.-F., Lü, H.-Z., Ma, Z.-W., Lu, P.-H., et al. (2010). Glutamine synthetase down-regulation reduces astrocyte protection against glutamate excitotoxicity to neurons. *Neurochem. Int.* 56, 577–584. doi: 10.1016/j.neuint.2009.12.021

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

frontiers in Pharmacology

MINI REVIEW
published: 21 November 2019
doi: 10.3389/fphar.2019.01346

Astrocyte-Derived Paracrine Signals: Relevance for Neurogenic Niche Regulation and Blood–Brain Barrier Integrity

Simona Federica Spampinato[1], Valeria Bortolotto[2], Pier Luigi Canonico[2], Maria Angela Sortino[1] and Mariagrazia Grilli[2]**

[1] Section of Pharmacology, Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy,
[2] Laboratory of Neuroplasticity, Department of Pharmaceutical Sciences, University of Piemonte Orientale, Novara, Italy

Astrocytes are essential for proper regulation of the central nervous system (CNS). Importantly, these cells are highly secretory in nature. Indeed they can release hundreds of molecules which play pivotal physiological roles in nervous tissues and whose abnormal regulation has been associated with several CNS disorders. In agreement with these findings, recent studies have provided exciting insights into the key contribution of astrocyte-derived signals in the pleiotropic functions of these cells in brain health and diseases. In the future, deeper analysis of the astrocyte secretome is likely to further increase our current knowledge on the full potential of these cells and their secreted molecules not only as active participants in pathophysiological events, but as pharmacological targets or even as therapeutics for neurological and psychiatric diseases. Herein we will highlight recent findings in our and other laboratories on selected molecules that are actively secreted by astrocytes and contribute in two distinct functions with pathophysiological relevance for the astroglial population: i) regulation of neural stem cells (NSCs) and their progeny within adult neurogenic niches; ii) modulation of the blood–brain barrier (BBB) integrity and function.

Keywords: astrocytes, blood–brain barrier, neural stem cells, neurogenesis, niche, paracrine signals, secretome

OPEN ACCESS

Edited by:
Stefania Ceruti,
University of Milan, Italy

Reviewed by:
Min-Yu Sun,
Washington University in St. Louis, United States
Carlos Spuch,
Instituto de Investigación Sanitaria Galicia Sur (IISGS), Spain

****Correspondence:***
Maria Angela Sortino
msortino@unict.it
Mariagrazia Grilli
mariagrazia.grilli@uniupo.it

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

Received: *02 September 2019*
Accepted: *24 October 2019*
Published: *21 November 2019*

Citation:
Spampinato SF, Bortolotto V, Canonico PL, Sortino MA and Grilli M (2019) Astrocyte-Derived Paracrine Signals: Relevance for Neurogenic Niche Regulation and Blood–Brain Barrier Integrity. Front. Pharmacol. 10:1346. doi: 10.3389/fphar.2019.01346

INTRODUCTION

Astrocytes are essential for brain homeostasis. They indeed support neurons both structurally and functionally by providing nutrients and neurotrophic factors, removing neurotransmitters and waste metabolites to ensure a homeostatic environment (Perez-Alvarez and Araque, 2013). Astrocytes regulate neurogenesis, axonal guidance, synaptogenesis (Allen and Lyons, 2018), as well as blood–brain barrier (BBB) function. Although still controversial, astrocytes may also release gliotransmitters to modulate synaptic transmission (Araque et al., 2014; Fiacco and McCarthy, 2018). Last but not least,

Abbreviations: ahNG, adult hippocampal neurogenesis; AJ, adherens junctions; aNG, adult neurogenesis; CCL, CC Chemokine Ligand; CXCL, C-X-C motif chemokine 12; DG, dentate gyrus; ECM, extracellular matrix; ICAM-1, intercellular adhesion molecule 1; IFNγ, interferon γ; IL-1, interleukin 1; IL-6, interleukin 6; LCN-2, lipocalin-2; NB, neuroblast; NPC, neural progenitor cell; NSC, neural stem cell; NVU, neurovascular unit; OB, olfactory bulb; PBMCs, peripheral blood mononuclear cells; SGZ, subgranular zone; SVZ, subventricular zone; TNFα, tumor necrosis factor α; TSP, thrombospondin; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

after brain injury, astrocytes are involved in neuroinflammatory responses in an attempt of repair and/or remodeling.

Astrocytes are highly secretory cells, with their secretome containing hundreds of molecules (Chen and Swanson, 2003; Dowell et al., 2009; Harada et al., 2015). Recent proteomic studies provided exciting insights into the contribution of astrocyte-derived signals in their pleiotropic functions in brain health and diseases (Jha et al., 2018). In this minireview, we will highlight recent findings on some molecules actively secreted by astrocytes and implicated in two specific functions, namely, regulation of neural stem cells (NSCs) and their progeny within adult neurogenic niches and modulation of BBB function. These apparently distant conditions are analyzed together as they share a strict dependence on astrocyte-secreted products.

ASTROCYTES AS KEY MODULATORS IN ADULT NEUROGENIC NICHES

The term adult neurogenesis (aNG) refers to the generation of new functionally integrated neurons in the adult brain. This peculiar form of neuroplasticity occurs in restricted areas of mammalian brain, the subventricular zone (SVZ) in the lateral ventricles and the subgranular zone (SGZ) in the hippocampal dentate gyrus (DG).

While the SVZ region is considered a poorly relevant neurogenic site in humans, neurogenesis occurring in the DG appears of physiological significance. Recently, the presence of thousands of adult-born neuroblasts (NBs) in the hippocampus of healthy people was described (Moreno-Jimenez et al., 2019). In this region, neural stem/progenitor cells (NSC/NPC) self-renew and give rise to transiently amplifying progenitor cells which, in turn, can generate NBs capable of terminal neuronal differentiation. Post-mitotic neuronal progeny can be functionally integrated as granule cells into the adult hippocampal circuitry (Bond et al., 2015; Kempermann et al., 2015). In recent years, adult hippocampal neurogenesis (ahNG) has attracted growing interest due to its potential involvement in cognition and memory, mood, and emotional behavior (Aimone et al., 2010; Eisch and Petrik, 2012; Aimone et al., 2014; Bortolotto et al., 2014). ahNG is profoundly dysregulated in several neuropsychiatric/neurodegenerative disorders opening to the possibility that it may participate in their pathophysiology or contribute to some associated symptoms, such as dementia and depressed mood (Grilli and Meneghini, 2012; Bortolotto and Grilli, 2016; Yun et al., 2016). Very recently, it has been reported that postmortem tissue from AD patients contained remarkably fewer DG NBs suggesting their degeneration in the disease (Moreno-Jimenez et al., 2019). This seminal paper confirmed previous key studies in the field (Spalding et al., 2013; Boldrini et al., 2018).

An important functional and anatomical concept in aNG is the "neurogenic niche," a permissive and instructive microenvironment which is crucial for preserving NSC functions, including their proliferative and differentiative properties (Ghosh, 2019). Although cell–cell contacts are relevant, paracrine signals originating from astrocytes within the niche appear very important. It was demonstrated that astrocytes are important neurogenic niche components which instruct NSC/NPC to adopt a neuronal fate (Song et al., 2002). Hence, the interest in the identity of astrocyte-secreted niche signals has been growing (Casse et al., 2018). We will now highlight key findings showing how astrocytes modulate aNG through release of different classes of secretory substances, as summarized in **Figure 1**.

Morphogens

Among the first candidate molecules identified for their role in aNG were morphogenic factors of the Wnt protein family. Several members, including Wnt3 and Wnt7, are expressed by hippocampal astrocytes together with Wnt receptors and Wnt/β-catenin signaling pathway components (Lie et al., 2005). Hippocampal niche astrocytes actively induce ahNG through secretion of Wnt proteins and activation of Wnt downstream signaling pathways. Overexpression of Wnt3 enhances neuronal differentiation, while blockade of Wnt signaling strongly reduces ahNG *in vivo* and *in vitro* (Lie et al., 2005). Moreno-Estelles demonstrated that Wnt7a released by astrocytes in the adult neurogenic niche is a key factor promoting NSC self-renewal (Moreno-Estelles et al., 2012).

Gliotransmitters

D-serine and Glutamate (Glu) were identified as molecules by which niche astrocytes regulate maturation, survival, and functional integration into local synaptic networks of adult-born neurons. To investigate the role of astrocytic exocytosis on aNG, SNAP Receptor protein (SNARE)-dependent exocytosis was genetically impaired in niche astrocytes (Sultan et al., 2015). Inhibition of vesicular release resulted in severely impaired integration and survival of newly generated hippocampal neurons, whereas developmentally born neurons appeared unaffected (Sultan et al., 2015). Adult-born neurons located within the territories of exocytosis-deficient astrocytes displayed reduced dendritic spine density and glutamatergic synaptic input, which correlated with decreased D-serine. Chronic administration of D-serine partially rescued defective phenotypes and restored N-methyl-D-aspartate (NMDA)-mediated synaptic transmission and dendritic maturation in mice with impaired astrocytic vesicular release (Sultan et al., 2015). The observation that rescue was only partial suggested that other molecules released by astrocytes could be important for maturation of adult-born hippocampal neurons. A critical role of vesicular Glu release from astrocytes was previously demonstrated in the SVZ where newly generated NBs migrate a long distance to reach their final destination, the olfactory bulb (OB). Platel et al. demonstrated that migrating NBs, which acquire functional NMDA receptor activity on their way to the OB, are ensheathed by astrocytes releasing glutamate in a Ca^{2+}-dependent manner (Platel et al., 2010). They showed that: i) increasing calcium in astrocytes induced NMDA receptor activity in NB; ii) blocking vesicular astrocytic release eliminated spontaneous NMDA receptor activity in NB; and iii) deletion of functional NMDA receptors in adult-born NB decreased generation and survival of newborn neurons in adult OB (Platel et al., 2010). Altogether, these findings correlate astrocyte-released Glu with generation, survival, and functional integration into local synaptic networks of adult-born OB neurons.

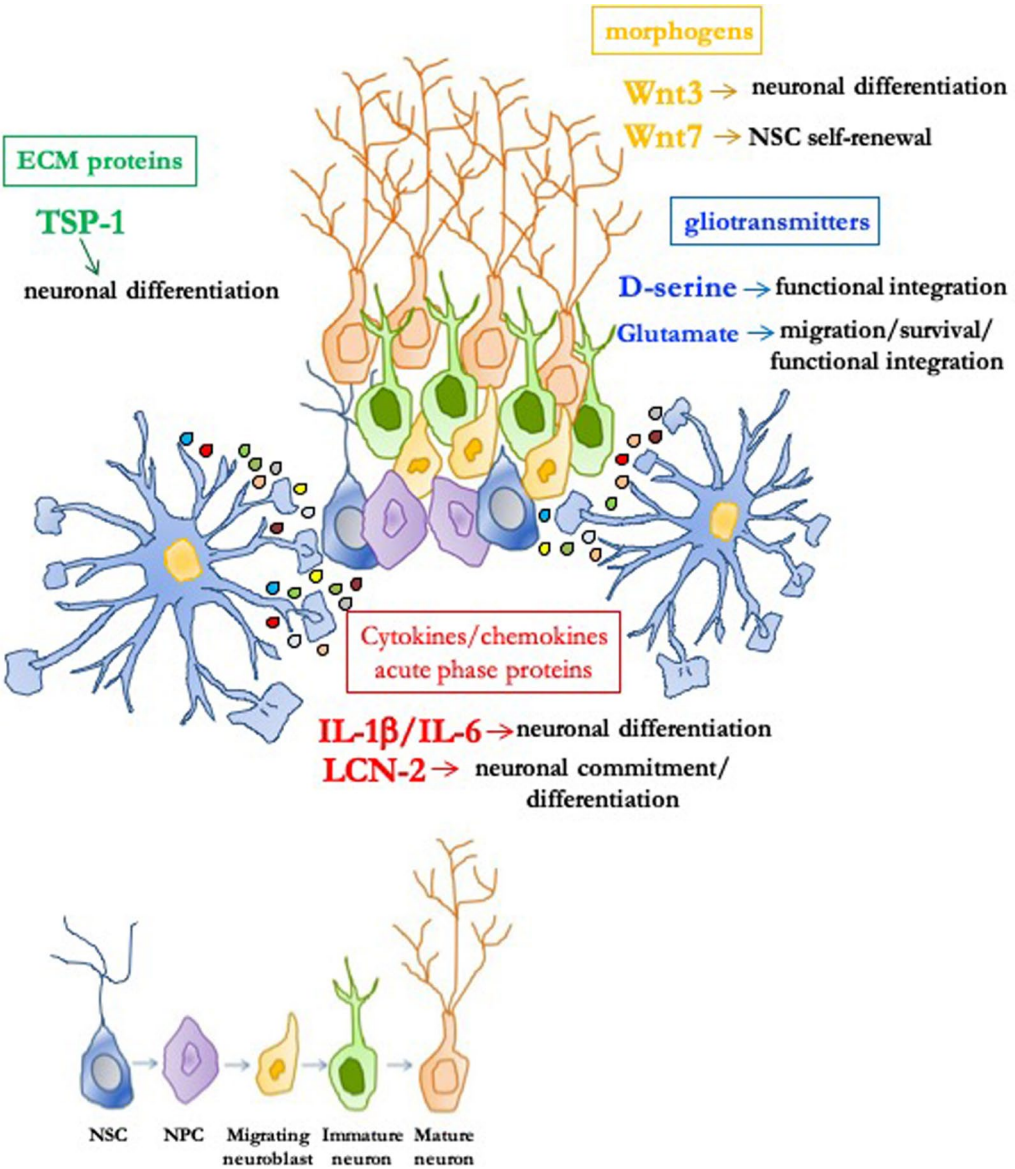

FIGURE 1 | Role of astrocyte-derived molecules in the adult neurogenic niche. In the permissive and instructive microenvironment of the neurogenic niche, astrocytes profoundly modulate adult neurogenesis through soluble signals. Neural stem/progenitor cells (NSC/NPC) self-renewal, neuronal commitment/differentiation, migration of neuroblasts, as well as survival and functional integration of newly born neurons can be affected by different classes of astrocytic-derived factors such as morphogens (i.e., Wnt3 and Wnt7), gliotransmitters (i.e., D-serine and glutamate), extracellular matrix (ECM) proteins [i.e., thrombospondin 1 (TSP-1)], and cytokines/chemokines/acute phase proteins [i.e., IL-1β, IL-6, and lipocalin-2 (LCN-2)].

Extracellular Matrix (ECM) Proteins

Several astrocyte-secreted ECM proteins modulate cellular functions. Usually these proteins are expressed at high levels during development and at lower levels in adulthood. Upon brain injury, their expression is upregulated, especially in reactive astrocytes, and they are often associated with CNS remodeling and synaptogenesis. Some ECM proteins play also an important role in the neurogenic microenvironment. The most investigated astrocyte-secreted matricellular proteins are thrombospondins (TSPs) which mediate cell–cell and cell–matrix interaction by binding other ECM components, membrane receptors, growth factors, and cytokines. TSP-1 represents a key astrocyte-derived pro-neurogenic factor which promotes neuronal differentiation of NSC (Lu and Kipnis, 2010). Adult TSP-1$^{-/-}$ mice exhibit reduced NSC proliferation and neuronal differentiation in both SVZ and SGZ (Lu and Kipnis, 2010). The voltage-gated calcium channel α2δ1 subunit was proposed to be a receptor which mediates TSP-1 synaptogenic effects (Eroglu et al., 2009). The α2δ1 subunit was also proven to be functionally expressed by adult hippocampal NPC and to mediate TSP-1 and pregabalin (an anticonvulsant/analgesic α2δ1 ligand) pro-neurogenic effects both *in vitro* and *in vivo* (Valente et al., 2012). These findings were further extended

in recent studies proposing a key role for nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling whose activation occurs in adult NSC *via* membrane receptors, including neurotransmitter receptors and α2δ1 (Meneghini et al., 2010; Bortolotto et al., 2017a; Bortolotto et al., 2019). NF-κB $p50^{-/-}$ mice exhibit strongly reduced ahNG *in vivo* (Denis-Donini et al., 2008) and *in vitro* (Meneghini et al., 2013; Valente et al., 2015; Bonini et al., 2016). Interestingly, TSP-1 promotes an increase in the percentage of newly formed neurons in wild type, but not in $p50^{-/-}$-derived ahNPC which have reduced α2δ1 expression levels (Cvijetic et al., 2017). Altogether, these data suggested that a disturbed astrocyte–NSC communication *via* TSP-1 may contribute to defects in ahNG in absence of p50.

Cytokines and Acute Phase Proteins

In contrast with the notion that inflammatory cytokines inhibit neuronal differentiation (Vallieres et al., 2002; Monje et al., 2003), IL-1β and IL-6, both highly expressed in neurogenic niches astrocytes, strongly promote NSC neuronal differentiation (Barkho et al., 2006). Lipocalin-2 (LCN-2) is an acute phase protein produced by and acting on astrocytes (Jha et al., 2015) which serves as "help-me" signal to activate astrocytes and microglia (Xing et al., 2014). Although its modulatory role in the CNS is not completely understood LCN-2 is commonly regarded as a deleterious signal (Ferreira et al., 2015) and a key target in regulating astrocyte reactivity. Indeed it has been demonstrated that knockdown of LCN-2 leads to reduced protein secretion from reactive astroglial cells, counteracting the perpetuation of inflammation in nearby astrocytes (Smith et al., 2018). LCN-2 is encoded by a NF-κB target gene (Uberti et al., 2000), and its expression is increased in the secretome of $p50^{-/-}$ astrocytes (Cvijetic et al., 2017; Bortolotto and Grilli, 2017b). Initially, based on these findings and its deleterious effects, our group hypothesized that overexpressed LCN-2 may contribute to impaired ahNG in $p50^{-/-}$ mice. To our surprise, LCN-2 promoted, in a concentration-dependent manner, neuronal differentiation of ahNPC. Under the same experimental conditions, LCN-2 had little effect on neuronal differentiation of $p50^{-/-}$ ahNPC which exhibited downregulation of the LCN-2 receptor 24p3R (Cvijetic et al., 2017). Altogether, these novel data proposed LCN-2 as a novel and unexpected astroglial-derived signal able to promote neuronal fate specification of ahNPC (Bortolotto and Grilli, 2017b). Recently, these findings were further extended by the demonstration that $LCN\text{-}2^{-/-}$ mice display deficits in proliferation and neuronal commitment of NSC and, in parallel, hippocampal dysfunction (Ferreira et al., 2018).

In summary, at present several astrocyte-derived signals which act as positive modulators of NSC and their progeny have been identified and characterized. Of note, little is currently known on soluble molecules of astrocytic origin which may exert negative effects on aNG. Anatomical and functional segregation along the hippocampal dorso-ventral axis is a well-established concept (Grilli et al., 1988; Tanti and Belzung, 2013), and marked differences in neurogenesis rate have been described in the dorsal compared to the ventral dentate gyrus (Piatti et al., 2011). It would be interesting to investigate whether subregional specificities in ahNG may also rely, at least in part, on different astrocyte-secreted molecules.

THE DUAL ROLE OF ASTROCYTIC-DERIVED FACTORS: FROM ENDOTHELIAL PROTECTION TO DISRUPTION OF BBB FUNCTION

The BBB is constituted by specialized endothelial cells, supported in their functions by astrocytes and pericytes, and is part of a more complex network, the neurovascular unit (NVU), that includes also microglia, neurons, and mast cells. Brain microvascular endothelial cells, the main anatomical BBB elements, express tight junctions (TJs) and adherens junctions (AJs) (Huber et al., 2001; Dejana and Giampietro, 2012), that allow a selective para- and transcellular movement of molecules and solutes across the barrier (Garg et al., 2008; Garcia et al., 2014). Trafficking through the BBB is regulated by specific transporters (Kastin and Pan, 2008), as well as by efflux pumps such as P-glycoprotein (P-gp) (Begley, 2004). The BBB contributes to make CNS a site of immune privilege, as low expression of adhesion molecules and tightness of cell-to-cell connections limit the access of pathogens and immune cells, preserving immune surveillance (Engelhardt and Ransohoff, 2005).

Astrocytes appear fundamental in BBB function. *In vitro*, barrier properties are lost in the absence of astrocytes (Ghazanfari and Stewart, 2001) and reestablished by astrocyte conditioned media or when astrocyte–endothelial cells contact is provided (Tao-Cheng et al., 1987; Neuhaus et al., 1991; Hayashi et al., 1997; Colgan et al., 2008). Further, endothelial cells derived from non-CNS districts, cultured in the presence of astrocytes or astrocyte-secreted factors, acquire BBB specific features, including expression of TJ or P-gp (Prat et al., 2001; Abbott et al., 2006).

Pericytes and radial glia, the major source of astrocyte precursors (McDermott et al., 2005), are essential in an early stage of barrier induction, whereas astrocytes play a major role later on, favoring barrier maturation and maintenance (Obermeier et al., 2013; Obermeier et al., 2016).

In pathological conditions, morphological changes in reactive astrocytes may induce loss of their interaction with endothelial cells (Alvarez et al., 2013). Depending on insult type, astrocytes undergo loss-of-function [e.g., failure of glutamate uptake (Broux et al., 2015)] and/or gain-of-function [production of a wide range of molecules including cytokines (Gimsa et al., 2013; Brambilla, 2019)]. All these events can lead to reduction or exacerbation of BBB damage. Herein we will analyze the crosstalk between astrocytes and endothelial cells in BBB function, focusing on few astrocytic soluble mediators that belong to the classes discussed above (**Figure 2**).

Morphogens

Sonic hedgehog (Shh) is one of the main mediators of BBB induction. It is expressed in astrocytes, and its receptor has been detected *in vivo* in mice and human blood vessels as well as in cultured BBB endothelial cells (Alvarez et al., 2011). Its genetic deletion results in reduced expression of endothelial junctional proteins and accumulation of solutes in CNS (Alvarez et al., 2011). Shh is overexpressed in astrocytes following an ischemic insult and reinforces junctional tightness (Liu et al., 2019) thus reducing BBB leakage and brain edema (Xia et al., 2013). Accordingly, Shh

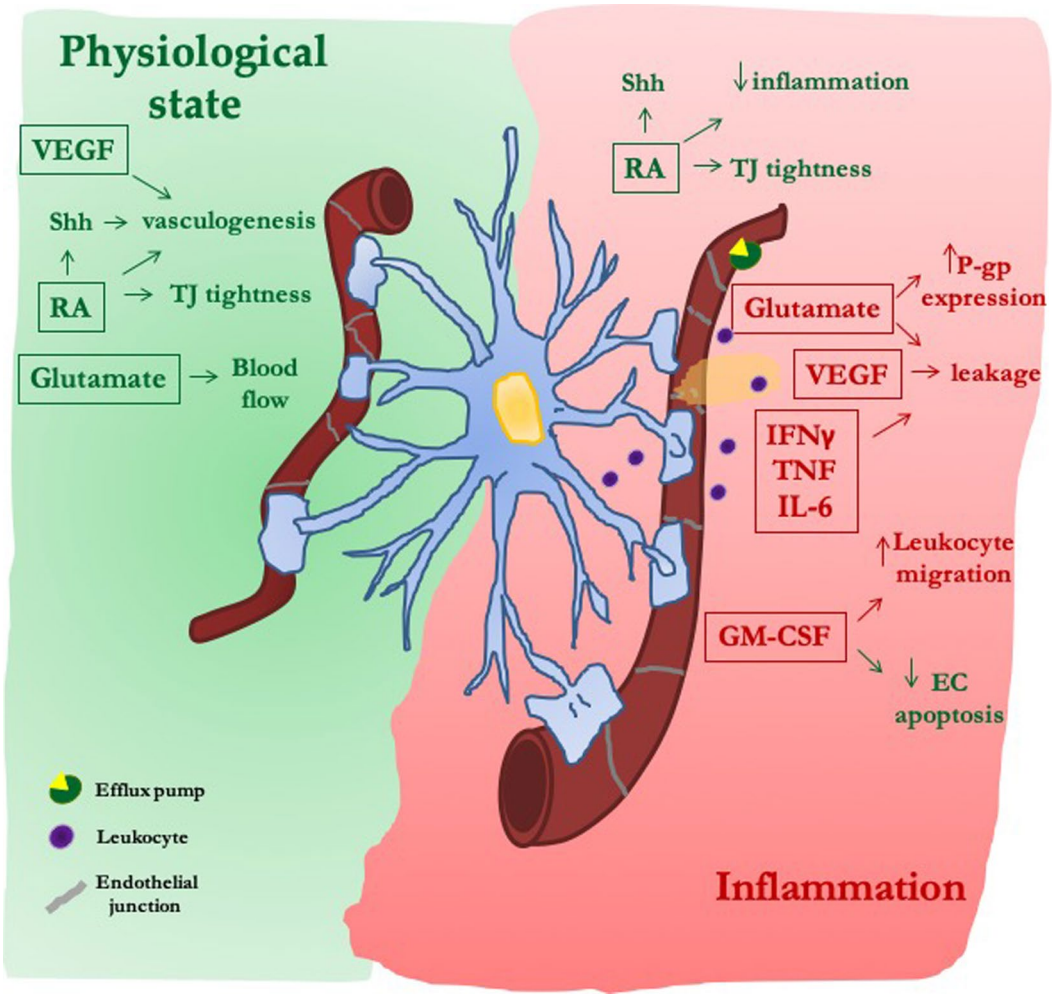

FIGURE 2 | The dual role of astrocytic-derived factors on blood–brain barrier (BBB). Under physiological conditions, astrocytes release morphogens [sonic hedgehog (Shh) and retinoic acid (RA)], trophic factors (VEGF), and gliotransmitters (Glu) that, reinforcing both the formation of new vessels and the tightness of their junctions, improve the proper endothelial function at the BBB. After inflammatory stimuli, secretion of morphogens (Shh and RA) is reactivated in an attempt to reduce the inflammatory-mediated damage on endothelial layer. On the contrary, VEGF and Glu induce junctional damage and BBB leakiness, as well as increased expression of efflux pumps [P-glycoprotein (P-gp)]. The secretion of cytokines and chemokines is further increased, thus facilitating BBB leakage and leukocyte migration.

mimetics promote immune-quiescence dampening leukocyte extravasation into the CNS, through the downregulation of adhesion molecules, as observed both *in vitro* (Alvarez et al., 2011) and *in vivo* (Singh et al., 2017). Retinoic acid (RA) is produced by radial glia and behaves as a morphogen playing a main role during brain development (Halilagic et al., 2007; Mizee et al., 2013). It is enhanced in reactive astrocytes after middle cerebral artery occlusion (Kong et al., 2015) and contributes to ameliorate barrier properties. RA indeed reinforces the expression of junctional proteins and P-gp in endothelial cells (Mizee et al., 2013) and reduces inflammatory genes (IL-6, CCL2, and VCAM-1) (Mizee et al., 2014). It also modifies ICAM-1 glycan composition (Chen et al., 2012), affecting the interaction of endothelial cells with PBMCs, an event that is modulated by astrocytes (Spampinato et al., 2019). Thus, beyond their physiological function in barrierogenesis, both Shh and RA play a role in the delay of BBB breakdown under pathological conditions.

Trophic Factors

The main vascular trophic factor is VEGF-A. In contrast to its main activity in promoting angiogenesis, proliferation, differentiation, and survival of endothelial cells during brain development (Esser et al., 1998; Zhao et al., 2015), in adulthood VEGF is a potent inducer of BBB disruption. Reactive astrocytes are VEGF-A primary source and increased BBB immunoreactivity is often observed in animal models of multiple sclerosis (Maharaj and D'Amore, 2007; Argaw et al., 2012), Alzheimer's disease (Zand et al., 2005), ischemia, and traumatic brain injury (Shore et al., 2004; Jiang et al., 2014; Wu et al., 2018). Acting either directly on its receptors on endothelial cells, (Argaw et al., 2012; Chapouly et al., 2015), or indirectly, through the modulation of matrix metalloproteinases (MMPs) (Michinaga et al., 2015; Spampinato et al., 2017), VEGF-A induces changes in the tightness of endothelial junctions, causing brain edema, as well as leukocyte adhesion and infiltration in the CNS. Accordingly, blockade of

VEGF-A through specific antibodies alleviates BBB disruption (Michinaga et al., 2018), whereas VEGF-A knockdown in astrocytes results in reduced endothelial expression of MMP9 and prevention of barrier leakage (Spampinato et al., 2017).

Cytokines and Chemokines

Cytokines released by reactive astrocytes in close proximity to the BBB induce TJ re-organization [TNF and IFNγ (Chaitanya et al., 2011), CCL2 (Yao and Tsirka, 2014)], and immune cells recruitment [CXCL10, CCL2, CCL5, IL-8, CXCL12 (Brambilla, 2019)], further contributing to neuroinflammation. By stimulating proteosomal degradation of junctional proteins (Chang et al., 2015), astrocyte-derived IL-6 increases barrier permeability and the release of chemokines, thus enhancing PBMCs' access into the CNS (Takeshita et al., 2017). Astrocytes may also mediate endothelial responses to cytokines. Their presence is in fact necessary for INFγ to affect barrier properties, whereas only slight effects are reported on endothelial cells cultured alone (Chaitanya et al., 2011). Conversely, astrocytes counteracted increased barrier permeability induced by TNF alone, or in association with IL-6, on induced plutipotent stem cells-derived endothelial cells. The modulation of BBB properties by astrocyte-derived factors appears to be the result of a complex balance. Indeed, stressed astrocytes release not only factors triggering barrier breakdown (i.e., IFNγ, IL-1β, CCL5, CCL2, and CCL4), but anti-inflammatory ones like IL-4 (Mantle and Lee, 2018).

In addition, among astrocyte-derived factors, granulocyte and macrophage colony-stimulating factor (GM-CSF) exhibits a dual and controversial role. While promoting TJ internalization and downregulation (Shang et al., 2016; Zhang et al., 2018) and monocyte migration through the BBB (Vogel et al., 2015), GM-CSF also protects endothelial cells from apoptosis (Spampinato et al., 2015), induces claudin-5 overexpression (Shang et al., 2016), and stimulates angiopoietin-1 release from pericytes, thereby reducing barrier permeability (Yan et al., 2017).

Gliotransmitters

Glu modifies BBB function through interaction with endothelial NMDA and metabotropic glutamate receptors. Activated astrocytes release large amounts of glutamate that act on endothelial NMDA receptors and promote oxidative stress (Scott et al., 2007), TJ disruption, and increased BBB permeability (Andras et al., 2007). Further, glutamate increases the expression of P-gp, as reported in endothelial cells cultured with astrocytes derived from amyotrophic lateral sclerosis (ALS) patients (Mohamed et al., 2019). This condition can justify "P-gp–mediated pharmacoresistance" (Mohamed et al., 2017), often observed in diseases including ALS and epilepsy (Avemary et al., 2013; Feldmann et al., 2013).

CONCLUDING REMARKS

Our current understanding of the role of astrocytes in adult mammalian brain is growing exponentially, unraveling a remarkable variety of functions under the control of these cells both under physiological and pathological conditions. In recent years, the fact that astrocytes execute many of their crucial functions in a paracrine manner is also providing fuel to major advancements in astrocyte biology. Several proteins identified in studies that have applied proteomics for comprehensive profiling of astrocyte-secreted proteins confirmed that many of them correlate with well-known astrocyte-mediated cell-to-cell communication pathways. In some cases, soluble signals released by astrocytes *in vitro* created the opportunity to propose novel unexpected roles for these molecules and astrocytes. Hopefully, in the future, deeper analysis of the astrocyte secretome may further increase our current knowledge on the full potential of these cells and their secreted molecules not only as active participants in pathophysiological events, but as pharmacological targets or even as therapeutics for CNS diseases.

AUTHOR CONTRIBUTIONS

All authors contributed to the discussion, preparation, and proofreading of the manuscript.

FUNDING

This work was partially supported by PRIN MIUR 2017. VB was supported by a SIF/MSD fellowship 2016. SFS was supported by a SIF/MSD fellowship 2017.

ACKNOWLEDGMENTS

The authors would like to apologize to all researchers whose relevant work could not be quoted, due to space limitations.

REFERENCES

Abbott, N. J., Ronnback, L., and Hansson, E. (2006). Astrocyte-endothelial interactions at the blood-brain barrier. *Nat. Rev. Neurosci.* 7 (1), 41–53. doi: 10.1038/nrn1824

Aimone, J. B., Deng, W., and Gage, F. H. (2010). Adult neurogenesis: integrating theories and separating functions. *Trends Cognit. Sci.* 14 (7), 325–337. doi: 10.1016/j.tics.2010.04.003

Aimone, J. B., Li, Y., Lee, S. W., Clemenson, G. D., Deng, W., and Gage, F. H. (2014). Regulation and function of adult neurogenesis: from genes to cognition. *Physiol. Rev.* 94 (4), 991–1026. doi: 10.1152/physrev.00004.2014

Allen, N. J., and Lyons, D. A. (2018). Glia as architects of central nervous system formation and function. *Sci.* 362 (6411), 181–185. doi: 10.1126/science.aat0473

Alvarez, J. I., Dodelet-Devillers, A., Kebir, H., Ifergan, I., Fabre, P. J., and Terouz, S., et al. (2011). The hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence. *Sci.* 334 (6063), 1727–1731. doi: 10.1126/science.1206936

Alvarez, J. I., Katayama, T., and Prat, A. (2013). Glial influence on the blood brain barrier. *Glia* 61 (12), 1939–1958. doi: 10.1002/glia.22575

Andras, I. E., Deli, M. A., Veszelka, S., Hayashi, K., Hennig, B., and Toborek, M. (2007). The NMDA and AMPA/KA receptors are involved in glutamate-induced alterations of occludin expression and phosphorylation in brain

endothelial cells. *J. Cereb. Blood Flow Metab.* 27 (8), 1431–1443. doi: 10.1038/sj.jcbfm.9600445

Araque, A., Carmignoto, G., Haydon, P. G., Oliet, S. H., Robitaille, R., and Volterra, A. (2014). Gliotransmitters travel in time and space. *Neuron* 81 (4), 728–739. doi: 10.1016/j.neuron.2014.02.007

Argaw, A. T., Asp, L., Zhang, J., Navrazhina, K., Pham, T., Mariani, J. N., et al. (2012). Astrocyte-derived VEGF-A drives blood-brain barrier disruption in CNS inflammatory disease. *J. Clin. Invest.* 122 (7), 2454–2468. doi: 10.1172/JCI60842

Avemary, J., Salvamoser, J. D., Peraud, A., Remi, J., Noachtar, S., Fricker, G., et al. (2013). Dynamic regulation of P-glycoprotein in human brain capillaries. *Mol. Pharm.* 10 (9), 3333–3341. doi: 10.1021/mp4001102

Barkho, B. Z., Song, H., Aimone, J. B., Smrt, R. D., Kuwabara, T., Nakashima, K., et al. (2006). Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation. *Stem Cells Dev.* 15 (3), 407–421. doi: 10.1089/scd.2006.15.407

Begley, D. J. (2004). ABC transporters and the blood-brain barrier. *Curr. Pharm. Des.* 10 (12), 1295–1312.

Boldrini, M., Fulmore, C. A., Tartt, A. N., Simeon, L. R., Pavlova, I., Poposka, V., et al. (2018). Human hippocampal neurogenesis persists throughout aging. *Cell Stem Cell* 22 (4), 589–599 e585. doi: 10.1016/j.stem.2018.03.015

Bond, A. M., Ming, G. L., Song, H. (2015). Adult mammalian neural stem cells and neurogenesis: five decades later. *Cell Stem Cell* 17 (4), 385–395. doi: 10.1016/j.stem.2015.09.003

Bonini, S. A., Mastinu, A., Maccarinelli, G., Mitola, S., Premoli, M., La Rosa, L. R., et al. (2016). Cortical structure alterations and social behavior impairment in p50-deficient mice. *Cereb Cortex* 26 (6), 2832–2849. doi: 10.1093/cercor/bhw037

Bortolotto, V., Bondi, H., Cuccurazzu, B., Rinaldi, M., Canonico, P. L., and Grilli, M. (2019). Salmeterol, a β2 adrenergic agonist, promotes adult hippocampal neurogenesis in a region-specific manner. *Front. Pharmacol.* 10, 1000. doi: 10.3389/fphar.2019.01000

Bortolotto, V., Cuccurazzu, B., Canonico, P. L., and Grilli, M. (2014). NF-kappaB mediated regulation of adult hippocampal neurogenesis: relevance to mood disorders and antidepressant activity. *BioMed. Res. Int.* 2014, 612798. doi: 10.1155/2014/612798

Bortolotto, V., and Grilli, M. (2016). Not only a bad guy: potential proneurogenic role of the RAGE/NF-kappaB axis in Alzheimer's disease brain. *Neural Regener. Res.* 11 (12), 1924–1925. doi: 10.4103/1673-5374.197130

Bortolotto, V., and Grilli, M. (2017b). Novel insights into the role of NF-κB p50 in astrocyte - mediated fate specification of adult neural progenitor cells. *Neural Regener. Res.* 12 (3), 354–357. doi: 10.4103/1673-5374.202919

Bortolotto, V., Mancini, F., Mangano, G., Salem, R., Xia, E., Del Grosso, E., et al. (2017a). Proneurogenic effects of trazodone in murine and human neural progenitor cells. *ACS Chem. Neurosci.* 8 (9), 2027–2038. doi: 10.1021/acschemneuro.7b00175

Brambilla, R. (2019). The contribution of astrocytes to the neuroinflammatory response in multiple sclerosis and experimental autoimmune encephalomyelitis. *Acta Neuropathol.* 137 (5), 757–783. doi: 10.1007/s00401-019-01980-7

Broux, B., Gowing, E., and Prat, A. (2015). Glial regulation of the blood-brain barrier in health and disease. *Semin. Immunopathol.* 37 (6), 577–590. doi: 10.1007/s00281-015-0516-2

Casse, F., Richetin, K., and Toni, N. (2018). Astrocytes' contribution to adult neurogenesis in physiology and Alzheimer's disease. *Front. Cell Neurosci.* 12, 432. doi: 10.3389/fncel.2018.00432

Chaitanya, G. V., Cromer, W. E., Wells, S. R., Jennings, M. H., Couraud, P. O., Romero, I. A., et al. (2011). Gliovascular and cytokine interactions modulate brain endothelial barrier in vitro. *J. Neuroinflammation* 8, 162. doi: 10.1186/1742-2094-8-162

Chang, C. Y., Li, J. R., Chen, W. Y., Ou, Y. C., Lai, C. Y., Hu, Y. H., et al. (2015). Disruption of in vitro endothelial barrier integrity by Japanese encephalitis virus-Infected astrocytes. *Glia* 63 (11), 1915–1932. doi: 10.1002/glia.22857

Chapouly, C., Tadesse Argaw, A., Horng, S., Castro, K., Zhang, J., Asp, L., et al. (2015). Astrocytic TYMP and VEGFA drive blood-brain barrier opening in inflammatory central nervous system lesions. *Brain* 138 (Pt 6), 1548–1567. doi: 10.1093/brain/awv077

Chen, C., Diao, D., Guo, L., Shi, M., Gao, J., Hu, M., et al. (2012). All-trans-retinoic acid modulates ICAM-1 N-glycan composition by influencing GnT-III levels and inhibits cell adhesion and trans-endothelial migration. *PloS One* 7 (12), e52975. doi: 10.1371/journal.pone.0052975

Chen, Y., and Swanson, R. A. (2003). Astrocytes and brain injury. *J. Cereb. Blood Flow Metab.* 23 (2), 137–149. doi: 10.1097/01.WCB.0000044631.80210.3C

Colgan, O. C., Collins, N. T., Ferguson, G., Murphy, R. P., Birney, Y. A., Cahill, P. A., et al. (2008). Influence of basolateral condition on the regulation of brain microvascular endothelial tight junction properties and barrier function. *Brain Res.* 1193, 84–92. doi: 10.1016/j.brainres.2007.11.072

Cvijetic, S., Bortolotto, V., Manfredi, M., Ranzato, E., Marengo, E., Salem, R., et al. (2017). Cell autonomous and noncell-autonomous role of NF-kappaB p50 in astrocyte-mediated fate specification of adult neural progenitor cells. *Glia* 65 (1), 169–181. doi: 10.1002/glia.23085

Dejana, E., and Giampietro, C. (2012). Vascular endothelial-cadherin and vascular stability. *Curr. Opin. Hematol.* 19 (3), 218–223. doi: 10.1097/MOH.0b013e3283523e1c

Denis-Donini, S., Dellarole, A., Crociara, P., Francese, M. T., Bortolotto, V., and Quadrato, G., et al. (2008). Impaired adult neurogenesis associated with short-term memory defects in NF-kappaB p50-deficient mice. *J. Neurosci.* 28 (15), 3911–3919. doi: 10.1523/JNEUROSCI.0148-08.2008

Dowell, J. A., Johnson, J. A., and Li, L. (2009). Identification of astrocyte secreted proteins with a combination of shotgun proteomics and bioinformatics. *J. Proteome Res.* 8 (8), 4135–4143. doi: 10.1021/pr900248y

Eisch, A. J., and Petrik, D. (2012). Depression and hippocampal neurogenesis: a road to remission? *Sci.* 338 (6103), 72–75. doi: 10.1126/science.1222941

Engelhardt, B., and Ransohoff, R. M. (2005). The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms. *Trends Immunol.* 26 (9), 485–495. doi: 10.1016/j.it.2005.07.004

Eroglu, C., Allen, N. J., Susman, M. W., O'Rourke, N. A., Park, C. Y., Ozkan, E., et al. (2009). Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. *Cell* 139 (2), 380–392. doi: 10.1016/j.cell.2009.09.025

Esser, S., Wolburg, K., Wolburg, H., Breier, G., Kurzchalia, T., Risau, W. (1998). Vascular endothelial growth factor induces endothelial fenestrations in vitro. *J. Cell Biol.* 140 (4), 947–959. doi: 10.1083/jcb.140.4.947

Feldmann, M., Asselin, M. C., Liu, J., Wang, S., McMahon, A., and Anton-Rodriguez, J., et al. (2013). P-glycoprotein expression and function in patients with temporal lobe epilepsy: a case-control study. *Lancet Neurol.* 12 (8), 777–785. doi: 10.1016/S1474-4422(13)70109-1

Ferreira, A. C., Da Mesquita, S., Sousa, J. C., Correia-Neves, M., Sousa, N., Palha, J. A., et al. (2015). From the periphery to the brain: Lipocalin-2, a friend or foe? *Prog. Neurobiol.* 131, 120–136. doi: 10.1016/j.pneurobio.2015.06.005

Ferreira, A. C., Santos, T., Sampaio-Marques, B., Novais, A., Mesquita, S. D., Ludovico, P., et al. (2018). Lipocalin-2 regulates adult neurogenesis and contextual discriminative behaviours. *Mol. Psychiatry* 23 (4), 1031–1039. doi: 10.1038/mp.2017.95

Fiacco, T. A., and McCarthy, K. D. (2018). Multiple lines of evidence indicate that gliotransmissiondoes not occur under physiological conditions. *J. Neurosci.* 38 (1), 3–13. doi: 10.1523/JNEUROSCI.0016-17.2017

Garcia, K. O., Ornellas, F. L., Martin, P. K., Patti, C. L., Mello, L. E., and Frussa-Filho, R., et al. (2014). Therapeutic effects of the transplantation of VEGF overexpressing bone marrow mesenchymal stem cells in the hippocampus of murine model of Alzheimer's disease. *Front. Aging Neurosci.* 6, 30. doi: 10.3389/fnagi.2014.00030

Garg, S. K., Banerjee, R., and Kipnis, J. (2008). Neuroprotective immunity: T cell-derived glutamate endows astrocytes with a neuroprotective phenotype. *J. Immunol.* 180 (6), 3866–3873. doi: 10.4049/jimmunol.180.6.3866

Ghazanfari, F. A., and Stewart, R. R. (2001). Characteristics of endothelial cells derived from the blood-brain barrier and of astrocytes in culture. *Brain Res.* 890 (1), 49–65. doi: 10.1016/s0006-8993(00)03053-5

Ghosh, H. S. (2019). Adult neurogenesis and the promise of adult neural stem cells. *J. Exp. Neurosci.* 13, 1179069519856876. doi: 10.1177/1179069519856876

Gimsa, U., Mitchison, N. A., and Brunner-Weinzierl, M. C. (2013). Immune privilege as an intrinsic CNS property: astrocytes protect the CNS against T-cell-mediated neuroinflammation. *Mediators Inflammation* 2013, 320519. doi: 10.1155/2013/320519

Grilli, M., and Meneghini, V. (2012). "NF-κB proteins in adult neurogenesis: relevance for learning and memory in physiology and pathology," in *Transcription factors CREB and NF-κB: involvement in synaptic plasticity and*

memory formation. Ed. Bentham Science Publishers. (Sharjah, United Arab Emirates), 79–96. doi: 10.2174/9781608052578112010100 79

Grilli, M., Nisoli, E., Memo, M., Missale, C., and Spano, P. (1988). Pharmacological characterization of D1 and D2 dopamine receptors in rat limbocortical areas. *II. Dorsal Hippocampus. Neurosci. Lett.* 87, 253–258. doi: 10.1016/0304-3940(88)90457-0

Halilagic, A., Ribes, V., Ghyselinck, N. B., Zile, M. H., Dolle, P., and Studer, M. (2007). Retinoids control anterior and dorsal properties in the developing forebrain. *Dev. Biol.* 303 (1), 362–375. doi: 10.1016/j.ydbio.2006.11.021

Harada, K., Kamiya, T., and Tsuboi, T. (2015). Gliotransmitter release from astrocytes: functional, developmental, and pathological implications in the brain. *Front. Neurosci.* 9, 499. doi: 10.3389/fnins.2015.00499

Hayashi, Y., Nomura, M., Yamagishi, S., Harada, S., Yamashita, J., and Yamamoto, H. (1997). Induction of various blood-brain barrier properties in non-neural endothelial cells by close apposition to co-cultured astrocytes. *Glia* 19 (1), 13–26.

Huber, J. D., Egleton, R. D., and Davis, T. P. (2001). Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier. *Trends Neurosci.* 24 (12), 719–725. doi: 10.1016/s0166-2236(00)02004-x

Jha, M. K., Kim, J. H., Song, G. J., Lee, W. H., Lee, I. K., Lee, H. W., et al. (2018). Functional dissection of astrocyte-secreted proteins: implications in brain health and diseases. *Prog. Neurobiol.* 162, 37–69. doi: 10.1016/j.pneurobio.2017.12.003

Jha, M. K., Lee, S., Park, D. H., Kook, H., Park, K. G., Lee, I. K., et al. (2015). Diverse functional roles of lipocalin-2 in the central nervous system. *Neurosci. Biobehav. Rev.* 49, 135–156. doi: 10.1016/j.neubiorev.2014.12.006

Jiang, S., Xia, R., Jiang, Y., Wang, L., and Gao, F. (2014). Vascular endothelial growth factors enhance the permeability of the mouse blood-brain barrier. *PloS One* 9 (2), e86407. doi: 10.1371/journal.pone.0086407

Kastin, A. J., and Pan, W. (2008). Blood-brain barrier and feeding: regulatory roles of saturable transport systems for ingestive peptides. *Curr. Pharm. Des.* 14 (16), 1615–1619. doi: 10.2174/138161208784705423

Kempermann, G., Song, H., and Gage, F. H. (2015). Neurogenesis in the Adult Hippocampus. *Cold Spring Harb Perspect. Biol.* 7 (9), a018812. doi: 10.1101/cshperspect.a018812

Kong, L., Wang, Y., Wang, X. J., Wang, X. T., Zhao, Y., Wang, L. M., et al. (2015). Retinoic acid ameliorates blood-brain barrier disruption following ischemic stroke in rats. *Pharmacol. Res.* 99, 125–136. doi: 10.1016/j.phrs.2015.05.014

Lie, D. C., Colamarino, S. A., Song, H. J., Desire, L., Mira, H., Consiglio, A., et al. (2005). Wnt signalling regulates adult hippocampal neurogenesis. *Nat.* 437 (7063), 1370–1375. doi: 10.1038/nature04108

Liu, S., Chang, L., and Wei, C. (2019). The sonic hedgehog pathway mediates Tongxinluo capsule-induced protection against blood-brain barrier disruption after ischaemic stroke in mice. *Basic Clin. Pharmacol. Toxicol.* 124 (6), 660–669. doi: 10.1111/bcpt.13186

Lu, Z., and Kipnis, J. (2010). Thrombospondin 1–a key astrocyte-derived neurogenic factor. *FASEB J.* 24 (6), 1925–1934. doi: 10.1096/fj.09-150573

Maharaj, A. S., and D'Amore, P. A. (2007). Roles for VEGF in the adult. *Microvasc Res.* 74 (2–3), 100–113. doi: 10.1016/j.mvr.2007.03.004

Mantle, J. L., and Lee, K. H. (2018). A differentiating neural stem cell-derived astrocytic population mitigates the inflammatory effects of TNF-alpha and IL-6 in an iPSC-based blood-brain barrier model. *Neurobiol. Dis.* 119, 113–120. doi: 10.1016/j.nbd.2018.07.030

McDermott, K. W., Barry, D. S., and McMahon, S. S. (2005). Role of radial glia in cytogenesis, patterning and boundary formation in the developing spinal cord. *J. Anat.* 207 (3), 241–250. doi: 10.1111/j.1469-7580.2005.00462.x

Meneghini, V., Bortolotto, V., Francese, M. T., Dellarole, A., Carraro, L., Terzieva, S., et al. (2013). High-mobility group box-1 protein and β-amyloid oligomers promote neuronal differentiation of adult hippocampal neural progenitors via receptor for advanced glycation end products/nuclear factor-κB axis: relevance for Alzheimer's disease. *J. Neurosci.* 33 (14), 6047–6059. doi: 10.1523/JNEUROSCI.2052-12.2013

Meneghini, V., Francese, M. T., Carraro, L., and Grilli, M. (2010). A novel role for the receptor for advanced glycation end-products in neural progenitor cells derived from adult SubVentricular Zone. *Mol. Cell Neurosci.* 45 (2), 139–150. doi: 10.1016/j.mcn.2010.06.005

Michinaga, S., Kimura, A., Hatanaka, S., Minami, S., Asano, A., Ikushima, Y., et al. (2018). Delayed administration of BQ788, an ETB antagonist, after experimental traumatic brain injury promotes recovery of blood-brain barrier function and a reduction of cerebral edema in mice. *J. Neurotrauma* 35 (13), 1481–1494. doi: 10.1089/neu.2017.5421

Michinaga, S., Seno, N., Fuka, M., Yamamoto, Y., Minami, S., Kimura, A., et al. (2015). Improvement of cold injury-induced mouse brain edema by endothelin ETB antagonists is accompanied by decreases in matrixmetalloproteinase 9 and vascular endothelial growth factor-A. *Eur. J. Neurosci.* 42 (6), 2356–2370. doi: 10.1111/ejn.13020

Mizee, M. R., Nijland, P. G., van der Pol, S. M., Drexhage, J. A., van Het Hof, B., Mebius, R., et al. (2014). Astrocyte-derived retinoic acid: a novel regulator of blood-brain barrier function in multiple sclerosis. *Acta Neuropathol.* 128 (5), 691–703. doi: 10.1007/s00401-014-1335-6

Mizee, M. R., Wooldrik, D., Lakeman, K. A., van het Hof, B., Drexhage, J. A., and Geerts, D., et al. (2013). Retinoic acid induces blood-brain barrier development. *J. Neurosci.* 33 (4), 1660–1671. doi: 10.1523/JNEUROSCI.1338-12.2013

Mohamed, L. A., Markandaiah, S., Bonanno, S., Pasinelli, P., and Trotti, D. (2017). Blood-brain barrier driven pharmacoresistance in amyotrophic lateral sclerosis and challenges for effective drug therapies. *AAPS J.* 19 (6), 1600–1614. doi: 10.1208/s12248-017-0120-6

Mohamed, L. A., Markandaiah, S. S., Bonanno, S., Pasinelli, P., and Trotti, D. (2019). Excess glutamate secreted from astrocytes drives upregulation of P-glycoprotein in endothelial cells in amyotrophic lateral sclerosis. *Exp. Neurol.* 316, 27–38. doi: 10.1016/j.expneurol.2019.04.002

Monje, M. L., Toda, H., and Palmer, T. D. (2003). Inflammatory blockade restores adult hippocampal neurogenesis. *Sci.* 302 (5651), 1760–1765. doi: 10.1126/science.1088417

Moreno-Estelles, M., Gonzalez-Gomez, P., Hortiguela, R., Diaz-Moreno, M., San Emeterio, J., Carvalho, A. L., et al. (2012). Symmetric expansion of neural stem cells from the adult olfactory bulb is driven by astrocytes via WNT7A. *Stem Cells* 30 (12), 2796–2809. doi: 10.1002/stem.1243

Moreno-Jimenez, E. P., Flor-Garcia, M., Terreros-Roncal, J., Rabano, A., Cafini, F., Pallas-Bazarra, N., et al. (2019). Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease. *Nat. Med.* 25 (4), 554–560. doi: 10.1038/s41591-019-0375-9

Neuhaus, J., Risau, W., and Wolburg, H. (1991). Induction of blood-brain barrier characteristics in bovine brain endothelial cells by rat astroglial cells in transfilter coculture. *Ann. N Y Acad. Sci.* 633, 578–580. doi: 10.1111/j.1749-6632.1991.tb15667.x

Obermeier, B., Daneman, R., and Ransohoff, R. M. (2013). Development, maintenance and disruption of the blood-brain barrier. *Nat. Med.* 19 (12), 1584–1596. doi: 10.1038/nm.3407

Obermeier, B., Verma, A., and Ransohoff, R. M. (2016). The blood-brain barrier. *Handb. Clin. Neurol.* 133, 39–59. doi: 10.1016/B978-0-444-63432-0.00003-7

Perez-Alvarez, A., and Araque, A. (2013). Astrocyte-neuron interaction at tripartite synapses. *Curr. Drug Targets* 14 (11), 1220–1224. doi: 10.2174/13894501113149990203

Piatti, V. C., Davies-Sala, M. G., Espósito, M. S., Mongiat, L. A., Trinchero, M. F., and Schinder, A. F. (2011). The timing for neuronal maturation in the adult hippocampus is modulated by local network activity. *J. Neurosci.* 31 (21), 7715–7728. doi: 10.1523/JNEUROSCI.1380-11.2011

Platel, J. C., Dave, K. A., Gordon, V., Lacar, B., Rubio, M. E., and Bordey, A. (2010). NMDA receptors activated by subventricular zone astrocytic glutamate are critical for neuroblast survival prior to entering a synaptic network. *Neuron* 65 (6), 859–872. doi: 10.1016/j.neuron.2010.03.009

Prat, A., Biernacki, K., Wosik, K., and Antel, J. P. (2001). Glial cell influence on the human blood-brain barrier. *Glia* 36 (2), 145–155.

Scott, G. S., Bowman, S. R., Smith, T., Flower, R. J., and Bolton, C. (2007). Glutamate-stimulated peroxynitrite production in a brain-derived endothelial cell line is dependent on N-methyl-D-aspartate (NMDA) receptor activation. *Biochem. Pharmacol.* 73 (2), 228–236. doi: 10.1016/j.bcp.2006.09.021

Shang, S., Yang, Y. M., Zhang, H., Tian, L., Jiang, J. S., Dong, Y. B., et al. (2016). Intracerebral GM-CSF contributes to transendothelial monocyte migration in APP/PS1 Alzheimer's disease mice. *J. Cereb. Blood Flow Metab.* 36 (11), 1978–1991. doi: 10.1177/0271678X16660983

Shore, P. M., Jackson, E. K., Wisniewski, S. R., Clark, R. S., Adelson, P. D., and Kochanek, P. M. (2004). Vascular endothelial growth factor is increased in cerebrospinal fluid after traumatic brain injury in infants and children. *Neurosurgery* 54 (3), 605–611. doi: 10.1227/01.neu.0000108642.88724.db

Singh, V. B., Singh, M. V., Piekna-Przybylska, D., Gorantla, S., Poluektova, L. Y., and Maggirwar, S. B. (2017). Sonic Hedgehog mimetic prevents leukocyte infiltration into the CNS during acute HIV infection. *Sci. Rep.* 7 (1), 9578. doi: 10.1038/s41598-017-10241-0

Smith, J. A., Braga, A., Verheyen, J., Basilico, S., Bandiera, S., Alfaro-Cervello, C., et al. (2018). RNA nanotherapeutics for the amelioration of astroglial reactivity. *Mol. Ther. Nucleic Acids* 10, 103–121. doi: 10.1016/j.omtn.2017.11.008

Song, H., Stevens, C. F., and Gage, F. H. (2002). Astroglia induce neurogenesis from adult neural stem cells. *Nat.* 417 (6884), 39–44. doi: 10.1038/417039a

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., et al. (2013). Dynamics of hippocampal neurogenesis in adult humans. *Cell* 153 (6), 1219–1227. doi: 10.1016/j.cell.2013.05.002

Spampinato, S. F., Merlo, S., Fagone, E., Fruciano, M., Barbagallo, C., Kanda, T., et al. (2019). Astrocytes modify migration of PBMCs induced by β-amyloid in a blood-brain barrier in vitro model. *Front. In Cell. Neurosci.* 13. doi: 10.3389/fncel.2019.00337

Spampinato, S. F., Merlo, S., Sano, Y., Kanda, T., and Sortino, M. A. (2017). Astrocytes contribute to Abeta-induced blood-brain barrier damage through activation of endothelial MMP9. *J. Neurochem.* 142 (3), 464–477. doi: 10.1111/jnc.14068

Spampinato, S. F., Obermeier, B., Cotleur, A., Love, A., Takeshita, Y., Sano, Y., et al. (2015). Sphingosine 1 phosphate at the blood brain barrier: Can the Modulation of S1P receptor 1 influence the response of endothelial cells and astrocytes to inflammatory stimuli? *PloS One* 10 (7), e0133392. doi: 10.1371/journal.pone.0133392

Sultan, S., Li, L., Moss, J., Petrelli, F., Casse, F., Gebara, E., et al. (2015). Synaptic integration of adult-born hippocampal neurons is locally controlled by astrocytes. *Neuron* 88 (5), 957–972. doi: 10.1016/j.neuron.2015.10.037

Takeshita, Y., Obermeier, B., Cotleur, A. C., Spampinato, S. F., Shimizu, F., Yamamoto, E., et al. (2017). Effects of neuromyelitis optica-IgG at the blood-brain barrier in vitro. *Neurol. Neuroimmunol Neuroinflamm* 4 (1), e311. doi: 10.1212/NXI.0000000000000311

Tanti, A., and Belzung, C. (2013). Neurogenesis along the septo-temporal axis of the hippocampus: Are depression and the action of antidepressants region-specific? *Neurosci.* 252, 234–252. doi: 10.1016/j.neuroscience.2013.08.017

Tao-Cheng, J. H., Nagy, Z., and Brightman, M. W. (1987). Tight junctions of brain endothelium in vitro are enhanced by astroglia. *J. Neurosci.* 7 (10), 3293–3299.

Uberti, D., Grilli, M., and Memo, M. (2000). Contribution of NF-kappaB and p53 in the glutamate-induced apoptosis. *Int. J. Dev. Neurosci.* 18 (4-5), 447–454. doi: 10.1016/s0736-5748(00)00018-6

Valente, M. M., Allen, M., Bortolotto, V., Lim, S. T., Conant, K., and Grilli, M. (2015). The MMP-1/PAR-1 axis enhances proliferation and neuronal differentiation of adult hippocampal neural progenitor cells. *Neural Plast* 2015, 646595. doi: 10.1155/2015/646595

Valente, M. M., Bortolotto, V., Cuccurazzu, B., Ubezio, F., Meneghini, V., Francese, M. T., et al. (2012). alpha2delta ligands act as positive modulators of adult hippocampal neurogenesis and prevent depression-like behavior induced by chronic restraint stress. *Mol. Pharmacol.* 82 (2), 271–280. doi: 10.1124/mol.112.077636

Vallieres, L., Campbell, I. L., Gage, F. H., and Sawchenko, P. E. (2002). Reduced hippocampal neurogenesis in adult transgenic mice with chronic astrocytic production of interleukin-6. *J. Neurosci.* 22 (2), 486–492.

Vogel, D. Y., Kooij, G., Heijnen, P. D., Breur, M., Peferoen, L. A., van der Valk, P., et al. (2015). GM-CSF promotes migration of human monocytes across the blood brain barrier. *Eur. J. Immunol.* 45 (6), 1808–1819. doi: 10.1002/eji.201444960

Wu, L., Ye, Z., Pan, Y., Li, X., Fu, X., Zhang, B., et al. (2018). Vascular endothelial growth factor aggravates cerebral ischemia and reperfusion-induced blood-brain-barrier disruption through regulating LOC102640519/HOXC13/ZO-1 signaling. *Exp. Cell Res.* 369 (2), 275–283. doi: 10.1016/j.yexcr.2018.05.029

Xia, Y. P., He, Q. W., Li, Y. N., Chen, S. C., Huang, M., Wang, Y., et al. (2013). Recombinant human sonic hedgehog protein regulates the expression of ZO-1 and occludin by activating angiopoietin-1 in stroke damage. *PloS One* 8 (7), e68891. doi: 10.1371/journal.pone.0068891

Xing, C., Wang, X., Cheng, C., Montaner, J., Mandeville, E., Leung, W., et al. (2014). Neuronal production of lipocalin-2 as a help-me signal for glial activation. *Stroke* 45 (7), 2085–2092. doi: 10.1161/STROKEAHA.114.005733

Yan, M., Hu, Y., Yao, M., Bao, S., and Fang, Y. (2017). GM-CSF ameliorates microvascular barrier integrity via pericyte-derived Ang-1 in wound healing. *Wound Repair Regener.* 25 (6), 933–943. doi: 10.1111/wrr.12608

Yao, Y., and Tsirka, S. E. (2014). Monocyte chemoattractant protein-1 and the blood-brain barrier. *Cell Mol. Life Sci.* 71 (4), 683–697. doi: 10.1007/s00018-013-1459-1

Yun, S., Donovan, M. H., Ross, M. N., Richardson, D. R., Reister, R., Farnbauch, L. A., et al. (2016). Stress-induced anxiety- and depressive-like phenotype associated with transient reduction in neurogenesis in adult nestin-CreERT2/diphtheria toxin fragment A transgenic mice. *PloS One* 11 (1), e0147256. doi: 10.1371/journal.pone.0147256

Zand, L., Ryu, J. K., and McLarnon, J. G. (2005). Induction of angiogenesis in the beta-amyloid peptide-injected rat hippocampus. *Neuroreport* 16 (2), 129–132. doi: 10.1097/00001756-200502080-00011

Zhang, H., Zhang, S., Zhang, J., Liu, D., Wei, J., Fang, W., et al. (2018). ZO-1 expression is suppressed by GM-CSF via miR-96/ERG in brain microvascular endothelial cells. *J. Cereb. Blood Flow Metab.* 38 (5), 809–822. doi: 10.1177/0271678X17702668

Zhao, F., Deng, J., Yu, X., Li, D., Shi, H., and Zhao, Y. (2015). Protective effects of vascular endothelial growth factor in cultured brain endothelial cells against hypoglycemia. *Metab. Brain Dis.* 30 (4), 999–1007. doi: 10.1007/s11011-015-9659-z

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

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 02 December 2019
doi: 10.3389/fphar.2019.01452

Exosomes From Astrocyte Processes: Signaling to Neurons

Arianna Venturini[1†‡], Mario Passalacqua[2‡], Simone Pelassa[1], Fabio Pastorino[3], Mariateresa Tedesco[4,5], Katia Cortese[6], Maria Cristina Gagliani[6], Giuseppina Leo[7], Guido Maura[1], Diego Guidolin[8], Luigi F. Agnati[7,9], Manuela Marcoli[1,10] and Chiara Cervetto[1]*

[1] Section of Pharmacology and Toxicology, Department of Pharmacy, University of Genova, Genova, Italy, [2] Section of Biochemistry, Department of Experimental Medicine, and Italian Institute of Biostructures and Biosystems, University of Genova, Genova, Italy, [3] Laboratory of Experimental Therapies in Oncology, IRCCS Istituto G. Gaslini, Genova, Italy, [4] 3BrainAG, Wädenswil, Switzerland, [5] Department of Informatics, Bioengineering, Robotics and System Engineering DIBRIS, University of Genova, Genova, Italy, [6] Section of Anatomy, Department of Experimental Medicine, University of Genova, Genova, Italy, [7] Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy, [8] Department of Neurosciences, University of Padova, Padova, Italy, [9] Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden, [10] Centre of Excellence for Biomedical Research CEBR, University of Genova, Genova, Italy

It is widely recognized that extracellular vesicles subserve non-classical signal transmission in the central nervous system. Here we assess if the astrocyte processes, that are recognized to play crucial roles in intercellular communication at the synapses and in neuron-astrocyte networks, could convey messages through extracellular vesicles. Our findings indicate, for the first time that freshly isolated astrocyte processes prepared from adult rat cerebral cortex, can indeed participate to signal transmission in central nervous system by releasing exosomes that by volume transmission might target near or long-distance sites. It is noteworthy that the exosomes released from the astrocyte processes proved ability to selectively target neurons. The astrocyte-derived exosomes were proven positive for neuroglobin, a protein functioning as neuroprotectant against cell insult; the possibility that exosomes might transfer neuroglobin to neurons would add a mechanism to the potential astrocytic neuroprotectant activity. Notably, the exosomes released from the processes of astrocytes maintained markers, which prove their parental astrocytic origin. This potentially allows the assessment of the cellular origin of exosomes that might be recovered from body fluids.

Keywords: adult astrocytes, astrocyte processes, cerebral cortex, ex-vivo, exosomes, extracellular vesicles, neuroglobin, neuron-astrocyte cocultures

Abbreviations: CNS, central nervous system; EVs, extracellular vesicles; GFAP, glial fibrillary protein; NGB, neuroglobin; BSA, bovine serum albumin; MAP-2, microtubule-associated protein 2; PFA, paraformaldehyde; PBS, phosphate buffer solution; β III Tub, β III Tubulin.

OPEN ACCESS

Edited by:
Juan Andrés Orellana, Pontifical Catholic University of Chile, Chile

Reviewed by:
Ursula Wyneken, University of the Andes, Chile
Edward J. Goetzl, University of California, San Francisco, United States

****Correspondence:***
Manuela Marcoli
marcoli@pharmatox.unige.it

†Present address:
Arianna Venturini, Telethon Institute of Genetics and Medicine, Pozzuoli, Italy

‡These authors have contributed equally to this work

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

Received: *24 July 2019*
Accepted: *13 November 2019*
Published: *02 December 2019*

Citation:
Venturini A, Passalacqua M, Pelassa S, Pastorino F, Tedesco M, Cortese K, Gagliani MC, Leo G, Maura G, Guidolin D, Agnati LF, Marcoli M and Cervetto C (2019) Exosomes From Astrocyte Processes: Signaling to Neurons. Front. Pharmacol. 10:1452. doi: 10.3389/fphar.2019.01452

INTRODUCTION

The relevance of neuron-astrocyte network function in the intercellular communication in central nervous system (CNS) as well as in the vulnerability to neurodegenerative and neuropsychiatric diseases is widely accepted (see Halassa and Haydon, 2010; Sofroniew, 2015; Verkhratsky et al., 2016). In the neuron-astrocyte networks the perisynaptic astrocyte processes function as sensors of transmitters in the extracellular environment —acted upon by neurotransmitters and gliotransmitters through a volume transmission mode of communication (see for reviews Agnati and Fuxe, 2000; Vizi, 2000)—and modulate neural activity by clearing glutamate and by releasing gliotransmitters (see Verkhratsky et al., 2016; Cervetto et al., 2018 and references therein); they also regulate extracellular space volume and coverage of synapses (Xie et al., 2013). Indeed, they represent the astrocyte compartment specially devoted to bidirectional neuron-astrocyte communication in the complex interaction involving pre- and post-synaptic elements (the tripartite synapse; Araque et al., 1999) with the extracellular matrix (the tetrapartite synapse; Thalhammer and Cingolani, 2014; Agnati et al., 2018) and to regulation of synapse plasticity.

An increasing amount of evidence indicates that extracellular vesicles (EVs) operate as carriers of signals in CNS. Intercellular communication through EVs is generally accepted as a mode of non-synaptic communication in CNS—the roamer-type of volume transmission—contributing to the role of the extracellular space in the signaling diffusion and codification in the brain (Agnati et al., 2014). Exosomes—EVs of about 30–100 nm diameter, released into the extracellular space upon fusion of multivesicular bodies with the plasma membrane—are recognized to play multiple roles in both physiological and pathological conditions in CNS (Février and Raposo, 2004; Raposo and Stoorvogel, 2013 and references therein). Various CNS cell types, including neurons, microglia and oligodendroglia, can release exosomes; while cultured astrocytes have been reported to secrete exosomes (see Taylor et al., 2007; Guescini et al., 2010; Wang et al., 2011; Wang et al., 2012; Guitart et al., 2016; Willis et al., 2017; Hira et al., 2018; Pascua-Maestro et al., 2019; Pei et al., 2019; Xu et al., 2019; see also Verkhratsky et al., 2016 and Lafourcade et al., 2016), less is known on the ability of astrocytes to release exosomes in neuron-astrocyte networks.

Here we investigate on the possibility that the processes of astrocytes might convey messages in non-classical mode through EVs. We report for the first time that astrocytic processes freshly prepared from adult rat cerebral cortex and originating from astrocytes that have matured in astrocyte-neuron networks, are provided with structures resembling multivesicular bodies and can release vesicles, which exhibit the features of exosomes. Therefore, although their subcellular origin cannot be directly demonstrated, the vesicles can be considered *bona-fide* exosomes (from now on, "exosomes"). Moreover, we report that the exosomes can transport neuroglobin (NGB). NGB, a protein produced mainly in neurons within the CNS but also detected in astrocytes, and exhibiting anti-oxidant, anti-apoptotic, and anti-inflammatory effects, might function as a neuroprotectant against hypoxic/ischemic insult, β-amyloid, or H_2O_2 toxicity (see Guidolin et al., 2014; Guidolin et al., 2016; Van Acker et al., 2019 and references therein). Noteworthy, the exosomes released from the astrocyte processes were able to selectively target neurons. The finding that astrocytic processes express and release NGB might contribute additional mechanisms to the astrocyte neuroprotective potential.

MATERIALS AND METHODS

Chemicals and Reagents

Percoll, bovine serum albumin (BSA), poly-L-ornithine, PKH67 fluorescent cell linker kit (catalog number PKH67GL MIDI67), and all the salts were from Sigma-Aldrich St. Louis, MO USA. The primary or secondary antibodies were from Sigma-Aldrich [mouse anti-synaptophysin, catalog number: S5768; rabbit anti-glial fibrillary protein (GFAP), catalog number: G9269; mouse anti-GFAP (clone G-A-5), catalog number: G3893; mouse anti-ezrin, catalog number: E8897; mouse anti-β-actin, catalog number: A2228; rabbit anti-β III tubulin, catalog number: SAB4500088], from Synaptic Systems, Goettingen, Germany [rabbit anti-microtubule-associated protein 2 (MAP2), catalog number: 188 003], from Merck Millipore Corporation, Darmstadt, Germany [mouse anti-oligodendrocyte (RIP), catalog number: MAB1580; mouse anti-integrin-αM (clone OX-42), catalog number: CBL 1512], from Santa Cruz Biotechnology Inc, Dallas, TX USA [rabbit anti-NGB (clone FL-151); catalog number: sc-30144] or from Thermo-Fisher Scientific Inc, Waltham, MA USA [mouse anti-Alix (clone 3A9), catalog number: MA1-83977; mouse anti-Tsg101 (clone 4A10), catalog number: MA1-23296; Alexa-Fluor 488 or 633 conjugated goat anti-rabbit or anti-mouse secondary antibodies]. The horseradish peroxidase-linked anti-rabbit or anti-mouse secondary antibodies were from Cell Signaling Technology Inc, Danvers, MA USA. Prolong Gold Antifade Mountant were from Molecular Probes, Eugene, OR USA; the microporous filters and the polyvinylidene difluoride membrane were bought from Merck Millipore Corporation, Darmstadt, Germany. The mini gel used for western blot were from Bio-Rad Laboratories, Hercules, CA USA; ECL-PLUS kit was from GE Healthcare, Milano, Italy; Neurobasal, DMEM, B27, Glutamax, and Pen-Strepto were from Gibco by Thermo-Fischer Scientific Inc.

Animals

Adult male rats (200–250 g, Sprague–Dawley) were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light-dark schedule (lights on 7 AM–7 PM). Food and water were freely available. To prepare primary neuronal cultures Sprague–Dawley rat embryos at the day 18 of gestation (E18) were used. The pregnant dams were anesthetized and the embryos were extracted by caesarian section.

Animal care and experimental procedures complied with the European Communities Parliament and Council Directive of 22 September 2010 (2010/63/EU) and with the Italian D.L. n. 26/2014, and were approved by the Italian Ministry of Health

(protocol number 26768 of November 2012 and protocol number 75F11.N.6JI of August 2018), in accordance with Decreto Ministeriale 116/1992. All efforts were made to minimize the number of animals used and their suffering, and no *in vivo* technique was used.

Preparation of Purified Astrocytic Processes

Purified astrocyte processes (gliosomes) were prepared from the cerebral cortex of adult male rats. Briefly, after decapitation, the tissue was rapidly removed and placed in ice-cold medium. Purified gliosomes were prepared by a discontinuous Percoll gradient according to Nakamura (Nakamura et al., 1993) as previously reported (see Cervetto et al., 2018). Briefly, rat cerebral cortices were homogenized in 10 volumes of 10 mM Tris/HCl pH 7.4 with 0.32 M sucrose, using a glass-Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged (5 min at 4°C and 1,000 g) to remove nuclei and debris and the supernatant stratified on a discontinuous Percoll gradient (2, 6, 10, and 20% (v/v) in Tris-buffered sucrose) and centrifuged for 5 min at 4°C and 33,000 g. The layer between 2% and 6% (v/v) Percoll (gliosomal fraction; purified astrocyte processes) was collected and washed by centrifugation. For release experiments, purified astrocyte processes were suspended in standard HEPES medium (mM: NaCl 128, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.0, and HEPES 10 with glucose 10, pH 7.4). Protein determinations were carried out using serum bovine albumin as the standard (see Cervetto et al., 2018).

Confocal Microscopy on Gliosomes and Synaptosomes

Immunofluorescence confocal microscopy on gliosomes and synaptosomes was performed according to sequential staining methods (see Cervetto et al., 2016; Cervetto et al., 2018). Briefly, gliosomes and synaptosomes were fixed and permeabilized in 2% paraformaldehyde (PFA)/0,1% Triton X-100 in phosphate buffer solution (PBS) pH 7.4 and then incubated in the diluted primary antibodies in 3% BSA in PBS (over-night, 4°C). The following primary antibodies were used: mouse anti-synaptophysin (1:1,000), rabbit anti-GFAP (1:1,000); mouse anti-RIP (1:10,000), and mouse anti-integrin-αM (1:25). After washing with PBS the preparations were incubated with the appropriate Alexa-Fluor 488 or 633 conjugated secondary antibodies (1:1,000). Gliosomes and synaptosomes were then smeared onto coverslips with anti-fade mounting medium (ProLong Gold). Images were collected by means of a three-channel TCS SP2 laser-scanning confocal microscope (Leica Wetzlar, Germany) using a plan apochromatic oil immersion objective 60×/numeric aperture 1.43. The ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) was used to count positive particles using 3D-counter object analyzed application (Threshold = 50 in all the fields—Fiji ImageJ). The percentage of GFAP, synaptophysin, RIP, and integrin-αM positive particles was estimated in three to five non-overlapping fields from three different preparations of gliosomes and synaptosomes, and are expressed as mean ± SEM.

Release Experiments and Extracellular Vesicle Isolation and Characterization

We collected the EVs released from the astrocyte processes essentially by applying the method used to collect the gliotransmitters released from isolated perfused astrocyte processes (gliosomes) or the neurotransmitters released from isolated perfused nerve terminals (synaptosomes). Briefly, gliosomes were stratified on microporous filters (MF-Millipore™, Thickness: 180μm; Pore size: 0.65μm; Merck-Millipore) at the bottom of parallel perfusion chambers at 37° C and continuously perfused (0.5 ml/min) with a standard medium as described previously (Cervetto et al., 2017; Cervetto et al., 2018). After 5-min perfusion, perfusate fractions were collected in a 10-min sample. The perfusate was pelleted by ultracentrifugation at 110,000 g for 90 min (Guescini et al., 2010) and the EVs were resuspended i) in PBS to perform nanosight analysis by using a dynamic light scattering; ii) in loading buffer for performing western blot analysis; iii) in diluent C, according to PKH67 kit technical instructions, to be labeled with the exosome dye PKH67 for assessing their ability to target cells.

Dynamic Light Scattering

To measure the size of the EVs released from astrocyte processes we performed the nanosight analysis on ultracentrifugation pellet resuspended in PBS using the Zetasizer Nano ZS90 particle sizer at a 90° fixed angle (Malvern Instruments, Worcestershire, United Kingdom), as previously described (Marimpietri et al., 2013). Nanosphere™ size standards with a mean diameter of 57 ± 4 nm (Thermo Scientific) were used for particle sizer calibration. The analysis was replicated on three different samples.

Western Blot

The western blot analysis was performed both on gliosomes, synaptosomes, and EVs. Proteins were denatured in Laemmli sample buffer and then subjected to a SDS-polyacrylamide gel electrophoresis (13% or 4–20% gradient mini gel) 200 V for 50 min (gliosomes: 5–20 μg/lane; synaptosomes: 10μg/lane; EVs: estimated amount of proteins: 2.53–6.32 μg/lane; Mini-Protean TGX Gel, Bio-Rad Laboratories), followed by electroblotting (100 V for 50 min) on polyvinylidene difluoride membrane (Immobilon-P PVDF; Millipore Corporation). The blot has been cut probing different regions of the same blot with multiple antibodies. Immunodetection was performed using the following primary antibodies: mouse anti-Alix (1:1,000); mouse anti-Tsg101 (1:800); rabbit anti-NGB (1:300); mouse anti-GFAP (1:1,000); mouse anti-ezrin (1:500); rabbit anti-MAP-2 (1:1,000); and rabbit anti-β III tubulin (1:1,000). Primary antibodies were incubated over-night at 4°C followed by washing and the application of horseradish peroxidase-linked anti-rabbit or anti-mouse (Cell Signaling Technology) secondary antibodies, incubated for 1 h at room temperature. Western blots were developed with the ECL-PLUS kit (GE Healthcare), according to the manufacturer's instructions. Band detection and densitometry were performed using the Chemi-Doc System and the quantity one software package (Bio-Rad

Laboratories). The membranes were stripped using Re-blot plus solution (Merck-Millipore Corporation) and re-probed with mouse anti-β-actin (1:10,000) also to estimate the amounts of proteins in exosomes. Developed films were analyzed using specific software (GelDoc; Bio-Rad Laboratories).

Electron Microscopy

Ultrastructural analysis of gliosomes and exosomes was performed by negative staining method. Briefly, 5µl drops of gliosomes or purified exosomes were placed onto formvar and carbon-coated copper grids and adsorbed for 20 min at room temperature. The excess of buffer was removed by using a filter paper. Then, grids were fixed in 2% PFA in PBS pH 7.2 for 5 min and washed out three times on large drops of distilled water. Grids were then incubated for 5 min at room temperature with 1% aqueous solution of uranyl acetate. Contrast enhancement was obtained by further incubating the grids with a mixture of 1% uranyl acetate and 1% methylcellulose for 5 min. After drying, grids with gliosomes or exosomes were immediately observed with a CM10 electron microscope (Philips, Eindhoven, The Netherlands). Digital images were taken with a Megaview II camera.

Labeling of Exosomes

For immunofluorescence analysis, the exosome pellet was resuspended in diluent C and stained with the dye PKH67 according to the producer's technical bulletin (Fitzner et al., 2011). The dye was gently pipetted with the sample, and after 5 min at room temperature, the staining reaction was stopped bringing the volume up to 35 ml with 10% BSA in PBS. Exosomes were pelleted by ultracentrifugation (110,000 g for 90 min) and resuspended in 150 µl of Neurobasal. In parallel as control condition, we prepared the control samples with equal volumes of PBS plus the same amount of diluent C, PKH67 dye, 10% BSA and PBS, and by omitting the exosomes, to exclude any non-specific labeling of cells by micelles of the aliphatic dye or by the excess of dye.

Neuron-Astrocyte Co-Cultures

Primary cortical cells were derived from Sprague–Dawley rat embryonic day 18 (E18). Culture preparation was performed as previously described (Chiappalone et al., 2006). Briefly, E18 timed pregnant Sprague–Dawley rat was euthanized by CO_2 and cervically dislocated in accordance with institutionally approved animal care. Embryos were dissected and cortices isolated in Hank's buffer solution without Ca^{2+} and Mg^{2+}. All tissue was collected and maintained in ice-cold buffer solution and, to obtain a single-cell suspension, cerebral cortices were enzymatically digested at 37°C with warm TrypLe Express for 15–18 min in a water bath. The digestion was stopped by adding medium (Neurobasal or DMEM) complemented with 10% FCS (fetal calf serum) for 3 min, after this interval the medium was carefully removed and the cortices, transferred in Neurobasal/B27 (supplemented with Glutamax and Pen-Strepto), were mechanically triturated with a sterile fire-polished Pasteur pipette. Single-cell suspension was well mixed, counted, and diluted. Finally, cells were plated on poly-L-ornithine (100 µg/ml) coated coverslips inserted into multiwells plates at the density around 5.0x 10^4cell/cm^2. The primary cultures were kept at 37°C in humidified atmosphere of 5% CO_2 in air. The culture medium was changed weekly, until the uptake experiments at 21 DIV, at the end of the 3 weeks of development of the *in vitro* culture the percentage composition of the cell population was distributed with 70 ± 15% of neurons and 30 ± 15% of glial cell (the percentage of neurons and astrocytes were estimated in three to five non-overlapping fields from three different cultures, and are expressed as mean ± SEM), consistent with previous findings (Chiappalone et al., 2006). Immunofluorescence assays were performed using rabbit anti-MAP-2 (1:500), mouse anti-GFAP (1:1,000), and DAPI dye. See a representative image acquired by epifluorescence microscopy in **Figure 3A**.

Cellular Uptake of Exosomes

Exosomes, made fluorescent with the PKH67 lipophilic dye, were dispersed (16 µl) in the same culture medium where the coverslips were immersed with the neuronal networks and incubated for 1 h at 37°C in humidified atmosphere of 5% CO_2 in air.

In parallel, as a negative control, the same volume (16 µl) of control sample was loaded onto other coverslips from the same neuronal preparation, and left to incubate for 1 h under the same conditions as above. After the time interval had elapsed, both the coverslips with the exosomes and those with control sample were washed repeatedly to remove the excess.

To evaluate the uptake capabilities of the exosomes, the experiments were repeated on three different neuronal preparations, developed *in vitro* for 3 weeks, and the biological sample was subjected to specific marking to confirm its neuronal/glial morphology and exosomes uptake. Briefly, the cells were fixed in 4% PFA, blocked with 3% BSA, and incubated primarily with rabbit or mouse primary antibodies (over-night at 4°C in humid chamber) and then with Alexa Fluor 546 donkey anti-mouse and 633 goat anti-rabbit (1h at room temperature). The following primary antibodies were used: rabbit anti-β III tubulin (1:500) or rabbit anti-MAP2 (1:500), or rabbit or mouse anti-GFAP (1:1,000). The excessive antibodies were washed by PBS. The glass coverslips were mounted with antifade mounting medium and observed using confocal microscopy (see above).

RESULTS

Gliosomes Obtained From Adult Rat Cerebral Cortex Are a Purified Preparation of Cerebrocortical Astrocyte Processes

At confocal microscopy, the astrocyte processes appeared labeled with the anti-GFAP antibody (a marker identifying astrocytes), and were negative for synaptophysin, integrin-αM, and RIP (markers for the nerve terminals, microglia, or oligodendrocytes, respectively. **Figures 1A–I**). As a control, we

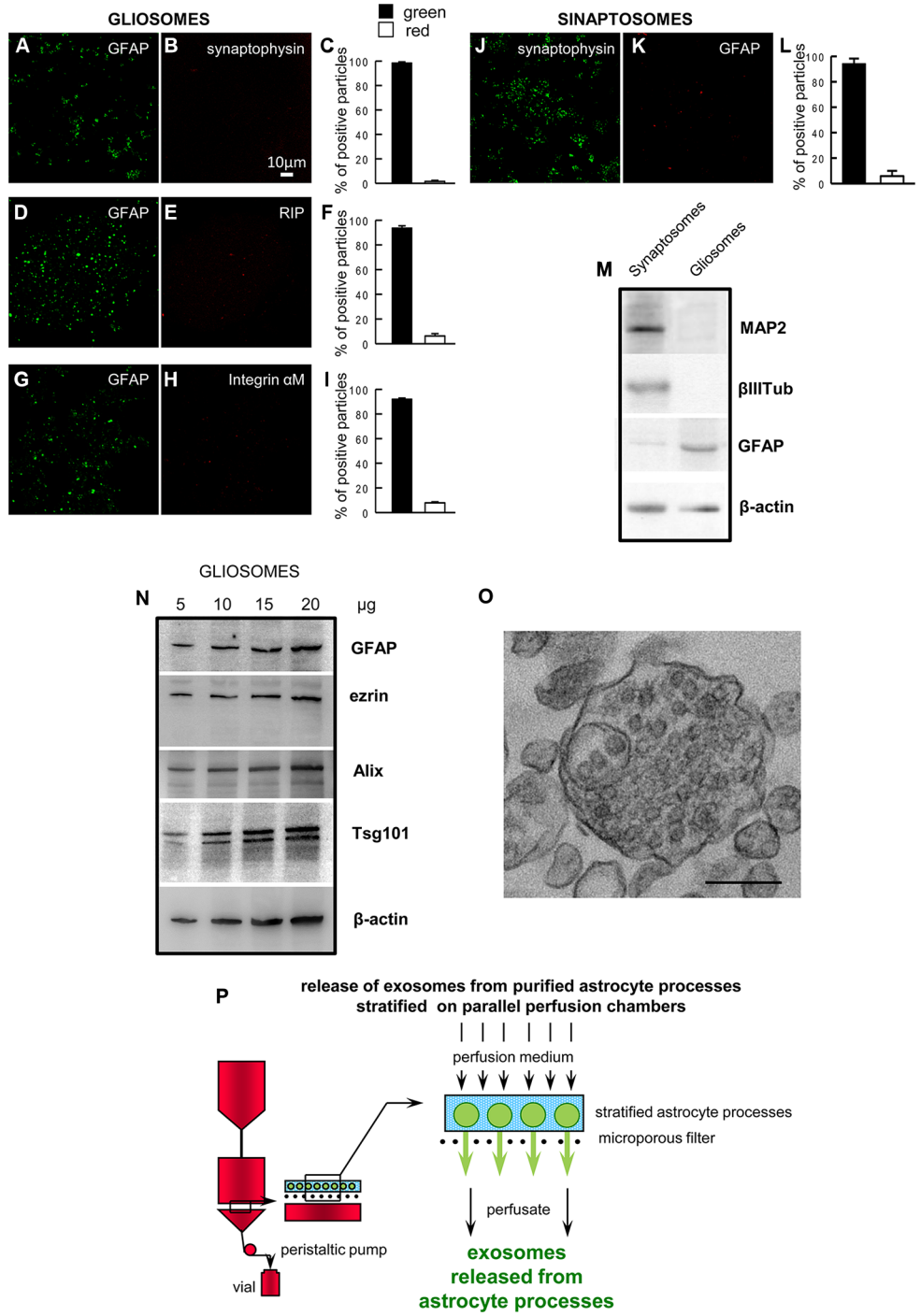

FIGURE 1 | Astrocyte processes obtained from adult rat cerebral cortex. Negligible contamination of gliosomes, positive for the specific glial marker GFAP **(A**, **D**, **G)**, by subcellular no-astrocytic particles. Immunofluorescent assay for synaptophysin **(B)**, RIP **(E)** or integrin-αM **(H)** markers for nerve terminals, microglia, and oligodendrocytes, respectively. As a positive control, the immunofluorescent assay for synaptophysin **(J)** was performed on cerebral cortical synaptosomes scarcely contaminated by subcellular GFAP-positive particles **(K)**. Bars **(C**, **F**, **I**, **L)** represent the percent of positive particles (% ± SEM of positive particles counted in three to five no-overlapping fields from n = 3 different preparations): GFAP (C, F, I, solid bars; L, empty bar), synaptophysin (C empty bar; L solid bar; L, empty bar), or RIP or integrin-αM (F or I, respectively; empty bar). Scale bars are indicated in the figures. Western blot analysis of gliosomes and synaptosomes **(M**, **N)**. The absence of cross-contamination of the astrocyte processes and nerve terminals is shown **(M)**: MAP2, β III tubulin, and GFAP proteins were used as selective markers for the synaptosome or gliosome preparations. Presence of the astrocytic markers GFAP and ezrin, and of the exosome markers Alix and Tsg101 in the gliosomes **(N)**. Electron microscopy image of a cortical astrocyte processes. A single gliosome is shown containing vesicles scattered in the cytoplasm and a multivesicular body **(O)**. Scale bars: 200 nm. Schematic of a perfusion unit of the apparatus allowing recovery of extracellular vesicles (exosomes) released from the processes during perfusion **(P)**. For other experimental details, see *Materials and Methods*.

also show that the nerve terminals (synaptosomes) prepared from rat cerebral cortex were positive for synaptophysin and negative for GFAP (**Figures 1J–L**). The Western blot analysis showed the absence of contamination by neural specific proteins (MAP2 and β III tubulin) in the gliosome preparation, and the negligible contamination of the cerebral cortical synaptosomes prepared in parallel (**Figure 1M**). The findings indicate that gliosomes are a purified preparation of processes of cerebrocortical astrocytes, negligibly contaminated by neuronal, microglial, or oligodendroglial particles. The processes were also analyzed by Western blot and were found to express the astrocytic markers GFAP and ezrin, the exosome endosomal-lysosomal sorting proteins Alix and Tsg101, and NGB (**Figure 1N**). The electron microscopy analysis on gliosomes revealed the presence of multivesicular bodies and scattered vesicles inside the astrocyte processes (**Figure 1O**).

Purified Astrocytic Processes Release Extracellular Vesicles Exhibiting the Characteristics of Exosomes

The EVs released and recovered in the perfusate from cerebrocortical astrocyte processes (see **Figure 1P** for a scheme of perfusion unit) were firstly analyzed using nanosight dynamic light-scattering analysis and electron microscopy imaging, and subsequently for the presence of the exosome specific protein markers Alix and Tsg101. At dynamic light-scattering analysis, the EVs showed a bell-shaped size distribution profile, peaking at mode 60 nm (range 50–75) (see in **Figure 2A** the tracing of a representative experiment from three different experiments with similar results). The observed size is consistent with the theoretical size of exosomes and previous observations (Skog et al., 2008). The typical cup shape appearance at the ultrastructural level and their size (electron microscopy images, **Figure 2B**) are consistent with previously reported exosome electron microscopy images (see Raposo and Stoorvogel, 2013). The EVs were verified for the presence of astrocytic markers, namely for GFAP and ezrin. Using western blot analysis, we obtained signaling for both GFAP and ezrin in the EVs recovered from the perfusion collected samples (**Figure 2C**), demonstrating the astrocytic source of the particles collected.

Both the exosome specific protein markers Alix and Tsg101 were present in the vesicles (**Figure 2D**), confirming that the EVs recovered in the perfusate from the processes exhibit the features of exosomes.

Gliosomes and exosomes were also labeled with anti-NGB antibody (**Figure 2E**), indicating that exosomes carry NGB protein.

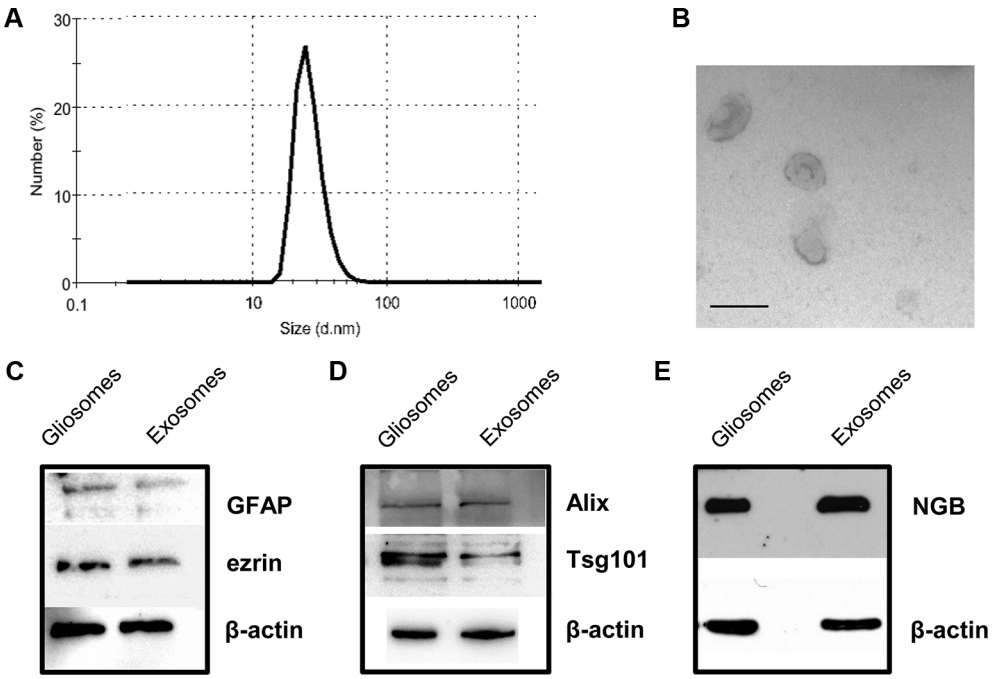

FIGURE 2 | Characterization of rat cerebrocortical astrocyte processes-released exosomes. Size distribution of exosomes released from the astrocyte processes, as assessed by the zetasizer nano ZS90 particle sizer. Curve shows a representative tracing (from three samples obtained from three different experiments with similar results) **(A)**. Electron microscopy images of vesicles released from astrocyte processes. Note the cup shape appearance and size, consistent with previously reported exosome electron microscopy images characteristics **(B**; scale bar: 100 nm). Presence of astrocytic markers, exosomal markers, and of NGB **(C–E)**. Western blot for the astrocytic markers GFAP and ezrin in gliosome preparation and in exosomes released from gliosomes **(C)**. Western blot for the exosomal markers Alix and Tsg101 in gliosome preparation and in gliosome-released exosomes **(D)**. Western blot for NGB in gliosome preparation and in gliosome-released exosomes **(E)**. For other experimental details, see *Materials and Methods*.

The Released Exosomes Selectively Target Neurons and Can Be Internalized by Neurons

The exosomes released and recovered in the perfusate from cerebrocortical astrocyte processes were able to target cells in neuron-astrocyte co-cultures. Notably, in the co-cultures, only GFAP-negative cells were targeted by the exosomes, while GFAP-positive astrocytes were not. In particular, we found that exosomes targeted cells exhibiting the morphological features of neurons (**Figure 3**); labeling with the neuronal markers MAP-2 and β III tubulin confirmed the selective transfer of astrocyte-released exosomes to neurons (**Figures 3B–I**; **Video 1**). Notably, confocal microscopy confirmed the ability of exosomes to be internalized rather than being attached to the surface of the neuronal membrane (**Figures 3B–H**); we found evidence for exosome presence inside the neurons both at their projections (**Figures 3B–H**) and at the perinuclear region (**Figure 3I**). Interesting to note, exosome traveling to perinuclear region was already reported in PC12 cells as well as in human-induced pluripotent stem cells or in human neuroblastoma cell lines (Tian et al., 2010; Sardar Sinha et al., 2018).

DISCUSSION

Our main finding is represented by the fact that exosomes can be released from astrocyte processes and selectively target neurons; these exosomes might transfer NGB of astrocytic origin. The relevance and the novelty of these findings are to be considered in light of the following considerations:

-Although it was already demonstrated that exosomes can be released from cultured astrocytes (Guescini et al., 2010), astrocytes in culture can only marginally mimic the behavior of astrocytes in situ. We here report on processes of astrocytes acutely prepared from adult rat cerebral cortex, thus reflecting the behavior of astrocyte processes in mature cerebrocortical neuron-astrocyte networks. Notably, these processes were positive for ezrin, an astrocytic cytoskeletal protein selective marker of the perisynaptic astrocyte processes, required for the astrocyte processes structural plasticity (see Cervetto et al., 2018 and references therein). In the perfusate from the processes we collected particles that proved to be positive for the specific protein markers for exosomes, the endosomal-lysosomal sorting proteins Alix and Tsg101, indicating that exosomes can be released from the processes. In fact, electron microscopy imaging indicated the presence of multivesicular bodies in the processes, consistent with their ability to release exosomes. Astrocyte processes might therefore participate in a roamer-type of volume transmission through the release of exosomes. Thus, the processes are capable of contributing in multiple modes to the signal transmission in CNS, both receiving messages and sending messages of different type, and presumably with different half-life and targets, such as the gliotransmitters (e.g. glutamate, that can be rapidly taken up and/or activate non synaptic glutamate receptors) and signals that may be transferred through EVs. Notably, perisynaptic processes exhibit plasticity and can rapidly change their morphology, modifying the coverage of pre- and postsynaptic elements at the synapses (Reichenbach et al., 2010; Bernardinelli et al., 2014); plasticity of perisynaptic processes has been reported to result in dramatic changes of the interstitial space during sleep or pharmacological anesthesia (Xie et al., 2013). We can hypothesize that the conceivable consequent opening of the synapses might reduce the "privacy" of synaptic wiring transmission in favor of volume transmission, suggesting that astrocyte signaling through volume transmission might have different relevance and functions depending on physiological cycles and the state of synaptic coverage. This would contribute to the shift from a neurocentric to a neuro-astrocentric view of the brain functioning, as the perisynaptic astrocyte processes may be the source for both classical volume transmission through the release of gliotransmitters, and for roamer-type volume transmission through the release of exosomes. By this way, the astrocyte processes might be capable of inducing transient phenotype changes in the receiving cells (see Agnati et al., 2014 and references therein).

The exosomes released from the astrocyte processes were found to target neurons. It was already shown that exosomes from cultured astrocytes could contact *co*-cultured neurons to promote neurite outgrowth of neighboring neurons and/or neuronal survival (see Janas et al., 2016 and references therein; Frühbeis et al., 2012; Caruso Bavisotto et al., 2019) and could protect neurons against ischemic damage (see Hira et al., 2018; Pei et al., 2019; Xu et al., 2019). This is however to our knowledge, the first evidence indicating that *ex-vivo* astrocytes —in particular, astrocyte processes acutely prepared from adult astrocytes that have matured in neuron-astrocyte network—can selectively communicate to neurons through exosomes. In the framework of the complex bidirectional signaling coordinating the function of the neuron-astrocyte networks the evidence that the astrocyte processes can release exosomes to target neurons adds a further mode of astrocyte-to-neuron communication that might be of significant (and so far uncovered) importance in physiological as well as in pathological conditions. As a matter of fact, exosomes transfer from astrocyte might result in a transient phenotype change of the receiving neurons, e.g. by enrichment in neuroprotective factors (see below) or by expression of receptors (see exosomes carrying functionally competent neurotransmitter receptors to receiving cells; Guescini et al., 2012) making neurons transiently able to recognize and decode extracellular signals, with possible relevant neuropharmacological implications. The ability of astrocyte-derived exosomes to effectively transfer signals and functions to neurons is a crucial point worth to be investigated in the future. Also, it remains not understood why some neurons (and their projections) are preferentially targeted by exosomes; further investigation is required to understand the neurochemical characteristics or the attracting pathways of the neurons/neuronal projections to which exosomes preferentially bind.

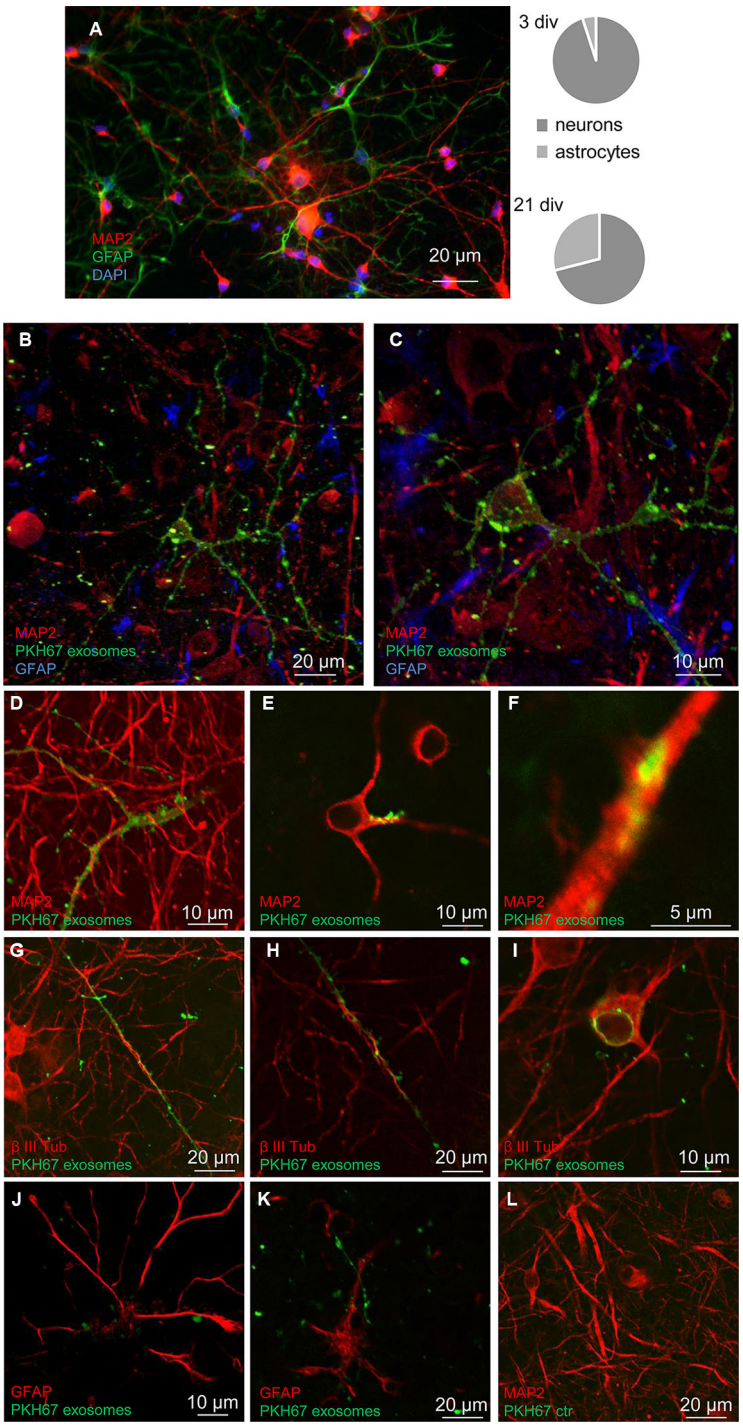

FIGURE 3 | Neurons: targets for the exosomes. Confocal images showing exosomes targeting neurons when added to a neuron-astrocyte co-culture. Characteristics of the neuron-astrocyte co-culture; see coexistence of GFAP-positive (green) astrocytes and MAP2-positive (red) neurons in a representative epifluorescence microscope image at 21 div and their relative distribution at 3 and 21 div culture **(A)**. DAPI stained was used to marker cellular nucleus. Scale bar is indicated in the figure. See that exosomes (marked with PKH67, green) preferentially contact GFAP-negative cells, while GFAP-positive astrocytes are not targeted (blue in B, C, red in J, K). Exosomes selectively target cells positive for the neuronal markers MAP2 (in **B**, **C**, **D**–**F)** or β III tubulin (red in G-I). The images are the merge of a single z stack of the two channels **(D**–**F**, **H**–**L)** or representative maximum intensity projections of the acquired z stacks of the two or three channels **(B**–**C**; **G)**. See in **Video 1** the z-axis analysis related to the panel B. Note internalization of exosomes **(D**–**I)**: evidence for exosome presence inside the neuronal projections **(D**–**H)** and at the perinuclear region **(I)**. A control sample prepared in parallel by omitting the exosome excluded the non-specific labeling of cells **(L)**. Scale bars are indicated in the figures. For other experimental details, see *Materials and Methods*.

-The exosomes were found to carry NGB. As a matter of fact, it was initially thought that NGB in mammals was expressed exclusively in neurons of the nervous system. NGB, however, was also observed in astrocytes and reactive astrocytes (see Della Valle et al., 2010 and references therein). In particular, it was hypothesized that NGB may be produced by astrocytes for secretion, possibly as a neuroprotective agent for neurons (Della Valle et al., 2010). Astrocytes, indeed, are recognized to play multiple roles in diverse pathological conditions in the brain, playing both neuroprotective and detrimental roles (see Verkhratsky et al., 2016) The possibility that astrocyte processes could release NGB through exosomes would allow them to send long-distance messages to cells, to transiently change their susceptibility to damage, and to participate in the beneficial effects of astrocytes in ischemic injury (see Verkhratsky et al., 2016 and references therein).

Actually, NGB can serve multiple crucial roles in cell defense and resistance to degeneration, and transferring NGB from astrocytes might contribute to protecting neurons. In this respect, it was reported that estradiol regulates NGB expression both in neurons and astrocytes through ERβ-mediated mechanisms and that this regulation of the expression of NGB may be part of the neuroprotective mechanisms activated by estradiol in astrocytes (see references in Guidolin et al., 2014; Guidolin et al., 2016). As a matter of fact, transferring signals through exosomes has been proposed to be involved in the participation of glial cells to neurodegeneration or neuroprotection (see Verkhratsky et al., 2016; Lafourcade et al., 2016); by supporting the ability of astrocyte-derived exosomes to target neurons, our findings indicate that astrocytes might participate to neuron neuroprotection by transferring NGB through this mode of astrocyte-neuron communication. Notably, the roles for exosomes in transferring protective signals to neurons were already suggested on the basis of data from cultured astrocytes (Taylor et al., 2007; Wang et al., 2011; Guitart et al., 2016; Hira et al., 2018; Pascua-Maestro et al., 2019; Pei et al., 2019; Xu et al., 2019).

-Exosomes, being released from a variety of cells, have been proposed as peripheral markers for diagnostic-prognostic purposes in various diseases. They have been also proposed as peripheral markers for CNS diseases; however, one of the problems in their reliability as markers, besides the correct classification of exosomes, is their origin (Raposo and Stoorvogel, 2013). It is to note that exosomes recovered in the blood and originating from astrocytes were reported to behave as marker for stress-induced disease (Gómez-Molina et al., 2019). Also, recently GFAP-positive exosomes originating from astrocytomas were found in the blood and were claimed to be of help to the glioma classification (Van Bodegraven et al., 2019). Furthermore, astrocyte-derived EVs were found in periphery in neuroinflammatory conditions or after brain focal radiation (Dickens et al., 2017; Willis et al., 2017; Cai et al., 2017). In addition to functioning as biomarkers of different pathological conditions, the astrocyte-derived exosomes in blood might also target peripheral organs in the brain-to-periphery signaling (Dickens et al., 2017; Cai et al., 2017; see also Gómez-Molina et al., 2019). Worthy of note, we here indicate that a subcellular region of astrocytes—the processes that are devoted to sending/receiving signals in the nervous system—might be primarily involved in signal communication through exosomes. Notably, the finding that astrocyte-derived exosomes are positive for astrocytic markers allows hypothesizing that analysis of these markers could help to understand the cellular origin of (parental cells originating the) exosomes that might be recovered in peripheral blood from healthy or diseased CNS.

CONCLUSIONS

In conclusion, our findings for the first time indicate that the astrocyte processes acutely prepared from astrocytes matured in a neuron-astrocyte network in CNS might participate to signal transmission by releasing exosomes, which, in turn might target near or long-distance targets by volume transmission. The exosomes released by the processes proved to selectively target neurons, adding a new non-conventional mode of astrocyte-to-neuron signal transmission, with unexplored impact on integrative communication in the CNS and neuropharmacological implications. Indeed, releasing NGB-carrying exosomes might be a mode for astrocytes to operate as a signal to protect neighboring cells in neuron-astrocyte networks. Also, our findings could help to understand the parental cell origin of the exosomes that might be recovered from peripheral blood.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Organismo Preposto al Benessere Animale OPBA, University of Genova and Italian Ministry of Health: protocol number 26768 of November 2012 (tissue preparation from humanely sacrificed adult rats); protocol number 75F11.N.6JI of August 2018 (primary neuron-astrocyte cultures from E18rats), in accordance with Decreto Ministeriale 116/1992. No *in vivo* experiment was performed.

AUTHOR CONTRIBUTIONS

GM, DG, LA, MM, and CC initiated the project. MM and CC designed the experiments. AV, SP, and CC performed the animal experiments. AV, SP, GL, and CC performed isolation and mark of exosomes. AV and MP performed the Western blot. FP performed the nanosight dynamic light-scattering analysis. KC and MG performed ultrastructural analysis. MT and CC

performed cell cultures and exosome uptake experiments. MP, MT, and CC performed cell imaging. AV, SP, GL, and CC analyzed the data. MP, FP, MT, GM, MM, and CC wrote the manuscript. DG and LA revised the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by the University of Genova [Grant number 020301002054 to MM, Grant numbers D31J1100003005 and D31J1100161005 to CC]; and the University of Padova [Grant number 60A06-0481/14 to DG]. The funding sources had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.01452/full#supplementary-material

VIDEO 1 | Neurons: targets for the exosomes. A z-axis analysis of astrocyte-derived exosomes marked with PKH67 (green) co-localization with MAP2 (red) in primary astrocyte neuron co-culture (scanning 0.14 μm z axis; 50 stacks). The merge images of each z field were imported as a sequence and then saved as a movie with three frames per second (ImageJ). GFAP (blue).

REFERENCES

Agnati, L. F., and Fuxe, K. (2000). Volume transmission as a key feature of information handling in the central nervous system possible new interpretative value of the Turing's B-type machine. *Prog. Brain Res.* 125, 3–19. doi: 10.1016/S0079-6123(00)25003-6

Agnati, L. F., Guidolin, D., Maura, G., Marcoli, M., Leo, G., Carone, C., et al. (2014). Information handling by the brain: proposal of a new "paradigm" involving the roamer type of volume transmission and the tunneling nanotube type of wiring transmission. *J. Neural Transm. (Vienna)* 12, 1431–1449. doi: 10.1007/s00702-014-1240-0

Agnati, L. F., Marcoli, M., Maura, G., Woods, A., and Guidolin, D. (2018). The brain as a "hyper-network": the key role of neural networks as main producers of the integrated brain actions especially *via* the "broadcasted" neuroconnectomics. *J. Neural Transm. (Vienna)* 125, 883–897. doi: 10.1007/s00702-018-1855-7

Araque, A., Parpura, V., Sanzgiri, R. P., and Haydon, P. G. (1999). Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci.* 22, 208–215. doi: 10.1016/S0166-2236(98)01349-6

Bernardinelli, Y., Muller, D., and Nikonenko, I. (2014). Astrocyte-synapse structural plasticity. *Neural Plast.* 2014, 232105. doi: 10.1155/2014/232105

Cai, S., Shi, G. S., Cheng, H. Y., Zeng, Y. N., Li, G., Zhang, M., et al. (2017). Exosomal mir-7 mediates bystander autophagy in lung after focal brain irradiation in mice. *Int. J. Biol. Sci.* 13, 1287–1296. doi: 10.7150/ijbs.18890

Caruso Bavisotto, C., Scalia, F., Marino Gammazza, A., Carlisi, D., Bucchieri, F., Conway de Macario, E., et al. (2019). Extracellular vesicle-mediated cell–cell communication in the nervous system: focus on neurological diseases. *Int. J. Mol. Sci.* 20, 434. doi: 10.3390/ijms20020434

Cervetto, C., Vergani, L., Passalacqua, M., Ragazzoni, M., Venturini, A., Cecconi, F., et al. (2016). Astrocyte-dependent vulnerability to excitotoxicity in spermine oxidase overexpressing mouse. *Neuromol. Med.* 18 (1), 50–68. doi: 10.1007/s12017-015-8377-3

Cervetto, C., Venturini, A., Passalacqua, M., Guidolin, D., Genedani, S., Fuxe, K., et al. (2017). A2A-D2 receptor-receptor interaction modulates gliotransmitter release from striatal astrocyte processes. *J. Neurochem.* 140, 268–279. doi: 10.1111/jnc.13885

Cervetto, C., Venturini, A., Guidolin, D., Maura, G., Passalacqua, M., Tacchetti, C., et al. (2018). Homocysteine and A2A-D2 receptor-receptor interaction at striatal astrocyte processes. *J. Mol. Neurosci.* 65, 456–466. doi: 10.1007/s12031-018-1120-4

Chiappalone, M., Bove, M., Vato, A., Tedesco, M., and Martinoia, S. (2006). Dissociated cortical networks show spontaneously correlated activity patterns during *in vitro* development. *Brain Res.* 1093, 41–53. doi: 10.1016/j.brainres.2006.03.049

Della Valle, B., Hempel, C., Kurtzhals, J. A., and Penkowa, M. (2010). *In vivo* expression of neuroglobin in reactive astrocytes during neuropathology in murine models of traumatic brain injury, cerebral malaria, and autoimmune encephalitis. *Glia* 58, 1220–1227. doi: 10.1002/glia.21002

Dickens, A. M., Tovar, Y. R. L. B., Yoo, S. W., Trout, A. L., Bae, M., Kanmogne, M., et al. (2017). Astrocyte-shed extracellular vesicles regulate the peripheral leukocyte response to inflammatory brain lesions. *Sci. Signal* 10, eaai7696. doi: 10.1126/scisignal.aai7696

Février, B., and Raposo, G. (2004). Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr. Opin. Cell Biol.* 16, 415–421. doi: 10.1016/j.ceb.2004.06.003

Fitzner, D., Schnaars, M., Van Rossum, D., Krishnamoorthy, G., Dibaj, P., Bakhti, M., et al. (2011). Selective transfer of exosomes from oligodendrocytes to microglia by micropinocytosis. *J. Cell Sci.* 124, 447–458. doi: 10.1242/jcs.074088

Frühbeis, C., Fröhlich, D., and Krämer-Albers, E.-M. (2012). Emerging roles of exosomes in neuron–glia communication. *Front. Physiol.* 3, 119. doi: 10.3389/fphys.2012.00119

Gómez-Molina, C., Sandoval, M., Ramirez, J. P., Varas-Godoy, M., Luarte, A., Lafourcade, C. A., et al. (2019). Small extracellular vesicles in rat serum contain astrocyte-derived protein biomarkers of repetitive stress. *Int. J. Neuropsychopharmacol.* 22, 232–246. doi: 10.1093/ijnp/pyy098

Guescini, M., Genedani, S., Stocchi, V., and Agnati, L. F. (2010). Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. *J. Neural Transm.* 117, 1–4. doi: 10.1007/s00702-009-0288-8

Guescini, M., Leo, G., Genedani, S., Carone, C., Pederzoli, F., Ciruela, F., et al. (2012). Microvesicle and tunneling nanotube mediated intercellular transfer of G-protein coupled receptors in cell cultures. *Exp. Cell Res.* 318, 603–613. doi: 10.1016/j.yexcr.2012.01.005

Guidolin, D., Agnati, L. F., Tortorella, C., Marcoli, M., Maura, G., Albertin, G., et al. (2014). Neuroglobin as a regulator of mitochondrial-dependent apoptosis: A bioinformatics analysis. *Int. J. Mol. Med.* 33, 111–116. doi: 10.3892/ijmm.2013.1564

Guidolin, D., Tortorella, C., Marcoli, M., Maura, G., and Agnati, L. F. (2016). Neuroglobin, a factor playing for nerve cell survival. *Int. J. Mol. Sci.* 17, 1817. doi: 10.3390/ijms17111817

Guitart, K., Loers, G., Buck, F., Bork, U., Schachner, M., and Kleene, R. (2016). Improvement of neuronal cell survival by astrocyte-derived exosomes under hypoxic and ischemic conditions depends on prion protein. *Glia* 64, 896–910. doi: 10.1002/glia.22963

Halassa, M. M., and Haydon, P. G. (2010). Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior. *Annu. Rev. Physiol.* 72, 335–355. doi: 10.1146/annurev-physiol-021909-135843

Hira, K., Ueno, Y., Tanaka, R., Miyamoto, N., Yamashiro, K., Inaba, T., et al. (2018). Astrocyte-derived exosomes treated with a Semaphorin 3A inhibitor enhance stroke recovery *via* prostaglandin D_2 synthase. *Stroke* 49, 2483–2494. doi: 10.1161/STROKEAHA.118.021272

Janas, A. M., Sapoń, K., Janas, T., Stowell, M. H. B., and Janas, T. (2016). Exosomes and other extracellular vesicles in neural cells and neurodegenerative diseases. *Biochim. Biophys. Acta* 1858, 1139–1151. doi: 10.1016/j.bbamem.2016.02.011

Lafourcade, C., Ramírez, J. P., Luarte, A., Fernández, A., and Wyneken, U. (2016). MiRNAs in Astrocyte-derived exosomes as possible mediators of neuronal plasticity. *J. Exp. Neurosci.* 2016, 10(s1. doi: 10.4137/JEN.S39916

Marimpietri, D., Petretto, A., Raffaghello, L., Pezzolo, A., Gagliani, C., Tacchetti, C., et al. (2013). Proteome profiling of neuroblastoma-derived exosomes reveal the

expression of proteins potentially involved in tumor progression. *PloS One* 8, e75054. doi: 10.1371/journal.pone.0075054

Nakamura, Y., Iga, K., Shibata, T., Shudo, M., and Kataoka, K. (1993). Glial plasmalemmal vesicles: a subcellular fraction from rat hippocampal homogenate distinct from synaptosomes. *Glia* 9, 48–56. doi: 10.1002/glia.440090107

Pascua-Maestro, R., Gonzalez, E., Lillo, C., Ganfornina, M. D., Falcon-Perez, J., and Sanchez, D. (2019). Extracellular vesicles secreted by astroglial cells transport Apolipoprotein D to neurons and mediate neuronal survival upon oxidative stress. *Front. Cell. Neurosci.* 12, 526. doi: 10.3389/fncel.2018.00526

Pei, X., Li, Y., Zhu, L., and Zhou, Z. (2019). Astrocyte-derived exosomes suppress autophagy and ameliorate neuronal damage in experimental ischemic stroke. *Exp. Cell Res.* 382, 111474. doi: 10.1016/j.yexcr.2019.06.019

Raposo, G., and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* 200, 373–383. doi: 10.1083/jcb.201211138

Reichenbach, A., Derouiche, A., and Kirchhoff, F. (2010). Morphology and dynamics of perisynaptic glia. *Brain Res. Rev.* 63, 11–25. doi: 10.1016/j.brainresrev.2010.02.003

Sardar Sinha, M., Ansell-Schultz, A., Civitelli, L., Hildesjo, C., Larsson, M., Lannfelt, L., et al. (2018). Alzheimer's disease pathology propagation by exosomes containing toxic amyloid-beta oligomers. *Acta Neuropathol.* 136, 41–56. doi: 10.1007/s00401-018-1868-1

Skog, J., Würdinger, T., Van Rijn, S., Meijer, D. H., Gainche, L., Sena-Esteves, M., et al. (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* 10, 1470–1476. doi: 10.1038/ncb1800

Sofroniew, M. V. (2015). Astrocyte barriers to neurotoxic inflammation. *Nat. Rev. Neurosci.* 16, 249–263. doi: 10.1038/nrn3898

Taylor, A. R., Robinson, M. B., Gifondorwa, D. J., Tytell, M., and Milligan, C. E. (2007). Regulation of heat shock protein 70 release in astrocytes: role of signaling kinases. *Dev. Neurobiol.* 67 (13), 1815–1829. doi: 10.1002/dneu.20559

Thalhammer, A., and Cingolani, L. A. (2014). Cell adhesion and homeostatic synaptic plasticity. *Neuropharmacology* 78, 23–30. doi: 10.1016/j.neuropharm.2013.03.015

Tian, T., Wang, Y., Wang, H., Zhu, Z., and Xiao, Z. (2010). Visualizing of the cellular uptake and intracellular trafficking of exosomes by live-cell microscopy. *J. Cell Biochem.* 111, 488–496. doi: 10.1002/jcb.22733

Van Acker, Z. P., Luyckx, E., and Dewilde, S. (2019). Neuroglobin expression in the brain: a story of tissue homeostasis preservation. *Mol. Neurobiol.* 56 (3), 2101–2122. doi: 10.1007/s12035-018-1212-8

Van Bodegraven, E. J., van Asperen, J. V., Robe, P. A. J., and Hol, E. M. (2019). Importance of GFAP isoform-specific analyses in astrocytoma. *Glia* 67, 1417–1433. doi: 10.1002/glia.23594

Verkhratsky, A., Matteoli, M., Parpura, V., Mothet, J. P., and Zorec, R. (2016). Astrocytes as secretory cells of the central nervous system: idiosyncrasies of vesicular secretion. *EMBO J.* 35, 239–257. doi: 10.15252/embj.201592705

Vizi, E. S. (2000). Role of high-affinity receptors and membrane transporters in nonsynaptic communication and drug action in the central nervous system. *Pharmacol. Rev.* 52, 63–89.

Wang, S., Cesca, F., Loers, G., Schweizer, M., Buck, F., Benfenati, F., et al. (2011). Synapsin I is an oligomannose-carrying glycoprotein, acts as an oligomannose-binding lectin, and promotes neurite outgrowth and neuronal survival when released *via* glia-derived exosomes. *J. Neurosci.* 31 (20), 7275–7290. doi: 10.1523/JNEUROSCI.6476-10.2011

Wang, G., Dinkins, M., He, Q., Zhu, G., Poirier, C., Campbell, A., et al. (2012). Astrocytes secrete exosomes enriched with proapoptotic ceramide and Prostate Apoptosis Response 4 (PAR-4): potential mechanism of apoptosis induction in Alzheimer Disease (AD). *J. Biol. Chem.* 287 (25), 21384–21395. doi: 10.1074/jbc.M112.340513

Willis, C. M., Ménoret, A., Jellison, E. R., Nicaise, A. M., Vella, A. T., and Crocker, S. J. (2017). A refined bead-free method to identify astrocytic exosomes in primary glial cultures and blood plasma. *Front. Neurosci.* 11, 335. doi: 10.3389/fnins.2017.00335

Xie, L., Kang, H., Xu, Q., Chen, M. J., Liao, Y., Thiyagarajan, M., et al. (2013). Sleep drives metabolite clearance from the adult brain. *Science* 342, 373–377. doi: 10.1126/science.1241224

Xu, L., Cao, H., Xie, Y., Zhang, Y., Du, M., Xu, X., et al. (2019). Exosome-shuttled miR-92b-3p from ischemic preconditioned astrocytes protects neurons against oxygen and glucose deprivation. *Brain Res.* 1717, 66–73. doi: 10.1016/j.brainres.2019.04.009

Conflict of Interest: Author MT was employed by company 3BrainAG, Wädenswil, Switzerland.

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

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 08 January 2020
doi: 10.3389/fphar.2019.01491

Modulation of Cytokine-Induced Astrocytic Endothelin-1 Production as a Possible New Approach to the Treatment of Multiple Sclerosis

Stéphanie Hostenbach[1*], *Miguel D'Haeseleer*[1,2,3], *Ron Kooijman*[2] *and Jacques De Keyser*[1,2,4]

[1] Department of Neurology, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel (VUB), Brussels, Belgium, [2] Center for Neurosciences, Vrije Universiteit Brussel (VUB), Brussels, Belgium, [3] Department of Neurology, National Multiple Sclerosis Centrum, Melsbroek, Belgium, [4] Department of Neurology, Universitair Medisch Centrum Groningen (UMCG), University of Groningen, Groningen, Netherlands

OPEN ACCESS

Edited by:
Juan Andrés Orellana, Pontifical Catholic University of Chile, Chile

Reviewed by:
Maria Vincenza Catania, Institute of Neurological Sciences (CNR), Italy
Fernando C. Ortiz, Universidad Autónoma de Chile, Chile

***Correspondence:**
Stéphanie Hostenbach
stephaniehostenbach@hotmail.com

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

Received: 26 June 2019
Accepted: 19 November 2019
Published: 08 January 2020

Citation:
Hostenbach S, D'Haeseleer M, Kooijman R and De Keyser J (2020) Modulation of Cytokine-Induced Astrocytic Endothelin-1 Production as a Possible New Approach to the Treatment of Multiple Sclerosis. Front. Pharmacol. 10:1491. doi: 10.3389/fphar.2019.01491

Background: In the human central nervous system (CN), resting astrocytes do not visually show endothelin-1 (ET-1)-like immunoreactivity. In patients with multiple sclerosis (MS), an inflammatory disorder of the CNS, high levels of ET-1 are found in reactive astrocytes in demyelinated plaques. ET-1 may contribute to the pathology of MS by interrupting the blood-brain-barrier, enhancing inflammatory responses, excitotoxicity and reducing cerebral blood flow.

Methods: We used the human astrocytoma cell line 1321N1 to investigate the role of inflammatory cytokines involved in MS lesions (IL-1β, TNF-α, IFN-γ, LPS, IL-10, TGF-β) on astrocytic ET-1 upregulation. Prucalopride, rolipram, fenofibrate, fluoxetine, simvastatin, daglutril, and resveratrol were investigated as potential candidate drugs to suppress cytokine-induced astrocytic ET-1 production. Effects on ET-1 production were measured using both ELISA and RT-qPCR.

Results and Conclusions: ET-1 secretion by astrocytoma cells was only stimulated by the pro-inflammatory cytokines IL-1β and TNF-α. Fluoxetine, simvastatin, and resveratrol significantly inhibited this IL-1β- and TNF-α-induced ET-1 production. Simvastatin and resveratrol significantly reduced ET-1 mRNA levels, indicating an effect at the level of transcription. Fluoxetine significantly reduced endothelin converting enzyme-1 mRNA levels, suggesting and effect at the level of protein-processing. The required concentrations of simvastatin (>0.1 μM) and resveratrol (>10 μM) cannot be achieved in humans using pharmacologically accepted doses. Fluoxetine exerted a significant inhibitory effect on ET-1 secretion at a concentration of 5 μM, which is pharmacologically achievable in human brain, but the effect was modest (<50% suppression) and probably not sufficient

to obtain a clinically relevant ET-1 effect. Our *in vitro* model can be a useful screening tool in the development of new drugs to suppress astrocytic ET-1 production. The effect of simvastatin was for the most part mediated *via* the mevalonate pathway, suggesting that this might be an interesting target for further drug development.

Keywords: multiple sclerosis, endothelin-1, astrocytes, cytokines, inflammation, fluoxetine, simvastatin, resveratrol

INTRODUCTION

Multiple sclerosis (MS) is a chronic disorder of the central nervous system (CNS) that is pathologically characterized by the appearance of focal inflammatory lesions associated with demyelination and gliosis (plaques), disseminated in place and time. In addition, degenerative processes take place, including a progressive diffuse axonal degeneration and hippocampal neuronal loss (Compston and Coles, 2008). Destructive immune responses play a key role in the generation of focal lesions. The mechanisms responsible for the degenerative processes, which largely determine long-term disability in patients with MS are less well understood.

High levels of endothelin-1 (ET-1) have been found both in plasma and cerebrospinal fluid (CSF) of MS patients (Speciale et al., 2000; Haufschild et al., 2001). The likely source of this ET-1 production are reactive astrocytes in focal MS lesions, which express high levels of ET-1, while resting astrocytes in human brain visually do not show ET-1 immunoreactivity (D'Haeseleer et al., 2013). Mice with astrocytic ET-1 overexpression developed more severe experimental allergic encephalomyelitis (EAE), which is an animal model for the inflammatory lesions in MS (Guo et al., 2014).

The increased levels of ET-1 produced by reactive astrocytes may contribute to the pathology of MS by interrupting the blood-brain-barrier (BBB), enhancing the inflammatory responses, promoting excitotoxicity, and lowering cerebral blood flow (CBF) (Hostenbach et al., 2016). The manner by which astrocytes affect myelination seems to correlate with their level of reactivity (Nash et al., 2011; Kiray et al., 2016), and ET-1 released from reactive astrocytes acts as negative regulator of the differentiation of oligodendrocyte progenitor cells and remyelination (Hammond et al., 2014). ET-1 is also a potent vasoconstrictor, and previous research has shown that CBF in MS patients is already globally impaired from the early stages of the disease (Law et al., 2004; D'haeseleer et al., 2011).

Chronic cerebral hypoperfusion on itself may contribute to the pathology of MS. Animals subjected to chronic cerebral hypoperfusion developed axonal degeneration, focal white matter lesions with apoptosis of oligodendrocytes, myelin breakdown, inflammatory reactions, gliosis (Tomimoto et al., 2003), and neuronal loss in the hippocampal CA1 region (Ohta et al., 1997), which are all pathological features of MS.

We have shown that the oral administration of a single dose of the ET antagonist bosentan in MS patients can restore their CBF to values found in healthy volunteers (D'Haeseleer et al., 2013). This finding formed the basis for this study. The aim was to test a number of cytokines, found in MS lesions, for their ability to induce ET-1 production in astrocytic cells *in vitro*. The most relevant cytokine-induced *in vitro* model will then be used to screen a series of existing compounds for human use that pass the blood-brain barrier and may have potential to suppress ET-1 production.

The synthesis of ET-1 is mainly regulated at the transcription and translation level resulting in a 212-amino acid protein, preproET-1, which is further processed by a furin-like proprotein convertase to an inactive intermediate, big ET-1, which is then cleaved by an endothelin-converting enzyme (ECE) or other proteases into ET-1 (Hostenbach et al., 2016).

A number of drugs have been shown to influence ET-1 synthesis in other cell lines by acting at different levels of ET-1 expression. Others may on mechanistic grounds be candidate drugs to inhibit cellular ET-1 synthesis. For our study, the following compounds were selected: simvastatin, resveratrol, fluoxetine, prucalopride, rolipram, fenofibrate, and daglutril.

Simvastatin has been shown to downregulate ET-1 expression in human fetal astrocytes transfected with HIV-Tat protein, and decrease the transcription rate of the *ET-1* gene in bovine endothelial cells (Hernandez-Perera et al., 2000; Chauhan et al., 2007).

Resveratrol inhibited ET-1 mRNA expression in cultured endothelial cells through attenuating the activator protein-1 binding site (AP-1) of the ET-1 promotor (Liu et al., 2003).

Fluoxetine activates protein kinase A (PKA) in astrocytes and the ET-1 promotor element FoxO1 is a physiological substrate for PKA by the mean of phosphorylation and thus inhibition of FoxO1 (Lee et al., 2011).

Prucalopride reduced interferon-γ-induced MHC class II and B7 costimulatory immunostaining in cultured astrocytes. Furthermore, the drug is known to enhance the intracellular cAMP production, which in turn can activate PKA (Zeinstra et al., 2006).

Rolipram is an inhibitor of cyclic nucleotide phosphodiesterase responsible for the inhibition of the degradation of cAMP, which in turn will activate PKA. The drug has been shown to prevent ET-1 induced actions in perfused lung tissue of rat (Held et al., 1997).

Fenofibrate inhibits ET-1 expression in human endothelial cells, through enhanced expression of the transcriptional Küppel-like factor 11 which inhibits the ET-1 promotor, and on the other hand through inhibition of glycogen synthase kinase-3 activity, which will also inhibit ET-1 expression.

Daglutril has an endopeptidase (endothelin-converting enzyme) inhibiting effect and was shown to antagonize ET-1 induced vasoconstrictor activity in isolated human vaginal tissue (Rahardjo et al., 2013).

MATERIALS AND METHODS

Regulation of ET-1 Production in Cultured Human Astrocytoma Cells

Astrocytoma Cell Line

The human astrocytoma cell line 1321N1 (gift from dr. Sarah Gerlo, Lab of Eukaryotic Gene Expression and Signal Transduction, Gent University, Belgium) was cultured in DMEM (Dulbecco's Modified Eagle's medium; Thermo Fisher, Belgium) with 10% FBS (Fetal Bovine Serum; Thermo Fisher, Belgium), 1% Fungizone (Thermo Fisher Belgium), and 1% Penstrep (Penicillin-Streptomycin-medium; Thermo Fisher Belgium) in a humidified 5% CO_2 atmosphere at 37°C. After approximately 1 week, they were fully grown and plated out in 12-well plates at a concentration of 30,000 cells per 2 ml DMEM. After 3 days, cells were confluent and used for the experiments described below.

Incubation With Inflammatory Cytokines

Cells were cultured for 6 h in either the absence or presence of inflammatory modulators, after which the supernatant was collected for the measurement of ET-1 and frozen at -80°C. A number of pro-inflammatory and anti-inflammatory cytokines were tested: TNF-α (Tumor Necrosis Factor alfa; Miltenyi Biotec, The Netherlands) at concentrations of 1, 10, 50, 100, and 250 ng/ml, IFN-γ (Interferon gamma; Life Technologies, Belgium) at a concentration of 100 ng/ml, IL-1β (Interleukin -1 beta; Life Technologies, Belgium) at 1, 10, 50, 100, and 250 ng/ml, LPS (Lipopolysacharide; Sigma Aldrich, Germany) at 0.5 and 10 μg/ml, thrombin (Sigma Aldrich, Germany) at 3 units/ml, IL6 (interleukin 6; R&D systems, Germany) at 10 ng/ml and 100 ng/ml, IL-10 (interleukin-10; Life Technologies, Belgium) at 10ng/ml and 100ng/ml and TGF-β (Transforming Growth Factor -beta; Sigma Aldrich, Belgium) at 10 ng/ml and 100 ng/ml. TNF-α, IL-1β, IL6 and IL10 were solved in LPS-free water (pharmacy University Hospital Brussels); LPS in PBS; thrombin in Ultrapure Water (Sartorius Biotech, type: Arium® Pro UV, Germany); IFN-γ in BSA (N,O-bis (trimethylsilyl) acetamide, Sigma Aldrich, Germany). The concentrations of the cytokines used were chosen according to previous experiments in cell cultures reported in the literature.

Incubation With Compounds to Suppress ET-1 Secretion

All drugs were dissolved in a proper dissolvent: prucalopride (Selleckchem, The Netherlands), rolipram (Tocris Bioscience, UK), fenofibrate (Sigma Aldrich, Germany), simvastatin (Merck Millipore, United Kingdom), daglutril SLV 306 (Axon Medchem, The Netherlands), resveratrol (Sigma Aldrich, Germany) in Dimethyl Sulfoxide (DMSO; Sigma Aldrich, Germany), and fluoxetine (Sigma Aldrich, Germany) in Ultrapure water (Sartorius Biotech, type: Arium® Pro UV, Germany).

Different concentrations of all the components were tested starting from active concentrations used in the literature. Prucalopride at concentrations of 50nM, 250 nM, 500nM; rolipram at 1μM, 5μM, 10μM; fenofibrate at 10μM, 50μM, 100μM; simvastatin at 1nM, 10nM, 100nM, 5μM, 25μM; daglutril at 1μM, 10μM, 50μM; resveratrol at 1μM, 10μM, 100μM, and fluoxetine at 1 μM, 5μM, and 10μM.

In the course of the experiments we also tested dibutyryl-cAMP (dbcAMP, Sigma Aldrich, Germany) in Ultrapure water at concentrations of 100μM, 250μM, and 500μM and mevalonate (Sigma Aldrich, Germany) in DMSO at concentrations of 10 μM and 100 μM.

To evaluate the effects of the selected drugs on ET-1 secretion, astrocytoma cells were incubated with the compound or vehicle for 24 h before the addition of TNF-α and IL-1β at a final concentration of 100 ng/ml each and supernatant for ET-1 measurements was taken 6 h after their administration. We used the combination of both cytokines because both are present in MS lesions.

Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of ET-1 in the supernatant of the cultured human astrocytoma cells were measured using the Endothelin Pan Specific ELISA kit® (R&D systems, Abingdon, UK), according to the manufacturer's instructions. This kit not only measures ET-1 but has a cross-reactivity with both ET-2 and ET-3. A study reported that neonatal rat astrocytes also produce ET-3 (Ehrenreich et al., 1991). We tested a specific Endothelin-1 ELISA kit (IBL International, Hamburg, Germany) and found that ET-1 concentrations were the same as the ET concentrations measured with the Pan Specific ELISA kit. Therefore, for the screening experiments we used the Endothelin Pan Specific ELISA kit® to reduce the costs.

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

RNA was isolated from cell pellets of cultured human astrocytoma cell line using the RNeasy Mini Kit® (Qiagen, Hilden, Germany), according to the manufacturer's instructions. cDNA was reversely transcribed using TaqMan™ Reverse Transcription Reagents (Thermo Fisher Scientific, Belgium). The expression of the transcripts for ET-1, ECE-1, and GAPDH were assessed using *TaqMan*™ gene expression assays with respectively following assay IDs: Hs00174961_m1, Hs01043735_m1, Hs00206701_m,1 and Hs02758991_g1. The mRNA levels of ET-1 and ECE-1 were normalized to GAPDH mRNA expression.

Statistical Analyses

Statistical analyses were performed using GraphPad Prisms 6.0b software. Data in all experiments are presented as the mean ± standard deviation (SD) of at least 4 independent experiments. Significant differences were tested with either the Mann Whitney U-test or the Kruskal-Wallis-test (including the Dunn's Multiple Comparisons Test). Values were considered statistically significant when $P < 0.05$.

RESULTS

Upregulation of ET-1 by Inflammatory Cytokines

Figure 1A shows that cultured astrocytoma cells produce very low basal levels of ET-1 and that TNF-α and IL-1β, both at a concentration of 100ng/ml, significantly increased ET-1 levels in the culture medium. No further increase of ET-1 levels was obtained at concentrations of 250ng/ml. In the Kruskal-Wallis-test, the increase in ET-1 was not significantly different between TNF-α and either IL-1β or the combination of both cytokines.

Effects of the other tested cytokines (INFγ, LPS, thrombin, IL-6, IL-10, TGF-β) were not statistically significant (not shown).

Effects of the Compounds on ET-1 Secretion

A concentration-dependent decrease in ET-1 secretion was found for simvastatin (**Figure 1B**), fluoxetine (**Figure 1C**), and resveratrol (**Figure 1D**). Simvastatin, fluoxetine, and resveratrol did not affect basal (noncytokine stimulated) ET-1 levels in the culture medium.

Incubations with prucalopride, rolipram, fenofibrate, and daglutril were without effect (not shown). Furthermore, in the course of the experiments with the drugs fluoxetine, prucalopride, and rolipram, we also tested the effect of dbcAMP on ET-1 secretion, since this component can activate PKA which in turn may regulate the ET-1 promoter element FoxO1. We tested dbcAMP in different concentrations, but there was no significant effect on ET-1 secretion (not shown). This indicates that drugs acting through the cAMP pathway have no effect on the ET-1 production, and that the positive effect of fluoxetine was likely obtained through a cAMP independent mechanism.

To test whether suppression the cholesterol synthesis pathway is key to the inhibition of ET-1 secretion by simvastatin, we tested the effect of mevalonate supplementation in the presence of simvastatin (**Figure 2**). Addition of mevalonate 10μM to the cells for the most part attenuated the inhibiting effect of simvastatin on ET-1 secretion, indicating that simvastatin decreases ET-1 levels, at least partially, through the mevalonate-pathway. Mevalonate alone did not have a significant effect. A higher concentration of mevalonate (100μM) could not further attenuate the inhibiting effect of simvastatin (not shown).

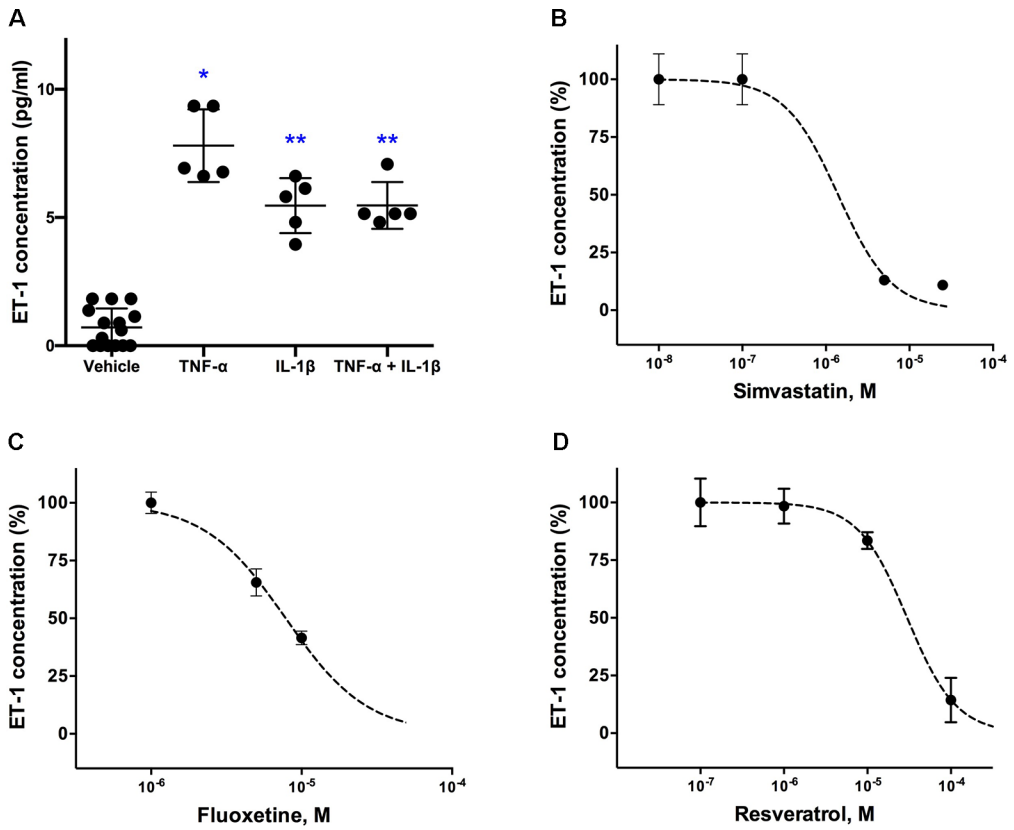

FIGURE 1 | Effects of **(A)** pro-inflammatory cytokines (TNF-α and IL-1β) on ET-1 secretion in cultured human astrocytoma cells and inhibitory concentration-response curves for **(B)** simvastatin, **(C)** fluoxetine and **(D)** resveratrol. Cytokines were administrated to obtain a final concentration of 100 ng/ml each and supernatant for ET-1 measurements was taken 6 h after their administration (n = 5). For the inhibitory experiments (n = 4), cells were pre-incubated with the drug for 24 h before their stimulation with the inflammatory cytokines. Data are presented as means ± SD. Dose-response curves were generated with GraphPad Prisms 6.0b software. $^{*}P < 0.001$ and $^{**}P < 0.05$ vs. the vehicle group.

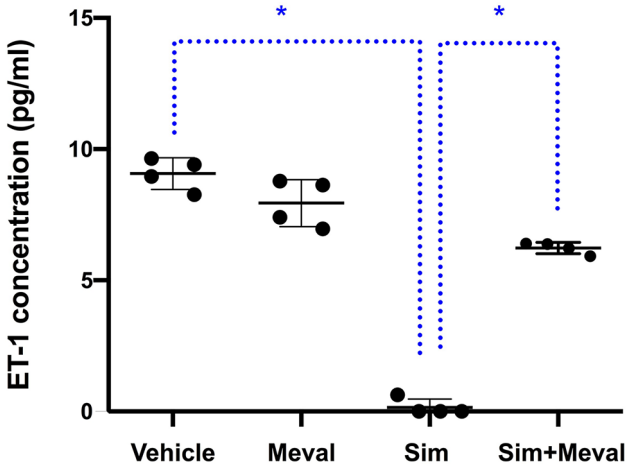

FIGURE 2 | Effects of mevalonate (Meval) 10μM on the inhibiting effect of 5μM simvastatin (Sim) on ET-1 secretion in cultured astrocytoma cells. Cells were pre-incubated with the compounds for 24 h before their stimulation with TNF-α and IL-1β. Mevalonate alone had no effect on ET-1 secretion but significantly attenuated the inhibiting effect of simvastatin on ET-1 secretion (n = 4). Data are presented as means ± SD. $P < 0.05$ vs. simvastatin. *$p = 0.0286$.

To assess at which level the production of ET-1 is regulated by fluoxetine, simvastatin, and resveratrol, we measured ET-1 mRNA levels. Both simvastatin (5 and 25 μM) and resveratrol (100 μM) significantly decreased the levels of ET-1 mRNA, indicating that these drugs act at the level of transcription (**Figure 3**). In contrast, fluoxetine 5 and 10μM was associated with an increase in ET-1 mRNA levels and a decrease in ECE-1 mRNA levels (**Figure 4**). Intracellular protein levels were not affected by fluoxetine (**Figure 5**). Taken together, our findings suggest that fluoxetine decreases ET-1 production by reducing ECE-1, which converts big ET-1 to ET-1.

DISCUSSION

We found that ET-1 secretion in human astrocytoma cells was stimulated by the pro-inflammatory cytokines IL-1β and TNF-α, which are known to be present in focal MS lesions (Bittner et al., 2014). The ET-1 promotor contains response elements for activator protein-1 (AP-1), which is the most important regulator for ET-1 (Stow et al., 2011), FoxO1 (Nicholson et al., 2010; Lee et al., 2011), and NF-κB (Morishita et al., 2014). The presence of the NF-κB response element can explain the stimulating effect of these pro-inflammatory cytokines.

We found no statistically significant difference in the level of ET-1 production by the astrocytoma cells using the highest concentration of TNF-α and the highest concentration of IL-1β. There was no additive effect by using both cytokines, which may be explained by the fact that each cytokine on itself already produced a maximal effect on ET-1 production, and it suggests that both cytokines act through the same mechanism.

Among the compounds tested, only simvastatin, resveratrol and fluoxetine significantly inhibited ET-1 production in human astrocytoma cells. Release of ET-1 by reactive astrocytes can be regulated at different levels, including transcription, translation, protein-processing or secretion of ET-1. The suppressive effect on ET-1 production by simvastatin and resveratrol was regulated at the mRNA level, whereas fluoxetine, at least partially, acted at the level of protein-processing.

We found that concentrations of 5 and 25 μM of simvastatin were needed to suppress transcription of the *ET-1* gene and production of ET-1. With both concentrations a decrease of 89% of the ET-1 concentrations was obtained. However, these concentrations can never be reached in human brain when pharmacological doses of simvastatin between 20 mg and 80 mg (high dose simvastatin) are used. Pleiotropic effects of statins in previous *in vitro* cell experiments not related to ET-1 production were also found at concentrations of 1–50 μM

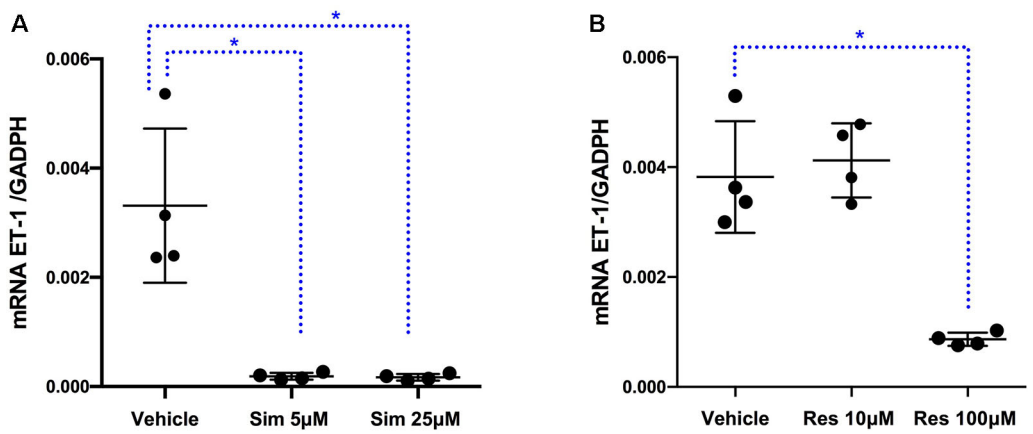

FIGURE 3 | Effects of **(A)** simvastatin (*$P < 0.05$) and **(B)** resveratrol (*$P < 0.05$) on ET-1 mRNA expression. Results were normalized to housekeeping GADPH mRNA expression. Cells were pre-incubated with the compounds for 24 h before their stimulation with TNF-α and IL-1β. RNA was isolated from the human astrocytoma cell pellets taken 6 h after their administration. Data are presented as means ± SD (n = 4).

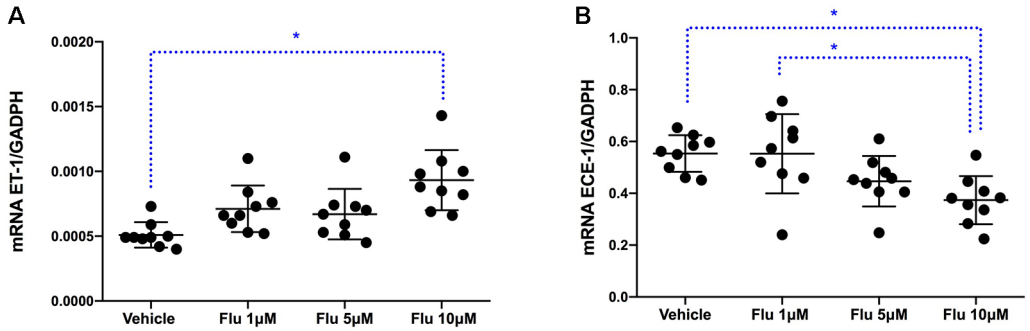

FIGURE 4 | Effects of fluoxetine on **(A)** ET-1 mRNA expression (*$P < 0.01$) and **(B)** ECE-1 mRNA expression (*$P < 0.01$). Results were normalized to housekeeping GADPH mRNA expression. Cells were pre-incubated with the compounds for 24 h before their stimulation with TNF-α and IL-1β. RNA was isolated from the human astrocytoma cell pellets taken 6 h after their administration. Data are presented as means ± SD (n = 9).

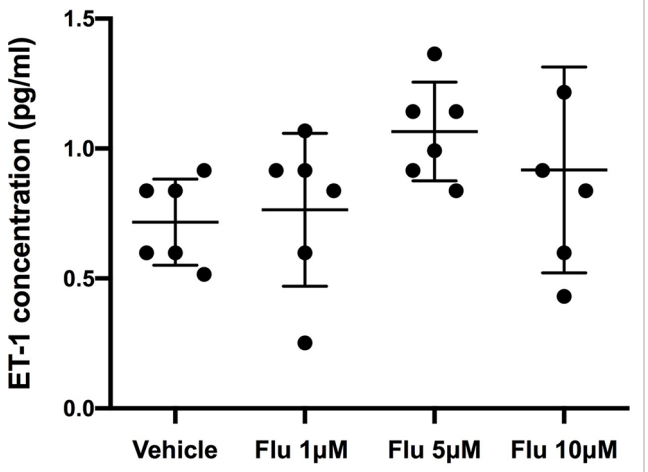

FIGURE 5 | Intracellular levels of ET-1 after the administration of different concentration of fluoxetine (Flu). Cells were pre-incubated with fluoxetine for 24 h before their stimulation with TNF-α and IL-1β. Concentrations of ET-1 were measured in cell lysates taken 6 h after their administration. There were no significant differences between the intracellular ET-1 concentrations. Data are presented as means ± SD (n = 6).

(Björkhem-Bergman et al., 2011). However, the mean concentration of statins in human serum after therapeutic doses is 1000 times lower (1–15 nM). Furthermore, only 1%–5% of this quantity is pharmacologically active and only one third of this serum concentration can reach the CNS (Björkhem-Bergman et al., 2011). Our results demonstrated that the effect of simvastatin was at least for the most part mediated *via* the mevalonate pathway, suggesting that this might be an interesting target for further drug development.

Simvastatin has been investigated in clinical trials in patients with MS. A meta-analysis performed in 2012 concluded that the addition of statins to interferon therapy did not significantly influence the relapse risk, disease progression, or EDSS scores in patients with relapsing remitting MS (Bhardwaj et al., 2012). A small study presented in 2014 suggested that simvastatin 80 mg a day in patients with secondary progressive MS might reduce the rate of whole-brain atrophy compared with placebo (Chataway et al., 2014). A phase 3 trial to confirm this effect in secondary progressive MS is ongoing in the UK (Williams et al., 2019). If simvastatin would have a beneficial effect in MS, our study suggests that it is not due to an effect on ET-1 production.

Fluoxetine concentrations of 5 and 10 μM significantly reduced ET-1 secretion, corresponding with a concentration decrease of 31% and 45%, respectively. Treatment of humans for 29 days with 40 mg fluoxetine resulted in brain fluoxetine levels of approximately 5 μM as assessed with magnetic resonance spectroscopy (Karson et al., 1993). A dose of 40 mg fluoxetine, which is often used in clinical practice, may thus be able to reduce ET-1 concentrations in brain, but the effect is probably too small to obtain a clinically significant effect, where an almost complete suppression of ET-1 should be achieved. Our findings that prucalopride, rolipram, and dbcAMP had no influence on astrocytic ET-1 secretion argue against the possibility that the effect of fluoxetine was mediated through the cAMP-dependent PKA pathway.

It has been shown that oral administration of fluoxetine in mice prevented EAE or ameliorated ongoing EAE. This was associated with a downregulation of different inflammatory cytokines (Il-6, IL-10, TNF-α, among others), indicating that this was the result of immunomodulatory effects of fluoxetine (Bhat et al., 2017). Preliminary evidence of a possible immunomodulatory effect of fluoxetine was also found in a small pilot study in patients with relapsing remitting MS. A daily dose of 20 mg fluoxetine tended to reduce the formation of new inflammatory lesions on magnetic resonance imaging of the brain compared to placebo (Mostert et al., 2008). Two randomized placebo-controlled trials with a daily dose of 40 mg of fluoxetine in patients with progressive MS, which reflects progressive axonal degeneration that proceeds rather independently of inflammation, failed to show a neuroprotective benefit (Wood, 2018; Cambron et al., 2019).

Resveratrol, a dietary antioxidant polyphenol is present in a number of regularly consumed plant species like berries, grapes and peanuts and is a major constituent of red wine. In a study with healthy volunteers, a single dose of 25 mg resveratrol was given as a dietary supplement. The concentration of the ^{14}C-

labelled resveratrol measured with high-performance liquid chromatography, 1 h after oral intake, was very low in the systemic circulation (about 2 μM), due to a very rapid and extensive metabolism by the bacterial flora in the human intestine. In another study where 5 g of resveratrol was administered orally to ten healthy volunteers, the maximum plasma concentration reached was 2, 36 μM (Walle et al., 2004). None of the *in vivo* pharmacokinetic studies in humans have shown plasma concentrations greater than 10 μM. In the brain, the concentration will probably be even lower. Resveratrol was well-tolerated and adverse reactions were mild at a dose of maximally 1 g a day; above this dose diarrhea was frequently reported. This dose can be assumed as the upper limit for clinical trials. In our study, we only found an inhibitory effect of resveratrol on ET-1 production by the astrocytoma cells at a concentration of 100 μM, but not at 10 μM.

A limitation of our study is that we used a human astrocytoma cell line as screening model, because they differ from primary human astrocytes and do not completely reflect the *in vivo* situation. However, the human astrocytoma cell line that we used is a well-established stable cell line capable of responding to cytokine exposure in a manner typical of reactive astrogliosis and is therefore a valuable cellular model in the assessment of *in vitro* drug screening. It is probably more relevant to the human response than existing animal cell-based models. It was our intention to confirm clinically significant positive findings in cultured human astrocytes. However, we did not proceed further because all results in the screening phase with the human astrocytoma cell line were disappointing.

CONCLUSION

Drugs that inhibit inflammation-induced ET-1 production in reactive astrocytes might widen the therapeutic arsenal in MS. Fluoxetine, simvastatin, and resveratrol, which are all drugs able to pass the blood-brain-barrier, suppressed inflammation-induced ET-1 secretion in cultured human astrocytoma cells. However, only fluoxetine exerted an effect at concentrations that are pharmacologically achievable in humans, but the effect was modest and probably insufficient to obtain a clinically relevant effect. Our *in vitro* model can be useful screening tool in the development of new drugs to suppress astrocytic ET-1 production, which must then be able to use in pharmacologically feasible doses. The mevalonate pathway might be an interesting target for further drug development. Suppressing astrocytic ET-1 production may be a potential therapeutic target in diverse other neurodegenerative disorders associated with reactive astrocytosis (Hostenbach et al., 2016). Due to the current lack of a suitable compound to suppress astrocytic ET-1 production we have started a phase 2 trial in MS patients with the ET-1 receptor antagonist bosentan (Hostenbach et al., 2019).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

SH, RK, and JK designed the experiments and interpreted the results. SH performed the experiments, analyzed the data, and wrote the manuscript. All authors discussed the results. MD'H, RK, and JK made critical revisions to the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

Research Foundation Flanders (FWO). SH is an FWO research fellow.

ACKNOWLEDGMENTS

We thank Anke De Smet (Vrije Universiteit Brussel) for the excellent technical assistance.

REFERENCES

Bhardwaj, S., Coleman, C. I., and Sobieraj, D. M. (2012). Efficacy of statins in combination with interferon therapy in multiple sclerosis: a meta-analysis. *Am. J. Health Syst. Pharm.* 69 (17), 1494–1499. doi: 10.2146/ajhp110675

Bhat, R., Mahapatra, S., Axtell, R. C., and Steinman, L. (2017). Amelioration of ongoing experimental autoimmune encephalomyelitis with fluoxetine. *J. Neuroimmunol.* 313, 77–81. doi: 10.1016/j.jneuroim.2017.10.012

Bittner, S., Afzali, A. M., Wiendl, H., and Meuth, S. G. (2014). Myelin oligodendrocyte glycoprotein (MOG35-55) induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice. *J. Vis. Exp.* (86). doi: 10.3791/51275

Björkhem-Bergman, L., Lindh, J. D., and Bergman, P. (2011). What is a relevant statin concentration in cell experiments claiming pleiotropic effects? *Br. J. Clin. Pharmacol.* 72 (1), 164–165. doi: 10.1111/j.1365-2125.2011.03907.x

Cambron, M., Mostert, J., D'Hooghe, M., Nagels, G., Willekens, B., Debruyne, J., et al. (2019). Fluoxetine in progressive multiple sclerosis: the FLUOX-PMS trial. *Mult. Scler.* 25 (13), 1728–1735. doi: 10.1177/1352458519843051

Chataway, J., Schuerer, N., Alsanousi, A., Chan, D., MacManus, D., Hunter, K., et al. (2014). Effect of high-dose simvastatin on brain atrophy and disability in secondary progressive multiple sclerosis (MS-STAT): a randomised, placebo-controlled, phase 2 trial. *Lancet* 383 (9936), 2213–2221. doi: 10.1016/S0140-6736(13)62242-4

Chauhan, A., Hahn, S., Gartner, S., Pardo, C. A., Netesan, S. K., McArthur, J., et al. (2007). Molecular programming of endothelin-1 in HIV-infected brain: role of Tat in up-regulation of ET-1 and its inhibition by statins. *FASEB J.* 21 (3), 777–789. doi: 10.1096/fj.06-7054com

Compston, A., and Coles, A. (2008). Multiple sclerosis. *Lancet.* 372 (9648), 1502–1517. doi: 10.1016/S0140-6736(08)61620-7

D'haeseleer, M., Cambron, M., Vanopdenbosch, L., and De Keyser, J. (2011). Vascular aspects of multiple sclerosis. *Lancet Neurol.* 10 (7), 657–666. doi: 10.1016/S1474-4422(11)70105-3

D'Haeseleer, M., Beelen, R., Fierens, Y., Cambron, M., Vanbinst, A. M., Verborgh, C., et al. (2013). Cerebral hypoperfusion in multiple sclerosis is reversible and mediated by endothelin-1. *Proc. Natl. Acad. Sci. U.S.A.* 110 (14), 5654–5658. doi: 10.1073/pnas.1222560110

Ehrenreich, H., Kehrl, J. H., Anderson, R. W., Rieckmann, P., Vitkovic, L., Coligan, J. E., et al. (1991). A vasoactive peptide, endothelin-3, is produced by and specifically binds to primary astrocytes. *Brain Res.* 538 (1), 54–58. doi: 10.1016/0006-8993(91)90375-6

Guo, Y., Chung, S. K., Siu, C. W., Kwan, S. C., Ho, P. W., Yeung, P. K., et al. (2014). Endothelin-1 overexpression exacerbate experimental allergic encephalomyelitis. *J. Neuroimmunol.* 276 (1–2), 64–70. doi: 10.1016/j.jneuroim.2014.08.616

Hammond, T. R., Gadea, A., Dupree, J., Kerninon, C., Nait-Oumesmar, B., Aguirre, A., et al. (2014). Astrocyte-Derived Endothelin-1 Inhibits Remyelination through Notch Activation. *Neuron* 81 (6), 1442. doi: 10.1016/j.neuron.2014.03.007

Haufschild, T., Shaw, S. G., Kesselring, J., and Flammer, J. (2001). Increased endothelin-1 plasma levels in patients with multiple sclerosis. *J. Neuroophthalmol.* 21 (1), 37–38. doi: 10.1097/00041327-200103000-00011

Held, H. D., Wendel, A., and Uhlig, S. (1997). Phosphodiesterase inhibitors prevent endothelin-1-induced vasoconstriction, bronchoconstriction, and thromboxane release in perfused rat lung. *Biochem. Biophys. Res. Commun.* 231 (1), 22–25. doi: 10.1006/bbrc.1996.6042

Hernandez-Perera, O., Perez-Sala, D., Soria, E., and Lamas, S. (2000). Involvement of Rho GTPases in the transcriptional inhibition of preproendothelin-1 gene expression by simvastatin in vascular endothelial cells. *Circ. Res.* 87 (7), 616–622. doi: 10.1161/01.RES.87.7.616

Hostenbach, S., D'haeseleer, M., Kooijman, R., and De Keyser, J. (2016). The pathophysiological role of astrocytic endothelin-1. *Prog. Neurobiol.* 144, 88–102. doi: 10.1016/j.pneurobio.2016.04.009

Hostenbach, S., Pauwels, A., Michiels, V., Raeymaekers, H., Van Binst, A. M., Van Merhaeghen-Wieleman, A., et al. (2019). Role of cerebral hypoperfusion in multiple sclerosis (ROCHIMS): study protocol for a proof-of-concept randomized controlled trial with bosentan. *Trials* 20 (1), 164. doi: 10.1186/s13063-019-3252-4

Karson, C. N., Newton, J. E., Livingston, R., Jolly, J. B., Cooper, T. B., Sprigg, J., et al. (1993). Human brain fluoxetine concentrations. *J. Neuropsychiatry Clin. Neurosci.* 5 (3), 322–329. doi: 10.1176/jnp.5.3.322

Kiray, H., Lindsay, S. L., Hosseinzadeh, S., and Barnett, S. C. (2016). The multifaceted role of astrocytes in regulating myelination. *Exp. Neurol.* 283 (Pt B), 541–549. doi: 10.1016/j.expneurol.2016.03.009

Law, M., Saindane, A. M., Ge, Y., Babb, J. S., Johnson, G., Mannon, L. J., et al. (2004). Microvascular abnormality in relapsing-remitting multiple sclerosis: perfusion MR imaging findings in normal-appearing white matter. *Radiology* 231 (3), 645–652. doi: 10.1148/radiol.2313030996

Lee, J. W., Chen, H., Pullikotil, P., and Quon, M. J. (2011). Protein kinase A-alpha directly phosphorylates FoxO1 in vascular endothelial cells to regulate expression of vascular cellular adhesion molecule-1 mRNA. *J. Biol. Chem.* 286 (8), 6423–6432. doi: 10.1074/jbc.M110.180661

Liu, J. C., Chen, J. J., Chan, P., Cheng, C. F., and Cheng, T. H. (2003). Inhibition of cyclic strain-induced endothelin-1 gene expression by resveratrol. *Hypertension.* 42 (6), 1198–1205. doi: 10.1161/01.HYP.0000103162.76220.51

Morishita, S., Oku, H., Horie, T., Tonari, M., Kida, T., Okubo, A., et al. (2014). Systemic simvastatin rescues retinal ganglion cells from optic nerve injury possibly through suppression of astroglial NF-κB activation. *PloS One* 9 (1), e84387. doi: 10.1371/journal.pone.0084387

Mostert, J. P., Admiraal-Behloul, F., Hoogduin, J. M., Luyendijk, J., Heersema, D. J., van Buchem, M. A., et al. (2008). Effects of fluoxetine on disease activity in relapsing multiple sclerosis: a double-blind, placebo-controlled, exploratory study. *J. Neurol. Neurosurg. Psychiatry* 79 (9), 1027–1031. doi: 10.1136/jnnp.2007.139345

Nash, B., Ioannidou, K., and Barnett, S. C. (2011). Astrocyte phenotypes and their relationship to myelination. *J. Anat.* 219 (1), 44–52. doi: 10.1111/j.1469-7580.2010.01330.x

Nicholson, S. K., Tucker, G. A., and Brameld, J. M. (2010). Physiological concentrations of dietary polyphenols regulate vascular endothelial cell expression of genes important in cardiovascular health. *Br. J. Nutr.* 103 (10), 1398–1403. doi: 10.1017/S0007114509993485

Ohta, H., Nishikawa, H., Kimura, H., Anayama, H., and Miyamoto, M. (1997). Chronic cerebral hypoperfusion by permanent internal carotid ligation produces learning impairment without brain damage in rats. *Neuroscience.* 79 (4), 1039–1050. doi: 10.1016/S0306-4522(97)00037-7

Rahardjo, H. E., Uckert, S., Taher, A., Sonnenberg, J. E., Kauffels, W., Rahardjo, D., et al. (2013). Effects of endopeptidase inhibition on the contraction-relaxation response of isolated human vaginal tissue. *J. Sex Med.* 10 (4), 951–959. doi: 10.1111/jsm.12064

Speciale, L., Sarasella, M., Ruzzante, S., Caputo, D., Mancuso, R., Calvo, M. G., et al. (2000). Endothelin and nitric. oxide levels in cerebrospinal fluid of patients with multiple sclerosis. *J. Neurovirol.* 6 (Suppl 2), S62–S66.

Stow, L. R., Jacobs, M. E., Wingo, C. S., and Cain, B. D. (2011). Endothelin-1 gene regulation. *FASEB J.* 25 (1), 16–28. doi: 10.1096/fj.10-161612

Tomimoto, H., Ihara, M., Wakita, H., Ohtani, R., Lin, J. X., Akiguchi, I., et al. (2003). Chronic cerebral hypoperfusion induces white matter lesions and loss of oligodendroglia with DNA fragmentation in the rat. *Acta Neuropathologica.* 106 (6), 527–534. doi: 10.1007/s00401-003-0749-3

Walle, T., Hsieh, F., DeLegge, M. H., Oatis, J. E.Jr., and Walle, U. K. (2004). High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab. Dispos.* 32 (12), 1377–1382. doi: 10.1124/dmd.104.000885

Williams, E., John, N., Blackstone, J., Brownlee, W., Frost, C., Greenwood, J., et al. (2019). TP1-11 MS-STAT2: a phase 3 trial of high dose simvastatin in secondary progressive multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry* 90 (3), e13–e1e. doi: 10.1136/jnnp-2019-ABN.40

Wood, H. (2018). Late-breaking news at ECTRIMS 2018. *Nat. Rev. Neurol.* 14 (12), 690. doi: 10.1038/s41582-018-0100-1

Zeinstra, E. M., Wilczak, N., Wilschut, J. C., Glazenburg, L., Chesik, D., Kroese, F. G., et al. (2006). 5HT4 agonists inhibit interferon-gamma-induced MHC class II and B7 costimulatory molecules expression on cultured astrocytes. *J. Neuroimmunol.* 179 (1–2), 191–195. doi: 10.1016/j.jneuroim.2006.06.012

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

frontiers in Pharmacology

REVIEW
published: 15 January 2020
doi: 10.3389/fphar.2019.01546

Connexins in Astrocyte Migration

Raúl Lagos-Cabré[1,2], *Francesca Burgos-Bravo*[1,2], *Ana María Avalos*[3] *and Lisette Leyton*[1,2*]

[1] Cellular Communication Laboratory, Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas (ICBM), Facultad de Medicina, Universidad de Chile, Santiago, Chile, [2] Advanced Center for Chronic Diseases (ACCDiS), Center for Studies on Exercise, Metabolism and Cancer (CEMC), Facultad de Medicina, Instituto de Ciencias Biomédicas (ICBM), Universidad de Chile, Santiago, Chile, [3] Facultad de Ciencias de la Salud, Instituto de Ciencias Biomédicas, Universidad Autónoma de Chile, Santiago, Chile

Astrocytes have long been considered the supportive cells of the central nervous system, but during the last decades, they have gained much more attention because of their active participation in the modulation of neuronal function. For example, after brain damage, astrocytes become reactive and undergo characteristic morphological and molecular changes, such as hypertrophy and increase in the expression of glial fibrillary acidic protein (GFAP), in a process known as astrogliosis. After severe damage, astrocytes migrate to the lesion site and proliferate, which leads to the formation of a glial scar. At this scar-forming stage, astrocytes secrete many factors, such as extracellular matrix proteins, cytokines, growth factors and chondroitin sulfate proteoglycans, stop migrating, and the process is irreversible. Although reactive gliosis is a normal physiological response that can protect brain cells from further damage, it also has detrimental effects on neuronal survival, by creating a hostile and non-permissive environment for axonal repair. The transformation of astrocytes from reactive to scar-forming astrocytes highlights migration as a relevant regulator of glial scar formation, and further emphasizes the importance of efficient communication between astrocytes in order to orchestrate cell migration. The coordination between astrocytes occurs mainly through Connexin (Cx) channels, in the form of direct cell-cell contact (gap junctions, GJs) or contact between the extracellular matrix and the astrocytes (hemichannels, HCs). Reactive astrocytes increase the expression levels of several proteins involved in astrocyte migration, such as $\alpha_v\beta_3$ Integrin, Syndecan-4 proteoglycan, the purinergic receptor P2X7, Pannexin1, and Cx43 HCs. Evidence has indicated that Cx43 HCs play a role in regulating astrocyte migration through the release of small molecules to the extracellular space, which then activate receptors in the same or adjacent cells to continue the signaling cascades required for astrocyte migration. In this review, we describe the communication of astrocytes through Cxs, the role of Cxs in inflammation and astrocyte migration, and discuss the molecular mechanisms that regulate Cx43 HCs, which may provide a therapeutic window of opportunity to control astrogliosis and the progression of neurodegenerative diseases.

Keywords: connexin 43, gap junctions, hemichannels, inflammation, scar-forming astrocytes, reactive astrocytes

OPEN ACCESS

Edited by:
Juan Andrés Orellana, Pontifical Catholic University of Chile, Chile

Reviewed by:
Luc Leybaert, Ghent University, Belgium
Juan C. Saez, Pontifical Catholic University of Chile, Chile

***Correspondence:**
Lisette Leyton
lleyton@med.uchile.cl

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

Received: *16 August 2019*
Accepted: *29 November 2019*
Published: *15 January 2020*

Citation:
Lagos-Cabré R, Burgos-Bravo F, Avalos AM and Leyton L (2020) Connexins in Astrocyte Migration. Front. Pharmacol. 10:1546. doi: 10.3389/fphar.2019.01546

INTRODUCTION

Astrocytes are the most numerous glial cells in the central nervous system (CNS) and comprise nearly half the volume of the adult mammalian brain (Agulhon, 2008; Filous and Silver, 2016). As such, astrocytes are critical for supporting neuronal structure and brain homeostasis (Chung et al., 2015). Additionally, astrocyte functions include metabolic regulation of neurons, synaptic support, establishment of the blood–brain barrier (BBB), and a defense mechanism that constrains an injured or damaged site (Brown and Ransom, 2007; Sofroniew and Vinters, 2010; Pekny, 2016).

During development, differentiating newborn astrocytes undergo migration in order to reach their final destination (Goldman, 1997), whereas astrocytes in the adult brain are quiescent under normal physiological conditions. These star-like cells are arranged in the brain as tiling domains, where they do not intermingle their processes (Halassa, 2007; Cao, 2010). This segregation of processes is thought to occur by contact inhibition during postnatal development and is lost in disease or post-injury conditions (Sofroniew, 2009).

Events occurring in response to brain damage involve the participation of glial cells and, particularly, astrocytes. During the first stages of the lesion, damaged axons are exposed to inhibitory molecules, such as those found in the myelin sheath of oligodendrocytes. Interaction of neuronal receptors with these myelin ligands results in low regenerative capacity of the injured neuronal processes (Cao, 2010). Additionally, astrocytes undergo varying morphological and molecular changes after damage, through a process called reactive gliosis (**Figure 1**) (Sofroniew, 2009; Burda and Sofroniew, 2014), which is triggered by different molecules derived from the blood, inflammatory cells, or released from injured cells, such as adenosine trisphosphate (ATP), endothelin-1, and the pro-inflammatory cytokines tumor necrosis factor (TNF), interleukin-1β (IL-1γ), interferon gamma (IFNγ) and IL-6 (Giulian, 1988; Ahmed, 2000; John et al., 2003; Gadea et al., 2008). The response of astrocytes during gliosis varies according to their proximity to the injured site. Thus, astrocytes close to the injury change from a quiescent to a reactive state, in which astrocytes suffer cellular hypertrophy, acquire a fibroblast-like amoeboid morphology, and increase the expression of diverse proteins, such as glial fibrillary acidic protein (GFAP), vimentin, nestin, and the inducible nitric oxide synthase (iNOS) (Miyake, 1988; Clarke, 1994; Lagos-Cabre, 2017). After severe injury, there is a pronounced hypertrophy of the astrocyte cell body and processes, and astrocytes migrate to the injured site, where they increase their proliferation. These notorious changes significantly decrease individual astrocyte domains and therefore, the processes arising from several astrocytes overlap and form the glial scar, which isolates the damaged tissue and protects the adjacent nerve cells from harmful molecules (Homkajorn et al., 2010). The confinement of the damaged area after an injury requires that astrocytes polarize and migrate to the affected zone, where they avoid propagation of the lesion by the uptake of extracellular signals, such as glutamate, free iron, cytokines, ATP, ADP, or adenosine (Bylicky et al., 2018). Interestingly, these are the same molecules that induce the reactive phenotype in the first place. Therefore, reactive gliosis not only protects CNS cells from further damage, but also exerts harmful effects on neuronal

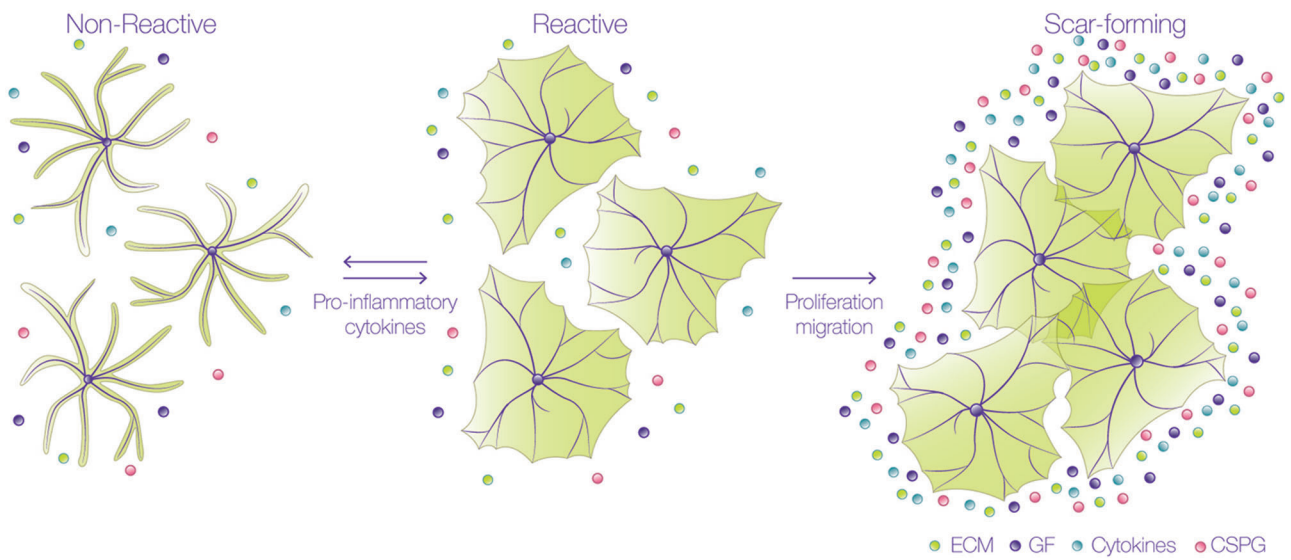

FIGURE 1 | Astrocytes undergo astrogliosis in a pro-inflammatory environment. Astrocytes change their morphology from a non-reactive into a reactive state when exposed to pro-inflammatory cytokines.They undergo hypertrophy and not only change their shape, but also their protein expression; up to this stage, the process is reversible and reactive astrocytes do not overlap their branches. These cells then proliferate and migrate to the lesion site to form the glial scar, where they secrete many factors, such as extracellular matrix (ECM) proteins, cytokines, growth factors (GF), and chondroitin sulfate proteoglycans (CSPG). In this scar-forming stage, astrocytes no longer move, and the process is irreversible.

survival and axonal regeneration (Pekny and Pekna, 2014; Sofroniew, 2014). Within 24 h after injury, and during the formation of the glial scar, astrocytes increase the secretion and deposition of chondroitin sulfate proteoglycans (CSPGs) into the extracellular matrix (ECM) which, together with the myelin-associated inhibitory molecules, create a hostile and non-permissive environment for axonal repair (Jones et al., 2003).

Reactive astrocytes can be classified as naïve (non-reactive), reactive, or scar-forming astrocytes, depending on their location and markers. Naïve and scar-forming astrocytes do not move, and astrocytes in the glial scar express N-Cadherin (Kanemaru, 2013). In contrast, reactive astrocytes (that move), express β-catenin, and metalloproteases, such as MMP2 and MMP13 (Verslegers, 2013). These important hallmarks suggest that there is a temporal sequence in the progression from naïve to reactive astrocytes, and then from reactive to scar-forming astrocytes. Since reactive astrocytes migrate to the injury site, isolate inflammatory cells and help repair tissue, this reactive stage constitutes a window of opportunity for interventions given that, up to this point, the process is reversible (**Figure 1**). These findings indicate that astrocyte migration is an important regulator of glial scar formation and highlight the relevance of studying the molecular mechanisms that regulate astrocyte motility. Additionally, in order for astrocytes to capture the signals of their surrounding microenvironment, they need to efficiently communicate with each other to orchestrate and synchronize, accordingly, each step of their movement. This coordination is achieved mainly by Connexin (Cx) channels, that can establish two distinct forms of communication: either through gap junctions (GJs), allowing direct cell-cell communication, or through hemichannels (HCs), that provide a pathway for the release and uptake of small molecules to and from extracellular compartments, respectively (Vicario, 2017). By sensing extracellular cues, astrocytes utilize their GJs or HCs in order to inform other cells of possible damage (Retamal, 2007). Furthermore, Cx HCs allow astrocytes to release molecules that can play a relevant role in autocrine/paracrine signaling in the brain (Retamal, 2007; Orellana et al., 2013; Alvarez, 2016; Lagos-Cabre, 2017), thereby potentiating important responses, such as cell migration (Alvarez, 2016; Lagos-Cabre, 2017).

The conversion of naïve astrocytes into motile and reactive cells observed after acute injury also occurs after stroke and neurodegenerative diseases, such as Alzheimer´s disease (AD) and Amyotrophic Lateral Sclerosis (ALS). Of note, reactive astrocytes up regulate the expression of several proteins that participate in astrocyte migration, such as $\alpha_v\beta_3$ Integrin, the heparin sulfate proteoglycan Syndecan-4, the purinergic P2X7 receptor (P2X7R), as well as Cx43 and Pannexin1 (Px1) HCs (Lagos-Cabre, 2017).

Astrocytes are the cells with the highest level of Cxs in the CNS (Nagy and Rash, 2000). The first evidence of astrocytic Cxs that particularly formed GJs was obtained *in situ* by freeze-fracture electron microscopy (Brightman and Reese, 1969; Dermietzel, 1974). Later, in 1991, Cx43 was found to be one of the major Cx subtypes in astrocytes (Dermietzel, 1991). The pivotal role of Cxs in astroglial connectivity was demonstrated with Cx43/Cx30 double knockout (KO) mice, in which intercellular communication was lost (Dermietzel, 2000). However, the first relationship between Cxs and astrocyte migration was discovered in Cx43 KO mouse fetuses, using organotypic brain slice cultures that showed an irregular distribution of astrocytes (Perez Velazquez, 1996). Importantly, this finding led to the idea that Cx43 played a relevant role in regulating astrocytic mobility. Since then, several studies have reported that Cxs affect astrocyte migration (Homkajorn et al., 2010; Kotini and Mayor, 2015; Lagos-Cabre, 2018).

The focus of this review will be on the ability of Cxs to form HCs in astrocytes, in particular Cx43 HCs, and how they control astrocyte migration by releasing small molecules to the extracellular space. These molecules activate receptors in the same or adjacent cells, which then continue the signaling cascades required for astrocytes to move. We will also compare the functions of HCs and GJs in cell communication and the interplay between these two cellular channels in the regulation of cell migration.

ASTROCYTES AND CELL COMMUNICATION

Astrocytes possess a characteristic star-like shape that distinguishes them from other non-neuronal cells of the glial family; however, despite the fact that astrocytes outnumber neurons and the other glia (i.e., microglia and oligodendrocytes) in rodents, their important role has always been undermined by neurons (Sosunov, 2014; Allen and Eroglu, 2017). In the human brain, there are many different types of astrocytes that can be identified by the combination of distinct cell markers, such as CD44, EAAT1, EAAT2, Aquaporin, and GFAP (Sosunov, 2014). The number of astrocytes in the human brain seems to vary according to the region, from 20–50%, and the exact ratio of total glial cells to neurons, although controversial, seems to be closer to one (von Bartheld et al., 2016).

The previous conception of astrocytes as being mere supporting cells for neurons is no longer valid. Today, it is known that astrocytes surround the pre- and post-synaptic membranes, thereby forming the "tripartite synapse" (Allen and Eroglu, 2017), and achieving functional integration and physical proximity to stimulate and regulate the activity of chemical synapses. Astrocytes also support and enhance the delivery of substrates required by neurons and act, for example, as a highway for glucose (Muller et al., 2018). Notably, and because astrocytes function primarily by anaerobic glycolysis, they can survive in low oxygen environments much longer than neurons. Astroglial Cx30 and Cx43 allow the diffusion of energy metabolites such as glucose and lactate and therefore, contribute to metabolic networks that are able to feed distant neurons in conditions such as hypoglycemia and/or high neuronal demand of energy

substrates (Rouach, 2008). Astrocytes can also assist the metabolic needs of neurons by buffering molecules such as glutamate, K^+, nitric oxide (NO), hydrogen peroxide (H_2O_2), and ammonia (Tsacopoulos and Magistretti, 1996; Dienel and Hertz, 2001; Aubert, 2007; Hertz et al., 2007). Astrocyte functions extend to the formation of the BBB by tightly apposing their end-feet to the endothelial cell vessels, thus helping with the maintenance of brain capillary permeability (Blanchette and Daneman, 2015; Zhao, 2015). In addition, astrocytes establish the principal defense mechanism after injury, surrounding the lesion site with their extended feet to avoid the propagation of damaging molecules (Ben Haim, 2015). To achieve all these functions, astrocytes need to sense and respond to signaling molecules, and then communicate with other astrocytes and their surroundings. Astrocytes display an extensive communication network by directly connecting cells through GJs, which are channels that consist of two facing connexons formed by a hexameric ring of Cxs, specifically Cx43/30 (Anders, 2014). Consequently, Cxs appear as one of the most important proteins related to cell communication in astrocytes, contributing to the coordination and maintenance of physiologic CNS function.

PROPERTIES OF CONNEXIN CHANNELS

All Cxs share a similar topology, with four alpha-helical transmembrane domains connected by two extracellular loops and one intracellular loop, and two cytoplasmic N- and C-terminal domains (Bennett, 2016). The principal feature of Cxs is the capacity to form GJs for the interchange of metabolites and second messengers between contacting cells, or HCs that participate in paracrine and autocrine cellular signaling. HCs are permeable to different types of small molecules < 1.2 kDa, depending on the Cx isoform involved (Giaume, 2013; Oyamada et al., 2013; Nielsen, 2017; Nielsen, 2017): ions such as Ca^{2+}, Na^+, and K^+; second messengers such as inositol 1,4,5 trisphosphate (IP_3), cAMP, and cGMP; metabolites such as ATP, glutamate, glucose, and glutathione; and other small molecules (Kumar and Gilula, 1996; Kang, 2008; Bosch and Kielian, 2014; De Bock, 2014). This permeability allows the communication between cells through a complex syncytial network. The long-distance mechanism described in the early 90's for astrocyte communication *via* the intercellular passage of Ca^{2+} waves through GJs (Cornell-Bell, 1990) is debatable at present. The velocity of transport of IP_3 through GJs for example, is 100-fold faster than that of Ca^{2+} itself (Allbritton et al., 1992; Hofer et al., 2002; De Bock, 2014), and because IP_3 might release Ca^{2+} from intracellular stores by activating IP_3 receptors (IP_3R) (Allbritton et al., 1992; Hofer et al., 2002) rather than by directly moving Ca^{2+} as initially thought, the passage of IP_3 molecules through GJ channels allows faster communication between cells. In the case of ATP released through HCs (Stout, 2002), accumulating evidence indicates that it activates purinergic receptors in the same (autocrine) or in neighboring (paracrine) cells, which induces the Ca^{2+} influx (Suadicani, 2004; Henriquez, 2011; Scemes and Spray, 2012; Alvarez, 2016; Lagos-Cabre, 2017) required for the propagation of Ca^{2+} (**Figure 2**).

The electrophysiological properties of Cxs are well known and their conductance allows to differentiate them and confirm the presence of specific Cxs in a given cell type (Retamal, 2007; Giaume, 2013). Cx HCs, also named connexons, can be formed

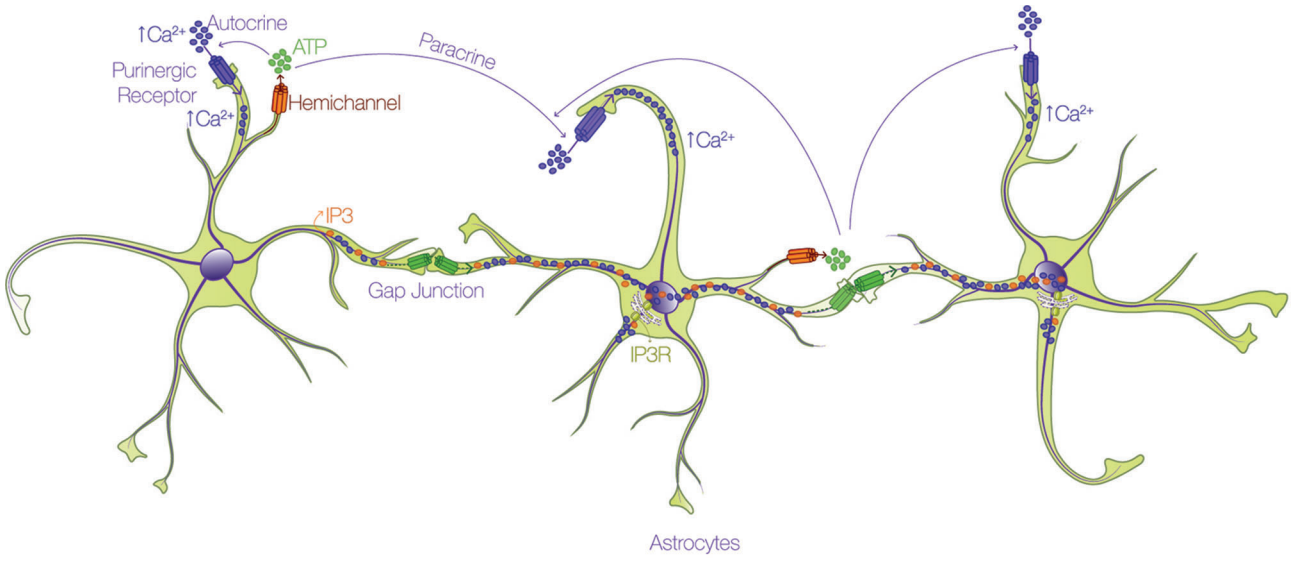

FIGURE 2 | Astrocytes form an interconnected network through calcium. Astrocytes utilize the propagation of intercellular calcium (Ca^{2+}) waves to achieve long-distance communication. There are two routes by which Ca^{2+} is mobilized through astrocytes: i) one pathway involves the passage of either IP_3 (orange dots) or Ca^{2+} (purple dots) through gap junction channels (green connexon) and ii) the other route depends on the release of ATP (green dots) through hemichannels (orange connexon), and subsequent activation of purinergic receptors (purple pore) in the same (autocrine) or in neighboring (paracrine) cells, which promote Ca^{2+} uptake.

by hexamers of the same (homomeric) or different (heteromeric) Cx subunits, while in the case of GJs, they are called homotypic or heterotypic when the two channels are formed by either homomeric or heteromeric connexons (Kumar and Gilula, 1996). On the other hand, the permeability of HCs and GJs also relies on subunit composition. For example, GJs formed by Cx40, Cx43 or Cx45 in cardiac cells show high permeability for several dyes with molecular weight above 400 Da, including Lucifer Yellow (LY) and propidium iodide (PI); in contrast, Cx30.2 shows no permeability for these two dyes (Rackauskas et al., 2007). The presence of Cx30.2, even in heterotypic GJs, precludes the permeability for LY or PI, suggesting that the presence of a non-permeable subunit is enough to completely modify the properties of GJs (Rackauskas et al., 2007). Interestingly, Cxs such as Cx30.2, which are permeable to small molecules, would be more adapted for electrical communication rather than metabolic transfer. Furthermore, GJs show selective permeability for biologically relevant molecules, such as second messengers; for instance, GJs formed by Cx43 have a 3-fold increase in permeability to cAMP compared to those formed by Cx26, and a 30-fold increase in permeability when compared to Cx36 channels, as tested in HeLa cells (Bedner, 2006). To add another level of complexity, Cxs can form homocellular GJs, such as neuron-neuron and astrocyte-astrocyte GJs, and heterocellular GJs, such as those formed between neurons and astrocytes (Nagy and Rash, 2000). Thus, the specific permeability properties and features of Cxs depend on the functionality of the distinct channels that they form. This specificity regulates channel conductance, electrical communication and metabolic coupling between cells (Vicario, 2017).

Since Cxs have a short life of only 1–5 h (Berthoud, 2004), the synthesis and delivery of new Cx proteins to the membrane is coupled to simultaneous GJ internalization, recycling to the membrane and Cx degradation (Segretain and Falk, 2004; Gilleron, 2009). Evidence has shown that Cxs can also be regulated by different types of post-translational modifications, like phosphorylation/dephosphorylation; and changes by oxidation, including effects of NO, hydrogen sulphide, or carbon monoxide, but not sulphur dioxide (Pogoda, 2016). Other modifications include acetylation, methylation, or ubiquitination (Pogoda, 2016). The stability of Cx43 on membranes depends, in part, on its interaction with the actin-associated proteins Zonula Occludens protein 1 (ZO-1) and Drebrin. The dissociation of Cx43/ZO-1 and Cx43/Drebrin from the cytoskeleton, through Src, has been found to promote Cx43 instability (Suh et al., 2012; Ambrosi, 2016; Sorgen, 2018).

Post-translational modification of Cxs is mainly represented by phosphorylation processes. Therefore, Cxs significantly interact with various protein kinases, as well as phosphatases. The cytoplasmic carboxy-terminal tail region of Cxs serves as a substrate for several kinases (Lampe and Lau, 2004; Marquez-Rosado, 2012), such as Cdk5 (Qi, 2016), ERK1/2 (De Vuyst, 2009), Akt (Park, 2007), PKA (Solan and Lampe, 2014), and PKC (Ek-Vitorin, 2006). The phosphorylation of Cx43 by Cdk5 on Ser279 and Ser282 decreases its membrane targeting and promotes its proteasomal degradation (Qi, 2016). GJs can be internalized after their ubiquitination as annular junctions in a clathrin-dependent process, and are sorted through the endosomal/lysosomal degradation pathway (Laird, 2006). In addition, Cx43 phosphorylation on S279/282 decreases GJ channel gating (Cottrell, 2003). On the other hand, Akt phosphorylates Cx43 in S373, forming larger GJs with higher communicational potential; this facilitates the turnover of GJs *via* the formation of an annular complex (Solan and Lampe, 2014). Moreover, Akt, PKA and PKC hierarchically phosphorylate Cx43 on various serine residues, thereby regulating the binding and release of ZO-1 from GJs, events that determine GJ function and endocytosis (Solan and Lampe, 2014; Thevenin, 2017).

Despite the large number of kinases that phosphorylate Cxs, HC activity is regulated only by PKA, PKC, MAPK and Akt (Pogoda, 2016). PKC-mediated phosphorylation of HCs formed by Cx43 abolishes sucrose and LY permeability by conformational changes in the structure of Cx43 (Bao et al., 2004). While phosphorylation by PKC closes Cx43 HCs, evidence from osteocyte cells indicates that their opening induced by shear stress depends on Cx43 phosphorylation by Akt on Ser369/Ser373 (Batra, 2014). Additionally, functional studies with lipid vesicles containing Cx43 HCs pre-loaded with fluorescent probes have indicated that phosphorylation of Cx43 by MAPK reduces the permeability of these liposomes (Kim, 1999). Given the large number of phosphorylation sites on Cx43, phosphatase-mediated dephosphorylation of Cx43 has been reported as an enhancer of HC permeability (Kim, 1999), while in GJs this post-translational modification enforces structural changes that reduce their functional coupling in astrocytes (Li et al., 2005). The role of serine/threonine phosphatases is to limit GJ conductance and enhance HC permeability. Thus, the regulation of Cx expression and activity has become a rich field of study for the analysis of their functional role in different physio-pathological conditions and today, GJs and HCs are not just viewed as mere connection proteins but rather as important regulators of cellular function.

ASTROCYTES AND CONNEXINS DURING INFLAMMATION

Different Cx isoforms are expressed in the brain. Thus far, 11 of the 21 Cx isoforms that have been described have been detected in the CNS (Mayorquin, 2018). Different types of astrocytes express several Cxs (Beyer, 2001; Giaume, 2013; Mansour, 2013; Bosch and Kielian, 2014), with Cx 30 and Cx43 being the mayor ones (Giaume and McCarthy, 1996). Additionally, Cx26, Cx30, Cx40, Cx45, and Cx46 mRNA has been detected in cultured astrocytes from Cx43 KO mice (Dermietzel, 2000), mRNA for Cx26, Cx30, Cx32, Cx40, and Cx43 has also been detected by single-cell RT-PCR in hippocampal astrocytes (Blomstrand, 2004), and GJs in cultured astrocytes are mainly composed of Cx43 (Dermietzel, 1991; Giaume, 1991). Cxs in astrocytes, oligodendrocytes, microglia and neurons are characterized according to the

developmental state, region and cell-type specific isoform expression, suggesting that Cxs play a critical role in the regulation and maintenance of various CNS functions (Lapato and Tiwari-Woodruff, 2018). Cx43 is ubiquitously expressed in astrocytes throughout the brain, and along with Cx26 and Cx30, contributes to the interconnection of the astrocyte network (Rash, 2001); however, Cx26 and Cx30 are less abundant in astrocytes (Contreras, 2004). This expression profile probably determines the autocrine and paracrine signaling interaction that mediates glial and neuroglial communication (Lapato and Tiwari-Woodruff, 2018). Importantly, Cx43 is upregulated under inflammatory conditions and in astrocytes derived from transgenic $hSOD^{G93A}$ mice, which is an animal model of ALS. The astrocytes of ALS mice exhibit increased number of GJs, active HCs, and elevated levels of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Almad, 2016; Lagos-Cabre, 2017). Additionally, pharmacological blockade of Cx43 with both GJ or HC blockers offers neuroprotection to motor neurons cultured with $hSOD^{G93A}$ astrocytes, suggesting a detrimental role of Cx43 in ALS neurodegenerative models (Almad, 2016). Blocking Cx43 has also shown protective effects in other neurodegenerative conditions, such as hypoxia and glaucoma (Vicario, 2017). Moreover, strategies combining Cx mimetic peptides to target glial and endothelial GJs and HCs with drugs that preclude electrical synaptic signaling pathways have been considered to improve survival of neurons in neurodegenerative diseases and injuries. These mimetic peptides have revealed a reduction in inflammatory signaling after blockage of Cx43 HC activity (Moore and O'Brien, 2015).

Given the extensive expression and regulation of Cxs in glial cells, there has been a significant interest in the role that they play in different neuropathologies. These diseases are not only specific to the CNS, but also involve the peripheral nervous system, among other systems (Abrams and Scherer, 2012). A number of these brain pathologies are associated with glial reactivity, and since Cx43 is highly expressed and regulated in astrocytes, relevant correlations of Cx43 changes are related with these pathologies (Giaume, 2013). Both in human tissue as well as in animal models, changes in Cx43 expression have been associated with ischemia and stroke, epilepsy, brain infection, inflammation and traumatic brain injury (Giaume, 2010). Furthermore, Cx43 also plays a relevant role in neurodegenerative diseases such as AD, Parkinson´s disease, ALS, Multiple sclerosis (Xing, 2019) and neuropsychiatric diseases, including major depressive disorder (Kim, 2018), highlighting the deleterious effect of compromising Cx43 functions in astrocytes.

On the other hand, we have reported in non-reactive astrocytes, that β_3 Integrin overexpression leads to increased Cx43 levels (Lagos-Cabre, 2017), suggesting that Cx43 regulatory elements are downstream of β_3 Integrin-induced signaling. This agrees with reports showing that β_3 Integrin can regulate the transcription factor NF-κB, which in turn, would regulate Cx43 expression by binding to its promoter (Alonso, 2010; Balasubramaniyan, 2013). Reports indicate that Cx expression is controlled by several common and well known transcription factors, such as Sp1, Sp3 and AP-1 (Oyamada et al., 2013). However, tissue-specific expression of Cxs is regulated by particular transcription factors, such as NKx2.5, Shox2, or Tbx5 for cardiac tissue Cxs; HNF1 and Mist for digestive system Cxs, and Wnt or Sox10 for neural tissue-related Cxs (Oyamada et al., 2013). In astrocytes, ciliary neurotrophic factor receptor α (CNTFRα) appears as a regulator of Cx43 expression by binding to CNTF-response elements (Ozog, 2004; Oyamada et al., 2013). Importantly, as previously mentioned, β_3 Integrin is upregulated under inflammatory conditions in the brain (Lagos-Cabre, 2017) and therefore, a clear link between Cx43 and the β_3 Integrin seems to exist in disease progression.

Pannexins (Pxs) are proteins similar to Cxs, but only structurally related and without sequence homology (Panchin, 2000). This protein family is composed of three members (Px1, Px2 and Px3), which are orthologues to insect innexins (Panchin, 2000; Baranova, 2004; Giaume, 2013). Despite the capacity of innexins to form GJs in insects, Pxs appear to form only HCs in mammals (Giaume, 2013). However, Pxs can form GJs when they are overexpressed in mammalian cells (Vanden Abeele, 2006). Px1 is the most studied and most ubiquitous Px. Px2 has been related to neuronal differentiation and tumor development processes, while Px3 has been involved in osteoblast and chondrocyte differentiation and sperm transportation (Bruzzone, 2003; Baranova, 2004; Turmel, 2011; Penuela et al., 2013). Interestingly, astrocytes express Px1 and Px2 (Giaume, 2013), and our own work indicates that Px1 is upregulated in astrocytes treated with the pro-inflammatory cytokine TNF or in astrocytes that overexpress β_3 Integrin (Lagos-Cabre, 2017). Therefore, an interesting possibility is that Px1, as observed for innexins, might form GJs in reactive astrocytes, in which Px1 is upregulated. In addition, Px1 participates, together with Cx43, in astrocyte migration induced by neuronal cues (Alvarez, 2016). Intriguingly, functional Px1 channels have been found in several blood components, such as red blood cells and platelets (Isakson, 2017). However, red blood cells lack Cx43 and do not promote vesicular release of ATP under physiological conditions (Locovei et al., 2006; Qiu, 2011); thus, the dynamic flow of red blood cells, which depends on the ATP released from the intracellular space, occurs through Px1 rather than Cx43 channels (Forsyth, 2011).

Astrocyte reactivity is a response to any pathological condition in the CNS, characterized not only by reactive gliosis, but also by the activation of mononuclear phagocytes, neuronal injury, and cell death, events which normally are linked to changes in the activity and regulation of several major CNS Cxs, such as Cx29, Cx30, Cx32, Cx36, Cx43, and Cx47 (Decrock, 2015; Belousov, 2017). Reactivity in astrocytes not only manifests with changes in cell morphology, but also at the level of expression and activity profile of various proteins, including Cxs and Pxs (Retamal, 2007; Homkajorn et al., 2010; Giaume, 2013; Bosch and Kielian, 2014; Ben Haim, 2015; Abudara, 2015; Alvarez, 2016; Almad, 2016; Garré, 2016; Grygorowicz et al., 2016; Lagos-Cabre, 2017; Yi et al., 2017). Interestingly, at least in the case of ALS and the animal model of multiple sclerosis (experimental autoimmune encephalomyelitis, EAE), it seems that the reactive phenotype in astrocytes is achieved at early stages of the disease, even before the appearance of early

symptoms (Levine, 1999; Grygorowicz et al., 2016). In the ALS mouse model, for example, astrocytes derived from the spinal cord of neonatal mice show reactive phenotype markers after 14 days of *in vitro* culture (Lagos-Cabre, 2017). Considering that in this animal model, the symptoms only appear after 3 months (Gurney, 1994; Rojas, 2014), reactive astrocytes may play an important role in the onset and progression of this neurodegenerative disease. Likewise, early appearance of astrogliosis markers has been recently reported in an induced EAE rat model (Grygorowicz et al., 2016). In this study, the authors show that as early as 2-4 days post induction of EAE, the levels of GFAP and S100β (another gliosis marker) are elevated, whereas the first symptoms manifest only after 10 days post EAE induction (Grygorowicz et al., 2016). These findings suggest that astrocyte reactivity is an early, if not the first step, in the onset of these diseases.

Intriguingly, the reactive phenotype is also achieved *in vitro* by the addition of pro-inflammatory cytokines such as IL-1β and TNF, or by the addition of conditioned medium from activated microglia (Retamal, 2007; Lagos-Cabre, 2017), suggesting that astrocytes in culture retain all the relevant components that can trigger the reactive response. Pro-inflammatory molecules not only upregulate astrocyte Cx43 and Px1, but also increase β_3 Integrin expression levels and induce astrocyte reactivity (Lagos-Cabre, 2017). Moreover, the reactive phenotype in astrocytes can also be achieved by overexpression of proteins in the absence of cytokine treatments. We have recently reported that by overexpressing β_3 Integrin, astrocytes increase the expression of reactivity markers, such as GFAP and iNOS, and attain a functional reactive phenotype by increasing Cx43, Px1, and P2X7R expression levels and ATP release. These changes make astrocytes responsive to external cues that promote cell polarization and migration (Lagos-Cabre, 2017; Lagos-Cabre, 2018). On the other hand, silencing of β_3 Integrin precludes stimulus-induced astrocyte migration even when the cells are treated with TNF (Lagos-Cabre, 2017). Additionally, Strużyńska's group described a temporally coincident elevated expression of Cx43, P2X7R and reactivity markers, where the sole blockade of P2X7R decreased astrogliosis and ameliorated EAE symptoms in an animal model (Grygorowicz et al., 2016). In the same line, Cx43 mimetic peptides have been reported to reduce astrogliosis and cytokine release, improving function after spinal cord injury (O'Carroll, 2013). These results, together with the recent findings that support the reversibility of astrocyte reactivity (Hara, 2017), indicate that the regulation of the signaling pathway that involves HC opening, ATP release, and the activation of the P2X7R might provide a therapeutic window of opportunity to control astrogliosis and the progression of neurodegenerative diseases.

Despite the capacity of Cxs to form GJs, HCs formed by these proteins seem to be mostly affected by a pro-inflammatory environment. For example, the strong reactivity of astrocytes observed in AD is accompanied by an increase in the activity of Cx43 HCs, which maintain the reactive phenotype by releasing toxic molecules to the extracellular space (Yi et al., 2017). In pilocarpine-induced status epilepticus mice, Cx43 and Cx40 levels increase in GFAP-positive astrocytes, effect that lasts for at least 2 months in the hippocampus (Wu, 2015). Similarly, in astrocytes treated with conditioned media from microglia activated by LPS, Giaume and co-workers found an increase in astrocyte permeability, along with a decrease in GJ communication (Retamal, 2007), demonstrating the importance of HCs -rather than GJs- during inflammation.

As stated above, increased levels of Cx43 during astrocyte reactivity help maintain the reactive phenotype of astrocytes and microglia by releasing ATP, glutamate and other molecules to the extracellular space, generating a positive feedback loop (Ben Haim, 2015). In the same context, high Cx43 levels in reactive astrocytes derived from ALS mice help sustain an increase in $[Ca^{2+}]_i$ induced by mechanical or ATP stimulation, which is abolished by a Cx43-blocking peptide (Almad, 2016). In agreement with these findings, mice with genetically reduced levels of Cx43 show attenuation of LPS-induced sepsis, which includes reduction of activated microglia and cytokine production (Zhou, 2015). These reports highlight Cx43 as a key element to maintain the astrocyte reactive phenotype by promoting ATP release and Ca^{2+} signals.

When spinal cord astrocytes are stimulated with fibroblast growth factor 1 (FGF-1), which stimulates astrocyte reactivity as well, increased Px1 and Cx43 HC opening induces cell permeability, ATP release and $[Ca^{2+}]_i$ increase (Garré, 2016). Interestingly, the opening of these HCs is prevented by the addition of a Phospholipase C gamma (PLCγ) inhibitor or by loading cells with BAPTA-AM (Garré, 2016), suggesting that Ca^{2+} signals likely derived from activation of IP_3R in the endoplasmic reticulum (ER) are involved in HC opening. Supporting this idea, *in vivo* studies have shown that after a brain cortex injury, the surrounding astrocytes become reactive. Interestingly, their reactivity can be prevented with BAPTA-AM, which reduces GFAP levels and glial scar formation (Gao, 2013), demonstrating the requirement of Ca^{2+} signals in this process. Similarly, in the astrocyte DITNC1 cell line, as well as in primary astrocytes treated with TNF, Ca^{2+} is released from the ER and ATP is released through HCs, in a complex signal transduction cascade that results in changes in cell shape and initiation of cell migration when stimulated with the neuronal protein Thy-1/CD90 (Henriquez, 2011; Alvarez, 2016; Lagos-Cabre, 2017; Lagos-Cabre, 2018). Thy-1/CD90 is a glycoprotein from the neuronal surface that binds to astrocytes by engaging $\alpha_v\beta_3$ Integrin and Syndecan-4 receptors, recruiting diverse focal adhesion proteins that include PLCγ. The activation of PLCγ results in DAG and IP_3 production and consequent IP_3R activation, Ca^{2+} release from the ER, and opening of Cx43 and Px1 HCs, which release ATP to the extracellular space. ATP then binds to the P2X7R, allowing Ca^{2+} entry and thus, inducing morphological changes and cell migration (Henriquez, 2011; Alvarez, 2016; Lagos-Cabre, 2017; Lagos-Cabre, 2018) (**Figure 3**). These findings demonstrate the ability of HCs to release molecules that sustain an increased $[Ca^{2+}]_i$ to maintain the astrocyte reactive phenotype and therefore, suggest that Ca^{2+} is a key player in the modulation of astrocyte reactivity. Since migration of astrocytes under either physiological or pathological conditions is a very complex process, future systematic studies are

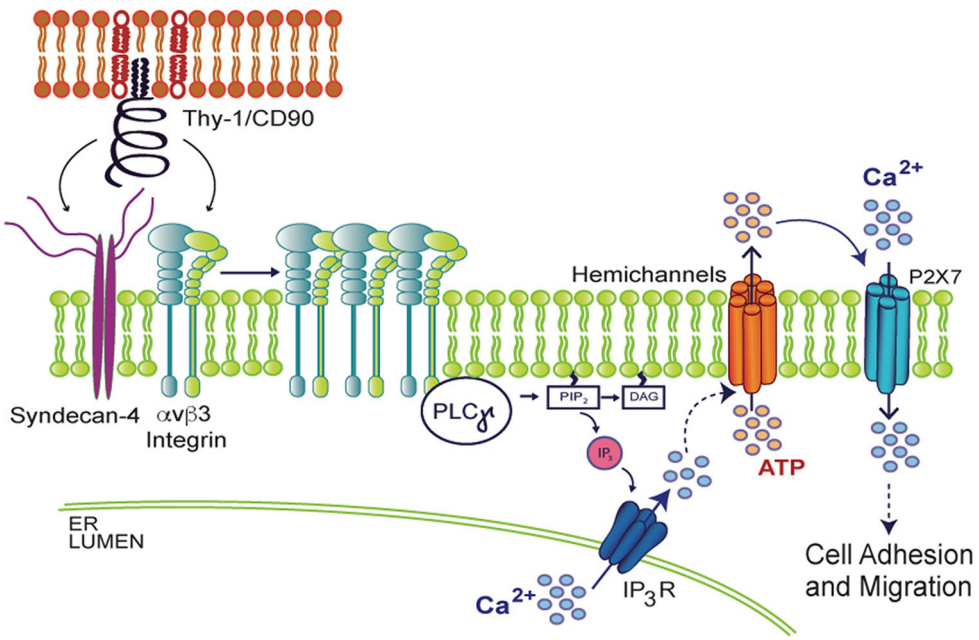

FIGURE 3 | Molecular mechanism involved in Thy-1/CD90-induced astrocyte adhesion and migration. In the context of neuron (upper red lipid bilayer) and astrocyte (lower green lipid bilayer) communication, neuronal Thy-1/CD90 interacts with both $\alpha_v\beta_3$ Integrin and Syndecan-4 astrocytic receptors, triggering PLCγ activation, IP_3 production, IP_3R activation, increase in cytosolic Ca^{2+}, and opening of hemichannels and subsequent ATP release. Extracellular ATP mediates P2X7R integrin-dependent transactivation, allowing Ca^{2+} entry, which results in morphological changes of astrocytes (increased adhesion) and later, cell migration.

needed to fully elucidate the relevant role of Ca^{2+} in astrocyte migration and reactivity.

REGULATION OF CELL MIGRATION BY CONNEXINS

Cell migration is an essential process for the development, maintenance and healing of multicellular organisms. By sensing their environment, cells polarize, extend filopodia and lamellipodia to the leading front, adhere to the ECM proteins through integrins and Syndecan-4, and form focal adhesions and bundles of actin microfilaments called stress fibers. These focal points of adhesion to ECM proteins, along with stress fibers, allow cells to contract their rear and promote forward cell movement (Ladoux and Mege, 2017). Until now, astrocyte migration has not been studied extensively and detailed mechanisms remain largely unknown. While single-cell migration has been studied in depth, collective cell migration is a less studied process that refers to the coordinated movement of cell groups, sheets, or chains (De Pascalis and Etienne-Manneville, 2017). However, collective cell migration cannot be simplified as a group of independent cells that move at the same speed and direction; but as a more complex phenomenon that can improve migration efficiency by rendering cells with specific features (Mayor and Etienne-Manneville, 2016). Just as single cells, migrating cell groups are equally relevant; they govern collective cell migration during embryonic development, wound healing, and cancer cell invasion, among other processes (Ladoux and Mege, 2017). Collective cell migration relies on both cell-environment, as well as cell-cell interactions, and on several proteins related to cell-cell communication, including proteins forming not only GJs, but also adherens junctions and tight junctions (Ladoux and Mege, 2017).

Cxs participate in the migration of astrocytes (Homkajorn et al., 2010; Alvarez, 2016; Lagos-Cabre, 2017) and several other cell types, such as neurons (Qi, 2016; Laguesse, 2017), cancer cells (Graeber and Hulser, 1998), keratinocytes (Jaraiz-Rodriguez, 2017) and bone marrow stromal cells (Jin, 2017). Reports have indicated that the mutant $Cx26^{S17F}$, related to keratitis-ichthyosis-deafness syndrome (KIDS), reduces GJ communication, and decreases collective migration of primary keratinocytes (Press, 2017). Interestingly, despite the fact that $Cx26^{S17F}$ mice show normal skin wound closure, their repaired zone is thicker than in controls, suggesting abnormal remodeling (Press, 2017). Similarly, wound-healing assays with HeLa cells that overexpress Cx26 show increased Rac1-dependent cell migration, along with downregulation of N-Cadherin (Polusani, 2016). It appears that the reduced levels of N-Cadherin release a break for cell migration that acts by "sequestering" Rac1 and other cellular components near the membrane; thus, when N-Cadherin levels go down, Rac1 is released and activated, allowing cell migration (Polusani, 2016). Of note, these authors also show that decreased levels of both N-Cadherin and cell migration

are dependent on Cx26-forming GJs, but not HCs (Polusani, 2016). Therefore, GJs seem important for the regulation of collective cell migration, in processes such as skin wound repair and tumor invasion.

On the other hand, Cx43 favors migration of projection neurons over radial glial cells in the developing brain (Laguesse, 2017). In this report, it is indicated that Cx43 favors cell-to-cell contact by interacting with elongator complex elements such as Elp1 and Elp3, allowing the acetylation of Cx43 and its membrane localization (Laguesse, 2017). Such membrane destination of Cx43 dependent on acetylation levels has also been reported in HeLa cells (Laguesse, 2017). An important observation in these studies is that channel activity was not required for neuronal migration. In other cases, the function of GJs as channels seems less clear, but cellular localization of Cx43 at the plasma membrane also seems to control cell migration by favoring cell adhesion. It will be important to determine if these "channel-dependent" or "channel-independent" functions require the presence of functional GJs or HCs, respectively, and whether or not these Cx structures acting as scaffolds are also important for cell migration (Kameritsch et al., 2012).

Accumulating evidence has also indicated that Cxs can enhance and inhibit cancer cell migration, depending on the stage of the disease and tissue involved (Kotini and Mayor, 2015). Cx26 and Cx43 expression levels are increased in invasive lesions and in lymph node metastases of breast cancer (Jamieson, 1998; Kanczuga-Koda, 2006). Overexpression of Cx43 in breast cancer metastatic cell lines enhances tumorigenesis without affecting GJ formation or cell motility (Li, 2008). Another report has indicated a correlation between Cx43 levels and metastatic potential in prostate cancer cells (Zhang, 2015), whereas in testicular cancer cells resistant to cisplatin, overexpression of Cx43 reduces migration/invasion of these cells (Wu, 2018). More importantly, the role of Cx43 in cell migration was first described in breast MCF-10A epithelial cells using a siRNA screening approach designed to identify genes that regulate cell motility (Simpson, 2008). In these cells, Cx43 controls migration and directionality, since knockdown of Cx43 leads to erratic, slow and reverse migration. This could be related to the increased capacity of MCF-10A cells to form protrusions, which results in cells with a more polygonal shape and diminished ability to migrate. Interestingly, a similar cellular shape has been observed in cardiac neural crest cells from $Cx43^{-/-}$ mice (Xu, 2006; Matsuuchi and Naus, 2013). Stachowiak and co-workers have shown that reincorporation of Cx43 through microvesicles derived from HeLa cells decreases migration of MDA-MB231 breast tumour cells (Ferrati, 2017). These Cx43-containing microvesicles are described to form GJs in these breast cancer cells, favoring the idea that functional GJs, rather than HCs, decrease cell migration. Considering these results, the role of Cx43 in cell migration still seems controversial. Perhaps, there is a critical amount of Cx43 at the plasma membrane that favors GJ formation, which might also determine the cellular ability to either move or remain stationary.

Accordingly, Cx43 has been involved in the inhibition of glioma cell migration (Jaraiz-Rodriguez, 2017). However, this effect relies on the interaction of Cx43 with c-Scr, and not on its activity as a channel or HC. In many cells, active c-Src phosphorylates and activates focal adhesion kinase (FAK), creating additional binding sites for protein-complex formation. These complexes induce formation of focal adhesions, which are essential for cells to adhere to a substrate and migrate (Dubash, 2009). Cx43 forms a complex with c-Src and inhibits Src activity by recruiting its inhibitor, C-terminal Src kinase (Csk), to the complex (Gonzalez-Sanchez, 2016). Therefore, Cx43 HCs could induce or repress cell motility by interacting with a different set of molecules, at least, in deregulated cells such as glioma cells and other cancer cells, where GJs can act as inhibitors of cell migration. Therefore, it seems clear that Cxs play an important role in cell migration in various cell types, but the final outcome is either membrane expression level- or cell-context-dependent.

In summary, despite available information concerning the mechanisms governing cell migration in various cell types, astrocyte migration still requires future research in order to better understand the molecular mechanisms that Cxs use to regulate motility, in order to serve as potential targets for the development of clinical interventions for astrogliosis and glioma metastasis.

CONNEXINS AND ASTROCYTE MIGRATION

Astrocytes in the adult brain are non-migratory cells; *i.e.*, are quiescent under normal physiological conditions. However, they can be activated to become migratory under pathological conditions such as trauma, ischemia, infection, inflammation and neurodegeneration (Zhan, 2017). Recent *in vivo* studies indicate that reactive astrocytes undergo hypertrophy, cell polarization, and cell migration (Bardehle, 2013; Moore and Jessberger, 2013; Sirko, 2013). Conversely, astrocytes reportedly undertake migration upon injury or other pro-inflammatory conditions to form a glial scar and repair the area of the lesion (Bush, 1999; Faulkner, 2004; Sofroniew, 2005; Chai, 2013). Results from embryonic brain slices of Cx43 KO mice show abnormal distribution of astrocytes when compared with the normal counterpart (Perez Velazquez, 1996; Kotini and Mayor, 2015). Similar experiments performed in a subline of Cx43 KO mice called "Shuffler", which exhibits defects in brain architecture and astrocyte distribution, strongly suggest migration defects of astrocytes lacking Cx43 (Wiencken-Barger, 2007; Kotini and Mayor, 2015). Our own findings with neonatal rat astrocytes activated *in vitro* by the addition of TNF or other cytokines, indicate that only reactive astrocytes move in response to external stimuli (Lagos-Cabre, 2017). In this context, Cx43 appears to be the most relevant HC-forming protein involved in reactive astrocyte migration, since the specific inhibitory peptide Gap19 abolishes HC opening and cell migration induced by neuronal Thy-1/CD90 (Alvarez, 2016; Lagos-Cabre, 2017). Therefore, pro-inflammatory signals that trigger astrocyte reactivity seem to be necessary for these cells to

move in response to extracellular cues, and their migration is related to the presence of Cx43.

Thy-1/CD90 activates its two receptors, $\alpha_v\beta_3$ Integrin and Syndecan-4, only in TNF-treated astrocytes (Leyton, 2001; Kong, 2013; Alvarez, 2016; Lagos-Cabre, 2017), likely because the expression levels of both receptors are enhanced upon pro-inflammatory conditions (Lagos-Cabre, 2017). Importantly, proteins upregulated by TNF treatment also include: Cx43, Px1, P2X7R, GFAP, and iNOS (Lagos-Cabre, 2017). The engagement of $\alpha_v\beta_3$ Integrin and Syndecan-4 by Thy-1/CD90 in reactive astrocytes triggers similar intracellular signaling pathways as those described for DITNC1 astrocytes (see **Figure 3**), including Ca^{2+} release from the ER, opening of Cx43 and Px1 HCs, ATP release, and P2X7R activation, with the consequent further increase in $[Ca^{2+}]_i$ required for cell migration (Abudara, 2015; Alvarez, 2016; Garré, 2016; Grygorowicz et al., 2016; Lagos-Cabre, 2017). However, this molecular mechanism seems to be necessary only for mature astrocytes, since the addition of conditioned media from microglia or IL-1β to astrocyte progenitor cultures reduces cell migration and spontaneous Ca^{2+} oscillations in these cells (Striedinger and Scemes, 2008). These astrocyte progenitors also show release of ATP to the extracellular medium, but in an exocytosis-dependent fashion that also depends on Ca^{2+} (Striedinger et al., 2007).

Despite the key role of ATP in astrocyte migration, the addition of different concentrations of extracellular ATP to non-reactive astrocytes only induces a graded reactive phenotype, including proliferation and stellation; however, under these conditions, astrocyte phenotype is not accompanied by an increase in GFAP and cells do not migrate in wound-healing assays (Adzic, 2017). These results indicate that even though ATP triggers various attributes of activated astrocytes, this is not sufficient to induce a full reactive phenotype in astrocytes. On the contrary, Wang and coworkers showed that astrocytes migrate after ATP or UTP treatment and increase their GFAP and $\alpha_v\beta_3/\beta_5$ Integrin levels, of which the latter is important for astrocyte migration after UTP treatment (Wang, 2005). In this study, the authors utilize primary astrocytes in culture, and suggest that they migrate because the nucleotides induce astrocyte reactivity, which is supported by the increased expression of GFAP and integrins. Despite the fact that Cxs were not investigated in these studies, by adding ATP (or UTP) to the extracellular medium of astrocytes and inducing astrocyte reactivity, Cx43 may also be upregulated (Lagos-Cabre, 2017), possibly explaining the effect observed in cell migration.

The localization of Cx43 is also modified in reactive astrocytes. Under normal conditions, Cx43 is mostly localized in intracellular vesicles, but after the addition of TNF, it localizes in a near-to-membrane zone (Lagos-Cabre, 2017); this result also supports the importance of HCs in astrocyte reactivity and migration. Indeed, the levels of Cx43 at the plasma membrane could regulate ATP release and, as a consequence, increase $[Ca^{2+}]_i$, which is necessary for cell migration (Alvarez, 2016; Lagos-Cabre, 2017). Consequently, any increase in $[Ca^{2+}]_i$ should lead to cell migration. In support of this assumption, Hayashi and coworkers observed that the increase in Ca^{2+} induced by ionomycin was necessary and sufficient to induce cell migration of leading edge mesodermal cells treated with this ionophore (Hayashi et al., 2018). Interestingly, our own results show that only partial cell migration (8.2 ± 1.8% wound closure compared to 2.7 ± 1.2% of control samples, **Figure 4**) is observed when astrocytes are treated with ionomycin in a wound healing assay, while after pre-treating with TNF, ionomycin significantly enhances migration (15.3 ± 4.1%), with respect to treatment with only ionomycin (**Figure 4**). Pre-incubation with BAPTA-AM completely abolishes astrocyte migration induced by ionomycin/TNF treatment (**Figure 4**), indicating the necessity of cytosolic Ca^{2+} for the response. However, although TNF alone

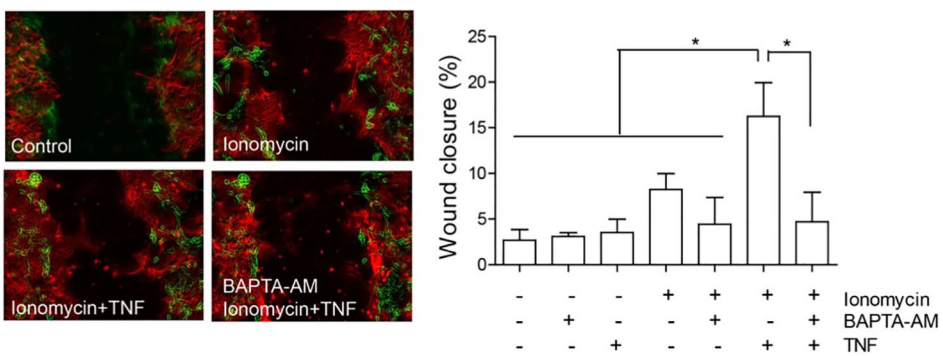

FIGURE 4 | Astrocyte migration induced by ionomycin. Primary astrocytes from rat cortexes were isolated and cultured as published before (Lagos-Cabre, 2017). Astrocytes were seeded in 24 well-plates and treated or not with 10 ng/ml of TNF for 48 h. Astrocytes were then subjected to a scratch with a pipette tip and floating cells were washed away before treatment addition. Left panel: Representative images of the wound-healing assay with pseudocolor of selected treatments. The green color represents cells in the wound edge at 0 h and the red color, cells of the same wound 24 h after treatment. Right panel: Wound-healing assay quantification of astrocyte migration 24 h after treating cells with 1 μM of ionomycin. Where indicated, cells were pre-incubated with 5 μM BAPTA-AM for 30 min, prior to ionomycin addition. Wound closure was higher in cells treated with ionomycin + TNF, revealing increase of migration. Values in the graph represent mean ± s.e.m. of three independent experiments. The results were analyzed using one-way ANOVA and Tukey's post-test. Statistical significance is indicated, *$p < 0.05$.

does not produce changes in either $[Ca^{2+}]_i$, ATP release, or cell migration (Sofroniew, 2014), it appears to prime the cells to respond to additional stimuli. Considering that TNF induces astrogliosis, these results suggest that the reactive phenotype is a key step for astrocytes to move and that $[Ca^{2+}]_i$ increase, although necessary, is not sufficient to induce astrocyte migration. This potentiation of the migratory effect induced by TNF is interesting and suggests that the increase in P2X7R protein levels (Lagos-Cabre, 2017) (or other Ca^{2+} channels) could explain the difference in migration by further increasing the Ca^{2+} influx induced by ionomycin. Alternatively, since astrocyte migration involves elevation of cytosolic Ca^{2+} *via* both ER release downstream of integrin activation and uptake of extracellular sources through ATP-gated-P2X7R pores (**Figure 3**), it is possible that Ca^{2+} is elevated at specific times and places. Therefore, the bulk of Ca^{2+} induction by ionomycin does not mimic all events that are triggered by physiological ligands, such as Thy-1/CD90. We have previously shown that astrocytes require TNF to respond to Thy-1/CD90, which induces a robust elevation of $[Ca^{2+}]_i$ by the release of ATP and activation of the P2X7R (Lagos-Cabre, 2017). Here, we confirmed that an increase in $[Ca^{2+}]_i$ alone only slightly affects astrocyte migration, an event that further requires the molecules that are overexpressed by TNF treatment (such as Cx HCs) to maintain, for example, a reactive phenotype, or to sustain a positive feedback loop between ATP release, P2X7R activation, and $[Ca^{2+}]_i$ increase. In our previous reports we have shown that two Ca^{2+} sources are needed to induce astrocyte migration: one dependent on Ca^{2+} released from internal stores and triggered by integrin engagement, which is necessary for Cx43 HC opening; and another related to ATP release and P2X7R activation (Henriquez, 2011; Alvarez, 2016; Lagos-Cabre, 2017). Thus, although ionomycin increases $[Ca^{2+}]_i$ in an artificial manner, low levels of Cx43 and P2X7R at the plasma membrane -due to the lack of pro-inflammatory signals- could explain the reduced effect of the ionophore on cell migration.

Alternatively, enhanced $[Ca^{2+}]_i$ induced by ionomycin might stimulate the opening of different pores, other than Cx HCs, but that share P2X7R properties, as has been reported in 2BH4 thymic epithelial cells and peritoneal macrophages (Faria, 2009). The nature of this pore was not determined and the authors indicated that pore activation induced by $[Ca^{2+}]_i$ depends on calmodulin, PLC, MAPK, and cytoskeleton components (Faria, 2009). According to the scratch assay results that we show here, primary astrocytes treated with ionomycin alone increase their migration, but to a level not as high as that in cells pre-treated with TNF (**Figure 4**). Thus, the key event seems to be a pro-inflammatory stimulus that, apart from provoking elevated levels of many surface proteins (Lagos-Cabre, 2017), could regulate distinct intracellular signaling pathways that might activate the alternative pore proposed by Farias and coworkers.

We have proposed that TNF elevates β_3 Integrin cluster formation in astrocytes by increasing the expression of $\alpha_v\beta_3$ Integrin at the plasma membrane. A low level of clustering could trigger signaling cascades involved in focal adhesion formation, including PLCγ activation and Ca^{2+} release *via* IP_3R activation to yet undetectable levels, but that prompts cells to quickly respond to stimuli like ionomycin. We have tested Cx43 HC opening by LY uptake in ionomycin-treated astrocytes and found that these HCs open even in the absence of TNF (Lagos-Cabre, 2018). These results suggest that: i) HC opening and astrocyte migration are two independent processes that can potentiate each other with Thy-1/CD90 stimulation (see above); ii) HC opening is only part of the mechanism required to be activated in order to trigger a response; and iii) the LY dye could be passing through a pore that is different from HCs, but that opens with ionomycin treatment (Faria, 2009). Additionally, the combination of ionomycin and Thy-1/CD90 induces lower migration levels than Thy-1/CD90 and TNF applied together in a Boyden chamber transmigration assay (Lagos-Cabre, 2018). The latter reinforces the idea that Thy-1/CD90 has a limited capacity to stimulate non-reactive cells and that ionomycin does not produce all the changes induced by TNF. Thus, astrocyte migration requires many molecular components increased by TNF and cannot be replaced by an artificial $[Ca^{2+}]_i$ increase, supporting the idea that Ca^{2+} is necessary, but not sufficient for astrocytes to migrate. Of note, the different elements that interact with each other to regulate astrocyte migration are also regulated by different signaling pathways related to various astrocytic functions. These mechanisms will provide insights for future research on astrocyte migration.

CONCLUDING REMARKS

In this review, we summarized studies related to cell migration and regulation of this process by Cxs. The information exposed here strongly suggests that astrocyte reactivity, as well as migration in a pro-inflammatory environment, relies predominantly on Cx HCs, rather than GJs.

Despite the similarity between Cxs, all of them show different properties that provide a broad spectrum of responses in any given situation. However, Cx43 probably emerges as the main Cx involved in astrocyte physiology, controlling its reactive phenotype, allowing migration and facilitating cell-cell communication with surrounding cells.

The effect of Cxs on migration is usually observed during inflammation, and the presence of Cx HCs is required to maintain the reactive phenotype of astrocytes after injury. Inhibition of Cx HCs with peptides or blockade of the P2X7R improves function after spinal cord injury or EAE symptoms, respectively, indicating that the modulation of this signaling pathway could provide a therapeutic opportunity to treat these conditions. Release of ATP by Cx HCs and intake of Ca^{2+} through the P2X7R are among the crucial steps for astrocyte reactivity and migration, demonstrating that these two processes are closely related, since only reactive astrocytes migrate.

The participation of Cxs in astrocyte migration is related to their function as channels and the communication that they mediate through cell-cell, as well as cell-ECM interactions. Importantly, the regulation of cell adhesion and cytoskeletal dynamics, both of which are relevant to cell migration, occurs by post-translational modifications of Cxs, which are induced by

kinases, phosphatases, and acetylases. In this scenario, both phosphorylation and acetylation regulate membrane localization of Cxs.

It is easy to speculate that GJs are not related to the maintenance of the negative conditions that characterize astrogliosis, since they exert their role in cell-cell communication and not in cell-ECM communication. We believe that single-cell migration is the operating mechanism during astrogliosis, and considering that GJs are reportedly more important for collective migration, and that this type of cell movement is not observed in the CNS after injury (Carbonell, 2005; Retamal, 2007), we propose that mostly HCs, rather than GJs, are related to astrocyte migration.

The presence of Cx HCs in astrocytes is not only important for the initiation of reactivity or migration, but also to maintain the reactive phenotype during longer periods, which then increases the negative effects of neurological diseases or pro-inflammatory conditions. This Cx role has been supported by several groups and could represent an important target for treatment or prevention of such pathologies. However, due to the importance of Cx HCs in astrocyte reactivity and migration, a specific treatment based on HC blockade should be pursued, especially for neurodegenerative diseases and astrocytoma/glioma treatment.

New studies able to discriminate between the two distinct Cx channel activities are necessary to enlighten the specific Cx roles in physiological and pathological conditions and for future development of interventions that will be able to ameliorate the detrimental effects of CNS injury and neurodegenerative diseases. The challenge will be to modulate reactive astrocytes according to the optimal regenerative responses desired, and to define the correct therapeutic window according to the specific stage of the pathology. These future therapeutic strategies should consider both pharmacological and nonpharmacological approaches to enrich the environment necessary for CNS regeneration (Pekny and Pekna, 2014). Furthermore, future research on the complex molecular mechanisms that regulate astrocyte migration is also needed for the development of clinical interventions for astrogliosis.

AUTHOR CONTRIBUTIONS

LL contributed in the conception and design of the work; FB-B and RL-C to the acquisition, analysis, and interpretation of data for the work. AA, RL-C, and LL contributed drafting the work. All authors critically revised the work for important intellectual content, provided approval for publication of the content, and agreed to be accountable for all aspects of the work.

FUNDING

LL was supported by Fondo Nacional de Desarrollo Científico y Tecnológico #1150744 (LL) and Comisión Nacional de Investigación Científica y Tecnológica-FONDAP #15130011 (LL). Fondo Nacional de Desarrollo Científico y Tecnológico #3140460 (RL-C). AA would like to thank the support of Project DIUA 169-2019 from Universidad Autónoma de Chile.

ACKNOWLEDGMENTS

LL would like to express gratitude for the professional drawing performed by Bonhomia Design.

REFERENCES

Abrams, C. K., and Scherer, S. S. (2012). Gap junctions in inherited human disorders of the central nervous system. *Biochim. Biophys. Acta* 1818 (8), 2030–2047. doi: 10.1016/j.bbamem.2011.08.015

Abudara, V., Roux, L., Dallerac, G., Matias, I., Dulong, J., Mothet, J. P., et al. (2015). Activated microglia impairs neuroglial interaction by opening Cx43 hemichannels in hippocampal astrocytes. *Glia* 63 (5), 795–811. doi: 10.1002/glia.22785

Adzic, M., Stevanovic, I., Josipovic, N., Laketa, D., Lavrnja, I., Bjelobaba, I. M., et al. (2017). Extracellular ATP induces graded reactive response of astrocytes and strengthens their antioxidative defense in vitro. *J. Neurosci. Res.* 95 (4), 1053–1066. doi: 10.1002/jnr.23950

Agulhon, C., Petravicz, J., McMullen, A. B., Sweger, E. J., Minton, S. K., Taves, S. R., et al. (2008). What is the role of astrocyte calcium in neurophysiology? *Neuron* 59 (6), 932–946. doi: 10.1016/j.neuron.2008.09.004

Ahmed, S. M., Rzigalinski, B. A., Willoughby, K. A., Sitterding, H. A., Ellis, E. F., et al. (2000). Stretch-induced injury alters mitochondrial membrane potential and cellular ATP in cultured astrocytes and neurons. *J. Neurochem.* 74 (5), 1951–1960. doi: 10.1046/j.1471-4159.2000.741951000000000.x

Allbritton, N. L., Meyer, T., and Stryer, L. (1992). Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. *Science* 258 (5089), 1812–1815. doi: 10.1126/science.1465619

Allen, N. J., and Eroglu, C. (2017). Cell Biology of Astrocyte-Synapse Interactions. *Neuron* 96 (3), 697–708. doi: 10.1016/j.neuron.2017.09.056

Almad, A. A., Doreswamy, A., Gross, S. K., Richard, J. P., Huo, Y., Haughey, N., et al. (2016). Connexin 43 in astrocytes contributes to motor neuron toxicity in amyotrophic lateral sclerosis. *Glia* 64 (7), 1154–1169. doi: 10.1002/glia.22989

Alonso, F., Krattinger, N., Mazzolai, L., Simon, A., Waeber, G., Meda, P., et al. (2010). An angiotensin II- and NF-kappaB-dependent mechanism increases connexin 43 in murine arteries targeted by renin-dependent hypertension. *Cardiovasc. Res.* 87 (1), 166–176. doi: 10.1093/cvr/cvq031

Alvarez, A., Lagos-Cabre, R., Kong, M., Cardenas, A., Burgos-Bravo, F., Schneider, P., et al. (2016). Integrin-mediated transactivation of P2X7R via hemichannel-dependent ATP release stimulates astrocyte migration. *Biochim. Biophys. Acta* 1863 (9), 2175–2188. doi: 10.1016/j.bbamcr.2016.05.018

Ambrosi, C., Ren, C., Spagnol, G., Cavin, G., Cone, A., Grintsevich, E. E., et al. (2016). Connexin43 Forms Supramolecular Complexes through Non-Overlapping Binding Sites for Drebrin, Tubulin, and ZO-1. *PloS One* 11 (6), e0157073. doi: 10.1371/journal.pone.0157073

Anders, S., Minge, D., Griemsmann, S., Herde, M. K., Steinhauser, C., Henneberger, C., et al. (2014). Spatial properties of astrocyte gap junction coupling in the rat hippocampus. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369 (1654), 20130600. doi: 10.1098/rstb.2013.0600

Aubert, A., Pellerin, L., Magistretti, P. J., and Costalat, R. (2007). A coherent neurobiological framework for functional neuroimaging provided by a model integrating compartmentalized energy metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 104 (10), 4188–4193. doi: 10.1073/pnas.0605864104

Balasubramaniyan, V., Dhar, D. K., Warner, A. E., Vivien Li, W. Y., Amiri, A. F., Bright, B., et al. (2013). Importance of Connexin-43 based gap junction in

cirrhosis and acute-on-chronic liver failure. *J. Hepatol.* 58 (6), 1194–1200. doi: 10.1016/j.jhep.2013.01.023

Bao, X., Reuss, L., and Altenberg, G. A. (2004). Regulation of purified and reconstituted connexin 43 hemichannels by protein kinase C-mediated phosphorylation of Serine 368. *J. Biol. Chem.* 279 (19), 20058–20066. doi: 10.1074/jbc.M311137200

Baranova, A., Ivanov, D., Petrash, N., Pestova, A., Skoblov, M., Kelmanson, I., et al. (2004). The mammalian pannexin family is homologous to the invertebrate innexin gap junction proteins. *Genomics* 83 (4), 706–716. doi: 10.1016/j.ygeno.2003.09.025

Bardehle, S., Kruger, M., Buggenthin, F., Schwausch, J., Ninkovic, J., Clevers, H., et al. (2013). Live imaging of astrocyte responses to acute injury reveals selective juxtavascular proliferation. *Nat. Neurosci.* 16 (5), 580–586. doi: 10.1038/nn.3371

Batra, N., Riquelme, M. A., Burra, S., Kar, R., Gu, S., and Jiang, J. X. (2014). Direct regulation of osteocytic connexin 43 hemichannels through AKT kinase activated by mechanical stimulation. *J. Biol. Chem.* 289 (15), 10582–10591. doi: 10.1074/jbc.M114.550608

Bedner, P., Niessen, H., Odermatt, B., Kretz, M., Willecke, K., and Harz, H. (2006). Selective permeability of different connexin channels to the second messenger cyclic AMP. *J. Biol. Chem.* 281 (10), 6673–6681. doi: 10.1074/jbc.M511235200

Belousov, A. B., Fontes, J. D., Freitas-Andrade, M., and Naus, C. C. (2017). Gap junctions and hemichannels: communicating cell death in neurodevelopment and disease. *BMC Cell Biol.* 18 (Suppl 1), 4. doi: 10.1186/s12860-016-0120-x

Ben Haim, L., Carrillo-de Sauvage, M. A., Ceyzeriat, K., and Escartin, C. (2015). Elusive roles for reactive astrocytes in neurodegenerative diseases. *Front. Cell Neurosci.* 9, 278. doi: 10.3389/fncel.2015.00278

Bennett, B. C., Purdy, M. D., Baker, K. A., Acharya, C., McIntire, W. E., Stevens, R. C., et al. (2016). An electrostatic mechanism for Ca(2+)-mediated regulation of gap junction channels. *Nat. Commun.* 7, 8770. doi: 10.1038/ncomms9770

Berthoud, V. M., Minogue, P. J., Laing, J. G., and Beyer, E. C. (2004). Pathways for degradation of connexins and gap junctions. *Cardiovasc. Res.* 62 (2), 256–267. doi: 10.1016/j.cardiores.2003.12.021

Beyer, E. C., Gemel, J., Martinez, A., Berthoud, V. M., Valiunas, V., Moreno, A. P., et al. (2001). Heteromeric mixing of connexins: compatibility of partners and functional consequences. *Cell Commun. Adhes.* 8 (4-6), 199–204. doi: 10.3109/15419060109080723

Blanchette, M., and Daneman, R. (2015). Formation and maintenance of the BBB. *Mech. Dev.* 138 Pt 1, 8–16. doi: 10.1016/j.mod.2015.07.007

Blomstrand, F., Venance, L., Siren, A. L., Ezan, P., Hanse, E., and Glowinski, J. (2004). Endothelins regulate astrocyte gap junctions in rat hippocampal slices. *Eur. J. Neurosci.* 19 (4), 1005–1015. doi: 10.1111/j.0953-816x.2004.03197.x

Bosch, M., and Kielian, T. (2014). Hemichannels in neurodegenerative diseases: is there a link to pathology? *Front. Cell Neurosci.* 8, 242. doi: 10.3389/fncel.2014.00242

Brightman, M. W., and Reese, T. S. (1969). Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.* 40 (3), 648–677. doi: 10.1083/jcb.40.3.648

Brown, A. M., and Ransom, B. R. (2007). Astrocyte glycogen and brain energy metabolism. *Glia* 55 (12), 1263–1271. doi: 10.1002/glia.20557

Bruzzone, R., Hormuzdi, S. G., Barbe, M. T., Herb, A., and Monyer, H. (2003). Pannexins, a family of gap junction proteins expressed in brain. *Proc. Natl. Acad. Sci. U.S.A.* 100 (23), 13644–13649. doi: 10.1073/pnas.2233464100

Burda, J. E., and Sofroniew, M. V. (2014). Reactive gliosis and the multicellular response to CNS damage and disease. *Neuron* 81 (2), 229–248. doi: 10.1016/j.neuron.2013.12.034

Bush, T. G., Puvanachandra, N., Horner, C. H., Polito, A., Ostenfeld, T., Svendsen, C. N., et al. (1999). Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron* 23 (2), 297–308. doi: 10.1016/S0896-6273(00)80781-3

Bylicky, M. A., Mueller, G. P., and Day, R. M. (2018). Mechanisms of endogenous neuroprotective effects of astrocytes in brain injury. *Oxid. Med. Cell Longev.* 2018, 6501031. doi: 10.1155/2018/6501031

Cao, Z., Gao, Y., Deng, K., Williams, G., Doherty, P., and Walsh, F. S. (2010). Receptors for myelin inhibitors: Structures and therapeutic opportunities. *Mol. Cell Neurosci.* 43 (1), 1–14. doi: 10.1016/j.mcn.2009.07.008

Carbonell, W. S., Murase, S., Horwitz, A. F., and Mandell, J. W. (2005). Migration of perilesional microglia after focal brain injury and modulation by CC chemokine receptor 5: an in situ time-lapse confocal imaging study. *J. Neurosci.* 25 (30), 7040–7047. doi: 10.1523/JNEUROSCI.5171-04.2005

Chai, R. C., Jiang, J. H., Wong, A. Y., Jiang, F., Gao, K., Vatcher, G., et al. (2013). AQP5 is differentially regulated in astrocytes during metabolic and traumatic injuries. *Glia* 61 (10), 1748–1765. doi: 10.1002/glia.22555

Chung, W. S., Allen, N. J., and Eroglu, C. (2015). Astrocytes control synapse formation, function, and elimination. *Cold Spring Harb. Perspect. Biol.* 7 (9), a020370. doi: 10.1101/cshperspect.a020370

Clarke, S. R., Shetty, A. K., Bradley, J. L., and Turner, D. A. (1994). Reactive astrocytes express the embryonic intermediate neurofilament nestin. *Neuroreport* 5 (15), 1885–1888. doi: 10.1097/00001756-199410000-00011

Contreras, J. E., Sanchez, H. A., Veliz, L. P., Bukauskas, F. F., Bennett, M. V., and Saez, J. C. (2004). Role of connexin-based gap junction channels and hemichannels in ischemia-induced cell death in nervous tissue. *Brain Res. Brain Res. Rev.* 47 (1-3), 290–303. doi: 10.1016/j.brainresrev.2004.08.002

Cornell-Bell, A. H., Finkbeiner, S. M., Cooper, M. S., and Smith, S. J. (1990). Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science* 247 (4941), 470–473. doi: 10.1126/science.1967852

Cottrell, G. T., Lin, R., Warn-Cramer, B. J., Lau, A. F., and Burt, J. M. (2003). Mechanism of v-Src- and mitogen-activated protein kinase-induced reduction of gap junction communication. *Am. J. Physiol. Cell Physiol.* 284 (2), C511–C520. doi: 10.1152/ajpcell.00214.2002

De Bock, M., Decrock, E., Wang, N., Bol, M., Vinken, M., Bultynck, G., et al. (2014). The dual face of connexin-based astroglial Ca(2+) communication: a key player in brain physiology and a prime target in pathology. *Biochim. Biophys. Acta* 1843 (10), 2211–2232. doi: 10.1016/j.bbamcr.2014.04.016

De Pascalis, C., and Etienne-Manneville, S. (2017). Single and collective cell migration: the mechanics of adhesions. *Mol. Biol. Cell* 28 (14), 1833–1846. doi: 10.1091/mbc.E17-03-0134

De Vuyst, E., Wang, N., Decrock, E., De Bock, M., Vinken, M., Van Moorhem, M., et al. (2009). Ca(2+) regulation of connexin 43 hemichannels in C6 glioma and glial cells. *Cell Calcium* 46 (3), 176–187. doi: 10.1016/j.ceca.2009.07.002

Decrock, E., De Bock, M., Wang, N., Bultynck, G., Giaume, C., Naus, C. C., et al. (2015). Connexin and pannexin signaling pathways, an architectural blueprint for CNS physiology and pathology? *Cell Mol. Life Sci.* 72 (15), 2823–2851. doi: 10.1007/s00018-015-1962-7

Dermietzel, R. (1974). Junctions in the central nervous system of the cat. 3. Gap junctions and membrane-associated orthogonal particle complexes (MOPC) in astrocytic membranes. *Cell Tissue Res.* 149 (1), 121–135. doi: 10.1007/bf00209055

Dermietzel, R., Hertberg, E. L., Kessler, J. A., and Spray, D. C. (1991). Gap junctions between cultured astrocytes: immunocytochemical, molecular, and electrophysiological analysis. *J. Neurosci.* 11 (5), 1421–1432. doi: 10.1523/JNEUROSCI.11-05-01421.1991

Dermietzel, R., Gao, Y., Scemes, E., Vieira, D., Urban, M., Kremer, M., et al. (2000). Connexin43 null mice reveal that astrocytes express multiple connexins. *Brain Res. Brain Res. Rev.* 32 (1), 45–56. doi: 10.1016/s0165-0173(99)00067-3

Dienel, G. A., and Hertz, L. (2001). Glucose and lactate metabolism during brain activation. *J. Neurosci. Res.* 66 (5), 824–838. doi: 10.1002/jnr.10079

Dubash, A. D., Menold, M. M., Samson, T., Boulter, E., Garcia-Mata, R., Doughman, R., et al. (2009). Chapter 1. Focal adhesions: new angles on an old structure. *Int. Rev. Cell Mol. Biol.* 277, 1–65. doi: 10.1016/S1937-6448(09)77001-7

Ek-Vitorin, J. F., King, T. J., Heyman, N. S., Lampe, P. D., and Burt, J. M. (2006). Selectivity of connexin 43 channels is regulated through protein kinase C-dependent phosphorylation. *Circ. Res.* 98 (12), 1498–1505. doi: 10.1161/01.RES.0000227572.45891.2c

Faria, R. X., Reis, R. A., Casabulho, C. M., Alberto, A. V., de Farias, F. P., Henriques-Pons, A., et al. (2009). Pharmacological properties of a pore induced by raising intracellular Ca2+. *Am. J. Physiol. Cell Physiol.* 297 (1), C28–C42. doi: 10.1152/ajpcell.00476.2008

Faulkner, J. R., Herrmann, J. E., Woo, M. J., Tansey, K. E., Doan, N. B., and Sofroniew, M. V. (2004). Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J. Neurosci.* 24 (9), 2143–2155. doi: 10.1523/JNEUROSCI.3547-03.2004

Ferrati, S., Gadok, A. K., Brunaugh, A. D., Zhao, C., Heersema, L. A., Smyth, H. D. C., et al. (2017). Connexin membrane materials as potent inhibitors of breast cancer cell migration. *J. R. Soc. Interface* 14 (133), 1–6. doi: 10.1098/rsif.2017.0313

Filous, A. R., and Silver, J. (2016). Targeting astrocytes in CNS injury and disease: A translational research approach. *Prog. Neurobiol.* 144, 173–187. doi: 10.1016/j.pneurobio.2016.03.009

Forsyth, A. M., Wan, J., Owrutsky, P. D., Abkarian, M., and Stone, H. A. (2011). Multiscale approach to link red blood cell dynamics, shear viscosity, and ATP release. *Proc. Natl. Acad. Sci. U.S.A.* 108 (27), 10986–10991. doi: 10.1073/pnas.1101315108

Gadea, A., Schinelli, S., and Gallo, V. (2008). Endothelin-1 regulates astrocyte proliferation and reactive gliosis via a JNK/c-Jun signaling pathway. *J. Neurosci.* 28 (10), 2394–2408. doi: 10.1523/JNEUROSCI.5652-07.2008

Gao, K., Wang, C. R., Jiang, F., Wong, A. Y., Su, N., Jiang, J. H., et al. (2013). Traumatic scratch injury in astrocytes triggers calcium influx to activate the JNK/c-Jun/AP-1 pathway and switch on GFAP expression. *Glia* 61 (12), 2063–2077. doi: 10.1002/glia.22577

Garré, J. M., Yang, G., Bukauskas, F. F., and Bennett, M. V. (2016). FGF-1 Triggers pannexin-1 hemichannel opening in spinal astrocytes of rodents and promotes inflammatory responses in acute spinal cord slices. *J. Neurosci.* 36 (17), 4785–4801. doi: 10.1523/JNEUROSCI.4195-15.2016

Giaume, C., and McCarthy, K. D. (1996). Control of gap-junctional communication in astrocytic networks. *Trends Neurosci.* 19 (8), 319–325. doi: 10.1016/0166-2236(96)10046-1

Giaume, C., Fromaget, C., el Aoumari, A., Cordier, J., Glowinski, J., and Gros, D. (1991). Gap junctions in cultured astrocytes: single-channel currents and characterization of channel-forming protein. *Neuron* 6 (1), 133–143. doi: 10.1016/0896-6273(91)90128-m

Giaume, C., Koulakoff, A., Roux, L., Holcman, D., and Rouach, N. (2010). Astroglial networks: a step further in neuroglial and gliovascular interactions. *Nat. Rev. Neurosci.* 11 (2), 87–99. doi: 10.1038/nrn2757

Giaume, C., Leybaert, L., Naus, C. C., and Saez, J. C. (2013). Connexin and pannexin hemichannels in brain glial cells: properties, pharmacology, and roles. *Front. Pharmacol.* 4, 88. doi: 10.3389/fphar.2013.00088

Giaume, C. N. (2013). C, Connexins, gap junctions, and glia. *WIREs Membr. Transp. Signal* 2, 10. doi: 10.1002/wmts.87

Gilleron, J., Carette, D., Fiorini, C., Benkdane, M., Segretain, D., and Pointis, G. (2009). Connexin 43 gap junction plaque endocytosis implies molecular remodelling of ZO-1 and c-Src partners. *Commun. Integr. Biol.* 2 (2), 104–106. doi: 10.4161/cib.7626

Giulian, D., Woodward, J., Young, D. G., Krebs, J. F., and Lachman, L. B. (1988). Interleukin-1 injected into mammalian brain stimulates astrogliosis and neovascularization. *J. Neurosci.* 8 (7), 2485–2490. doi: 10.1523/JNEUROSCI.08-07-02485.1988

Goldman, J. E., Zerlin, M., Newman, S., Zhang, L., and Gensert, J. (1997). Fate determination and migration of progenitors in the postnatal mammalian CNS. *Dev. Neurosci.* 19 (1), 42–48. doi: 10.1159/000111184

Gonzalez-Sanchez, A., Jaraiz-Rodriguez, M., Dominguez-Prieto, M., Herrero-Gonzalez, S., Medina, J. M., and Tabernero, A. (2016). Connexin43 recruits PTEN and Csk to inhibit c-Src activity in glioma cells and astrocytes. *Oncotarget* 7 (31), 49819–49833. doi: 10.18632/oncotarget.10454

Graeber, S. H., and Hulser, D. F. (1998). Connexin transfection induces invasive properties in HeLa cells. *Exp. Cell Res.* 243 (1), 142–149. doi: 10.1006/excr.1998.4130

Grygorowicz, T., Welniak-Kaminska, M., and Struzynska, L. (2016). Early P2X7R-related astrogliosis in autoimmune encephalomyelitis. *Mol. Cell Neurosci.* 74, 1–9. doi: 10.1016/j.mcn.2016.02.003

Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., Alexander, D. D., et al. (1994). Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 264 (5166), 1772–1775. doi: 10.1126/science.8209258

Halassa, M. M., Fellin, T., Takano, H., Dong, J. H., and Haydon, P. G. (2007). Synaptic islands defined by the territory of a single astrocyte. *J. Neurosci.* 27 (24), 6473–6477. doi: 10.1523/JNEUROSCI.1419-07.2007

Hara, M., Kobayakawa, K., Ohkawa, Y., Kumamaru, H., Yokota, K., Saito, T., et al. (2017). Interaction of reactive astrocytes with type I collagen induces astrocytic scar formation through the integrin-N-cadherin pathway after spinal cord injury. *Nat. Med.* 23 (7), 818–828. doi: 10.1038/nm.4354

Hayashi, K., Yamamoto, T. S., and Ueno, N. (2018). Intracellular calcium signal at the leading edge regulates mesodermal sheet migration during Xenopus gastrulation. *Sci. Rep.* 8 (1), 2433. doi: 10.1038/s41598-018-20747-w

Henriquez, M., Herrera-Molina, R., Valdivia, A., Alvarez, A., Kong, M., Munoz, N., et al. (2011). ATP release due to Thy-1-integrin binding induces P2X7-mediated calcium entry required for focal adhesion formation. *J. Cell Sci.* 124 (Pt 9), 1581–1588. doi: 10.1242/jcs.073171

Hertz, L., Peng, L., and Dienel, G. A. (2007). Energy metabolism in astrocytes: high rate of oxidative metabolism and spatiotemporal dependence on glycolysis/glycogenolysis. *J. Cereb. Blood Flow Metab.* 27 (2), 219–249. doi: 10.1038/sj.jcbfm.9600343

Hofer, T., Venance, L., and Giaume, C. (2002). Control and plasticity of intercellular calcium waves in astrocytes: a modeling approach. *J. Neurosci.* 22 (12), 4850–4859. doi: 10.1523/JNEUROSCI.22-12-04850.2002

Homkajorn, B., Sims, N. R., and Muyderman, H. (2010). Connexin 43 regulates astrocytic migration and proliferation in response to injury. *Neurosci. Lett.* 486 (3), 197–201. doi: 10.1016/j.neulet.2010.09.051

Isakson, B. (2017). A review of the current state of pannexin channels as they relate to the blood vessel wall. *J. Med. Sci.* 37 (5), 7. doi: 10.4103/jmedsci.#jmedsci_43_17

Jamieson, S., Going, J. J., D'Arcy, R., and George, W. D. (1998). Expression of gap junction proteins connexin 26 and connexin 43 in normal human breast and in breast tumours. *J. Pathol.* 184 (1), 37–43. doi: 10.1002/(SICI)1096-9896(199801)184:1<37::AID-PATH966>3.0.CO;2-D

Jaraiz-Rodriguez, M., Tabernero, M. D., Gonzalez-Tablas, M., Otero, A., Orfao, A., Medina, J. M., et al. (2017). A short region of connexin43 reduces human glioma stem cell migration, invasion, and survival through Src, PTEN, and FAK. *Stem Cell Rep.* 9 (2), 451–463. doi: 10.1016/j.stemcr.2017.06.007

Jin, J., Wang, T., Wang, Y., Chen, S., Li, Z., Li, X., et al. (2017). SRC3 expressed in BMSCs promotes growth and migration of multiple myeloma cells by regulating the expression of Cx43. *Int. J. Oncol.* 51 (6), 1694–1704. doi: 10.3892/ijo.2017.4171

John, G. R., Lee, S. C., and Brosnan, C. F. (2003). Cytokines: powerful regulators of glial cell activation. *Neuroscientist* 9 (1), 10–22. doi: 10.1177/1073858402239587

Jones, L. L., Margolis, R. U., and Tuszynski, M. H. (2003). The chondroitin sulfate proteoglycans neurocan, brevican, phosphacan, and versican are differentially regulated following spinal cord injury. *Exp. Neurol.* 182 (2), 399–411. doi: 10.1016/s0014-4886(03)00087-6

Kameritsch, P., Pogoda, K., and Pohl, U. (2012). Channel-independent influence of connexin 43 on cell migration. *Biochim. Biophys. Acta* 1818 (8), 1993–2001. doi: 10.1016/j.bbamem.2011.11.016

Kanczuga-Koda, L., Sulkowski, S., Lenczewski, A., Koda, M., Wincewicz, A., and Baltaziak, M. (2006). Increased expression of connexins 26 and 43 in lymph node metastases of breast cancer. *J. Clin. Pathol.* 59 (4), 429–433. doi: 10.1136/jcp.2005.029272

Kanemaru, K., Kubota, J., Sekiya, H., Hirose, K., Okubo, Y., and Iino, M. (2013). Calcium-dependent N-cadherin up-regulation mediates reactive astrogliosis and neuroprotection after brain injury. *Proc. Natl. Acad. Sci. U.S.A.* 110 (28), 11612–11617. doi: 10.1073/pnas.1300378110

Kang, J., Kang, N., Lovatt, D., Torres, A., Zhao, Z., Lin, J., et al. (2008). Connexin 43 hemichannels are permeable to ATP. *J. Neurosci.* 28 (18), 4702–4711. doi: 10.1523/JNEUROSCI.5048-07.2008

Kim, D. Y., Kam, Y., Koo, S. K., and Joe, C. O. (1999). Gating connexin 43 channels reconstituted in lipid vesicles by mitogen-activated protein kinase phosphorylation. *J. Biol. Chem.* 274 (9), 5581–5587. doi: 10.1074/jbc.274.9.5581

Kim, R., Healey, K. L., Sepulveda-Orengo, M. T. , and Reissner, K. J. (2018). Astroglial correlates of neuropsychiatric disease: From astrocytopathy to astrogliosis. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 87 (Pt A), 126–146. doi: 10.1016/j.pnpbp.2017.10.002

Kong, M., Munoz, N., Valdivia, A., Alvarez, A., Herrera-Molina, R., Cardenas, A., et al. (2013). Thy-1-mediated cell-cell contact induces astrocyte migration through the engagement of alphaVbeta3 integrin and syndecan-4. *Biochim. Biophys. Acta* 1833 (6), 1409–1420. doi: 10.1016/j.bbamcr.2013.02.013

Kotini, M., and Mayor, R. (2015). Connexins in migration during development and cancer. *Dev. Biol.* 401 (1), 143–151. doi: 10.1016/j.ydbio.2014.12.023

Kumar, N. M., and Gilula, N. B. (1996). The gap junction communication channel. *Cell* 84 (3), 381–388. doi: 10.1016/s0092-8674(00)81282-9

Ladoux, B., and Mege, R. M. (2017). Mechanobiology of collective cell behaviours. *Nat. Rev. Mol. Cell Biol.* 18 (12), 743–757. doi: 10.1038/nrm.2017.98

Lagos-Cabre, R., Alvarez, A., Kong, M., Burgos-Bravo, F., Cardenas, A., Rojas-Mancilla, E., et al. (2017). alphaVbeta3 Integrin regulates astrocyte reactivity. *J. Neuroinflammation* 14 (1), 194. doi: 10.1186/s12974-017-0968-5

Lagos-Cabre, R., Brenet, M., Diaz, J., Perez, R. D., Perez, L. A., Herrera-Molina, R., et al. (2018). Intracellular Ca(2+) increases and connexin 43 hemichannel opening are necessary but not sufficient for Thy-1-induced astrocyte migration. *Int. J. Mol. Sci.* 19 (8), 1–16. doi: 10.3390/ijms19082179

Laguesse, S., Close, P., Van Hees, L., Chariot, A., Malgrange, B., and Nguyen, L. (2017). Loss of Elp3 impairs the acetylation and distribution of connexin-43 in the developing cerebral cortex. *Front. Cell Neurosci.* 11, 122. doi: 10.3389/fncel.2017.00122

Laird, D. W. (2006). Life cycle of connexins in health and disease. *Biochem. J.* 394 (Pt 3), 527–543. doi: 10.1042/BJ20051922

Lampe, P. D., and Lau, A. F. (2004). The effects of connexin phosphorylation on gap junctional communication. *Int. J. Biochem. Cell Biol.* 36 (7), 1171–1186. doi: 10.1016/S1357-2725(03)00264-4

Lapato, A. S., and Tiwari-Woodruff, S. K. (2018). Connexins and pannexins: at the junction of neuro-glial homeostasis & disease. *J. Neurosci. Res.* 96 (1), 31–44. doi: 10.1002/jnr.24088

Levine, J. B., Kong, J., Nadler, M., and Xu, Z. (1999). Astrocytes interact intimately with degenerating motor neurons in mouse amyotrophic lateral sclerosis (ALS). *Glia* 28 (3), 215–224. doi: 10.1002/(SICI)1098-1136(199912)28:3<215::AID-GLIA5>3.0.CO;2-C

Leyton, L., Schneider, P., Labra, C. V., Ruegg, C., Hetz, C. A., Quest, A. F., et al. (2001). Thy-1 binds to integrin beta(3) on astrocytes and triggers formation of focal contact sites. *Curr. Biol.* 11 (13), 1028–1038. doi: 10.1016/s0960-9822(01)00262-7

Li, W., Hertzberg, E. L., and Spray, D. C. (2005). Regulation of connexin43-protein binding in astrocytes in response to chemical ischemia/hypoxia. *J. Biol. Chem.* 280 (9), 7941–7948. doi: 10.1074/jbc.M410548200

Li, Z., Zhou, Z., Welch, D. R., and Donahue, H. J. (2008). Expressing connexin 43 in breast cancer cells reduces their metastasis to lungs. *Clin. Exp. Metastasis* 25 (8), 893–901. doi: 10.1007/s10585-008-9208-9

Locovei, S., Bao, L., and Dahl, G. (2006). Pannexin 1 in erythrocytes: function without a gap. *Proc. Natl. Acad. Sci. U.S.A.* 103 (20), 7655–7659. doi: 10.1073/pnas.0601037103

Mansour, H., McColm, J. R., Cole, L., Weible, M., Korlimbinis, A., and Chan-Ling, T. (2013). Connexin 30 expression and frequency of connexin heterogeneity in astrocyte gap junction plaques increase with age in the rat retina. *PloS One* 8 (3), e57038. doi: 10.1371/journal.pone.0057038

Marquez-Rosado, L., Solan, J. L., Dunn, C. A., Norris, R. P., and Lampe, P. D. (2012). Connexin43 phosphorylation in brain, cardiac, endothelial and epithelial tissues. *Biochim. Biophys. Acta* 1818 (8), 1985–1992. doi: 10.1016/j.bbamem.2011.07.028

Matsuuchi, L., and Naus, C. C. (2013). Gap junction proteins on the move: connexins, the cytoskeleton and migration. *Biochim. Biophys. Acta* 1828 (1), 94–108. doi: 10.1016/j.bbamem.2012.05.014

Mayor, R., and Etienne-Manneville, S. (2016). The front and rear of collective cell migration. *Nat. Rev. Mol. Cell Biol.* 17 (2), 97–109. doi: 10.1038/nrm.2015.14

Mayorquin, L. C., Rodriguez, A. V., Sutachan, J. J., and Albarracin, S. L. (2018). Connexin-Mediated Functional and Metabolic Coupling Between Astrocytes and Neurons. *Front. Mol. Neurosci.* 11, 118. doi: 10.3389/fnmol.2018.00118

Miyake, T., Hattori, T., Fukuda, M., Kitamura, T., and Fujita, S. (1988). Quantitative studies on proliferative changes of reactive astrocytes in mouse cerebral cortex. *Brain Res.* 451 (1-2), 133–138. doi: 10.1016/0006-8993(88)90757-3

Moore, D. L., and Jessberger, S. (2013). All astrocytes are not created equal–the role of astroglia in brain injury. *EMBO Rep.* 14 (6), 487–488. doi: 10.1038/embor.2013.54

Moore, K. B., and O'Brien, J. (2015). Connexins in neurons and glia: targets for intervention in disease and injury. *Neural Regener. Res.* 10 (7), 1013–1017. doi: 10.4103/1673-5374.160092

Muller, M. S., Fouyssac, M., and Taylor, C. W. (2018). Effective glucose uptake by human astrocytes requires its sequestration in the endoplasmic reticulum by glucose-6-phosphatase-beta. *Curr. Biol.* 28 (21), 3481–3486 e4. doi: 10.1016/j.cub.2018.08.060

Nagy, J. I., and Rash, J. E. (2000). Connexins and gap junctions of astrocytes and oligodendrocytes in the CNS. *Brain Res. Brain Res. Rev.* 32 (1), 29–44. doi: 10.1016/s0165-0173(99)00066-1

Nielsen, B. S., Hansen, D. B., Ransom, B. R., Nielsen, M. S., and MacAulay, N. (2017). Connexin hemichannels in astrocytes: an assessment of controversies regarding their functional characteristics. *Neurochem. Res.* 42 (9), 2537–2550. doi: 10.1007/s11064-017-2243-7

Nielsen, B. S., Alstrom, J. S., Nicholson, B. J., Nielsen, M. S., and MacAulay, N. (2017). Permeant-specific gating of connexin 30 hemichannels. *J. Biol. Chem.* 292 (49), 19999–20009. doi: 10.1074/jbc.M117.805986

O'Carroll, S. J., Gorrie, C. A., Velamoor, S., Green, C. R., and Nicholson, L. F. (2013). Connexin43 mimetic peptide is neuroprotective and improves function following spinal cord injury. *Neurosci. Res.* 75 (3), 256–267. doi: 10.1016/j.neures.2013.01.004

Orellana, J. A., Montero, T. D., and von Bernhardi, R. (2013). Astrocytes inhibit nitric oxide-dependent Ca(2+) dynamics in activated microglia: involvement of ATP released via pannexin 1 channels. *Glia* 61 (12), 2023–2037. doi: 10.1002/glia.22573

Oyamada, M., Takebe, K., and Oyamada, Y. (2013). Regulation of connexin expression by transcription factors and epigenetic mechanisms. *Biochim. Biophys. Acta* 1828 (1), 118–133. doi: 10.1016/j.bbamem.2011.12.031

Ozog, M. A., Bernier, S. M., Bates, D. C., Chatterjee, B., Lo, C. W., and Naus, C. C. (2004). The complex of ciliary neurotrophic factor-ciliary neurotrophic factor receptor alpha up-regulates connexin43 and intercellular coupling in astrocytes via the Janus tyrosine kinase/signal transducer and activator of transcription pathway. *Mol. Biol. Cell* 15 (11), 4761–4774. doi: 10.1091/mbc.E04-03-0271

Panchin, Y., Kelmanson, I., Matz, M., Lukyanov, K., Usman, N., and Lukyanov, S. (2000). A ubiquitous family of putative gap junction molecules. *Curr. Biol.* 10 (13), R473–R474. doi: 10.1016/s0960-9822(00)00576-5

Park, D. J., Wallick, C. J., Martyn, K. D., Lau, A. F., Jin, C., and Warn-Cramer, B. J. (2007). Akt phosphorylates Connexin43 on Ser373, a "mode-1" binding site for 14-3-3. *Cell Commun. Adhes.* 14 (5), 211–226. doi: 10.1080/15419060701755958

Pekny, M., and Pekna, M. (2014). Astrocyte reactivity and reactive astrogliosis: costs and benefits. *Physiol. Rev.* 94 (4), 1077–1098. doi: 10.1152/physrev.00041.2013

Pekny, M., Pekna, M., Messing, A., Steinhauser, C., Lee, J. M., Parpura, V., et al. (2016). Astrocytes: a central element in neurological diseases. *Acta Neuropathol.* 131 (3), 323–345. doi: 10.1007/s00401-015-1513-1

Penuela, S., Gehi, R., and Laird, D. W. (2013). The biochemistry and function of pannexin channels. *Biochim. Biophys. Acta* 1828 (1), 15–22. doi: 10.1016/j.bbamem.2012.01.017

Perez Velazquez, J. L., Frantseva, M., Naus, C. C., Bechberger, J. F., Juneja, S. C., Velumian, A., et al. (1996). Development of astrocytes and neurons in cultured brain slices from mice lacking connexin43. *Brain Res. Dev. Brain Res.* 97 (2), 293–296. doi: 10.1016/s0165-3806(96)00156-3

Pogoda, K., Kameritsch, P., Retamal, M. A., and Vega, J. L. (2016). Regulation of gap junction channels and hemichannels by phosphorylation and redox changes: a revision. *BMC Cell Biol.* 17 (Suppl 1), 11. doi: 10.1186/s12860-016-0099-3

Polusani, S. R., Kalmykov, E. A., Chandrasekhar, A., Zucker, S. N., and Nicholson, B. J. (2016). Cell coupling mediated by connexin 26 selectively contributes to reduced adhesivity and increased migration. *J. Cell Sci.* 129 (23), 4399–4410. doi: 10.1242/jcs.185017

Press, E., Alaga, K. C., Barr, K., Shao, Q., Bosen, F., Willecke, K., et al. (2017). Disease-linked connexin26 S17F promotes volar skin abnormalities and mild wound healing defects in mice. *Cell Death Dis.* 8 (6), e2845. doi: 10.1038/cddis.2017.234

Qi, G. J., Chen, Q., Chen, L. J., Shu, Y., Bu, L. L., Shao, X. Y., et al. (2016). Phosphorylation of connexin 43 by Cdk5 modulates neuronal migration during embryonic brain development. *Mol. Neurobiol.* 53 (5), 2969–2982. doi: 10.1007/s12035-015-9190-6

Qiu, F., Wang, J., Spray, D. C., Scemes, E., and Dahl, G. (2011). Two non-vesicular ATP release pathways in the mouse erythrocyte membrane. *FEBS Lett.* 585 (21), 3430–3435. doi: 10.1016/j.febslet.2011.09.033

Rackauskas, M., Verselis, V. K., and Bukauskas, F. F. (2007). Permeability of homotypic and heterotypic gap junction channels formed of cardiac connexins mCx30.2, Cx40, Cx43, and Cx45. *Am. J. Physiol. Heart Circ. Physiol.* 293 (3), H1729–H1736. doi: 10.1152/ajpheart.00234.2007

Rash, J. E., Yasumura, T., Davidson, K. G., Furman, C. S., Dudek, F. E., and Nagy, J. I. (2001). Identification of cells expressing Cx43, Cx30, Cx26, Cx32 and Cx36

in gap junctions of rat brain and spinal cord. *Cell Commun. Adhes.* 8 (4-6), 315–320. doi: 10.3109/15419060109080745

Retamal, M. A., Froger, N., Palacios-Prado, N., Ezan, P., Saez, P. J., Saez, J. C., et al. (2007). Cx43 hemichannels and gap junction channels in astrocytes are regulated oppositely by proinflammatory cytokines released from activated microglia. *J. Neurosci.* 27 (50), 13781–13792. doi: 10.1523/JNEUROSCI.2042-07.2007

Rojas, F., Cortes, N., Abarzua, S., Dyrda, A., and van Zundert, B. (2014). Astrocytes expressing mutant SOD1 and TDP43 trigger motoneuron death that is mediated via sodium channels and nitroxidative stress. *Front. Cell Neurosci.* 8, 24. doi: 10.3389/fncel.2014.00024

Rouach, N., Koulakoff, A., Abudara, V., Willecke, K., and Giaume, C. (2008). Astroglial metabolic networks sustain hippocampal synaptic transmission. *Science* 322 (5907), 1551–1555. doi: 10.1126/science.1164022

Scemes, E., and Spray, D. C. (2012). Extracellular K(+) and astrocyte signaling via connexin and pannexin channels. *Neurochem. Res.* 37 (11), 2310–2316. doi: 10.1007/s11064-012-0759-4

Segretain, D., and Falk, M. M. (2004). Regulation of connexin biosynthesis, assembly, gap junction formation, and removal. *Biochim. Biophys. Acta* 1662 (1-2), 3–21. doi: 10.1016/j.bbamem.2004.01.007

Simpson, K. J., Selfors, L. M., Bui, J., Reynolds, A., Leake, D., Khvorova, A., et al. (2008). Identification of genes that regulate epithelial cell migration using an siRNA screening approach. *Nat. Cell Biol.* 10 (9), 1027–1038. doi: 10.1038/ncb1762

Sirko, S., Behrendt, G., Johansson, P. A., Tripathi, P., Costa, M., Bek, S., et al. (2013). Reactive glia in the injured brain acquire stem cell properties in response to sonic hedgehog. [corrected]. *Cell Stem Cell* 12 (4), 426–439. doi: 10.1016/j.stem.2013.01.019

Sofroniew, M. V., and Vinters, H. V. (2010). Astrocytes: biology and pathology. *Acta Neuropathol.* 119 (1), 7–35. doi: 10.1007/s00401-009-0619-8

Sofroniew, M. V. (2005). Reactive astrocytes in neural repair and protection. *Neuroscientist* 11 (5), 400–407. doi: 10.1177/1073858405278321

Sofroniew, M. V. (2009). Molecular dissection of reactive astrogliosis and glial scar formation. *Trends Neurosci.* 32 (12), 638–647.doi: 10.1016/j.tins.2009.08.002

Sofroniew, M. V. (2014). Astrogliosis. *Cold Spring Harb. Perspect. Biol.* 7 (2), a020420. doi: 10.1101/cshperspect.a020420

Solan, J. L., and Lampe, P. D. (2014). Specific Cx43 phosphorylation events regulate gap junction turnover in vivo. *FEBS Lett.* 588 (8), 1423–1429. doi: 10.1016/j.febslet.2014.01.049

Sorgen, P. L., Trease, A. J., Spagnol, G., Delmar, M., and Nielsen, M. S. (2018). Protein(-)Protein Interactions with Connexin 43: Regulation and Function. *Int. J. Mol. Sci.* 19 (5), 1–21. doi: 10.3390/ijms19051428

Sosunov, A. A., Wu, X., Tsankova, N. M., Guilfoyle, E., McKhann, G. M., and Goldman, J. E. (2014). Phenotypic heterogeneity and plasticity of isocortical and hippocampal astrocytes in the human brain. *J. Neurosci.* 34 (6), 2285–2298. doi: 10.1523/JNEUROSCI.4037-13.2014

Stout, C. E., Costantin, J. L., Naus, C. C., and Charles, A. C. (2002). Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. *J. Biol. Chem.* 277 (12), 10482–10488. doi: 10.1074/jbc.M109902200

Striedinger, K., and Scemes, E. (2008). Interleukin-1beta affects calcium signaling and in vitro cell migration of astrocyte progenitors. *J. Neuroimmunol.* 196 (1-2), 116–123. doi: 10.1016/j.jneuroim.2008.03.014

Striedinger, K., Meda, and Scemes, E. (2007). Exocytosis of ATP from astrocyte progenitors modulates spontaneous Ca2+ oscillations and cell migration. *Glia* 55 (6), 652–662. doi: 10.1002/glia.20494

Suadicani, S. O., Flores, C. E., Urban-Maldonado, M., Beelitz, M., and Scemes, E. (2004). Gap junction channels coordinate the propagation of intercellular Ca2+ signals generated by P2Y receptor activation. *Glia* 48 (3), 217–229. doi: 10.1002/glia.20071

Suh, H. N., Kim, M. O., and Han, H. J. (2012). Laminin-111 stimulates proliferation of mouse embryonic stem cells through a reduction of gap junctional intercellular communication via RhoA-mediated Cx43 phosphorylation and dissociation of Cx43/ZO-1/drebrin complex. *Stem Cells Dev.* 21 (11), 2058–2070. doi: 10.1089/scd.2011.0505

Thevenin, A. F., Margraf, R. A., Fisher, C. G., Kells-Andrews, R. M., and Falk, M. M. (2017). Phosphorylation regulates connexin43/ZO-1 binding and release, an important step in gap junction turnover. *Mol. Biol. Cell* 28 (25), 3595–3608. doi: 10.1091/mbc.E16-07-0496

Tsacopoulos, M., and Magistretti, P. J. (1996). Metabolic coupling between glia and neurons. *J. Neurosci.* 16 (3), 877–885. doi: 10.1046/j.1460-9568.2000.00973.x

Turmel, P., Dufresne, J., Hermo, L., Smith, C. E., Penuela, S., Laird, D. W., et al. (2011). Characterization of pannexin1 and pannexin3 and their regulation by androgens in the male reproductive tract of the adult rat. *Mol. Reprod. Dev.* 78 (2), 124–138. doi: 10.1002/mrd.21280

Vanden Abeele, F., Bidaux, G., Gordienko, D., Beck, B., Panchin, Y. V., Baranova, A. V., et al. (2006). Functional implications of calcium permeability of the channel formed by pannexin 1. *J. Cell Biol.* 174 (4), 535–546. doi: 10.1083/jcb.200601115

Verslegers, M., Lemmens, K., Van Hove, I., and Moons, L. (2013). Matrix metalloproteinase-2 and -9 as promising benefactors in development, plasticity and repair of the nervous system. *Prog. Neurobiol.* 105, 60–78. doi: 10.1016/j.pneurobio.2013.03.004

Vicario, N., Zappala, A., Calabrese, G., Gulino, R., Parenti, C., Gulisano, M., et al. (2017). Connexins in the central nervous system: physiological traits and neuroprotective targets. *Front. Physiol.* 8, 1060. doi: 10.3389/fphys.2017.01060

von Bartheld, C. S., Bahney, J., and Herculano-Houzel, S. (2016). The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting. *J. Comp. Neurol.* 524 (18), 3865–3895. doi: 10.1002/cne.24040

Wang, M., Kong, Q., Gonzalez, F. A., Sun, G., Erb, L., Seye, C., et al. (2005). P2Y nucleotide receptor interaction with alpha integrin mediates astrocyte migration. *J. Neurochem.* 95 (3), 630–640. doi: 10.1111/j.1471-4159.2005.03408.x

Wiencken-Barger, A. E., Djukic, B., Casper, K. B., and McCarthy, K. D. (2007). A role for Connexin43 during neurodevelopment. *Glia* 55 (7), 675–686. doi: 10.1002/glia.20484

Wu, X. L., Tang, Y. C., Lu, Q. Y., Xiao, X. L., Song, T. B., and Tang, F. R. (2015). Astrocytic Cx 43 and Cx 40 in the mouse hippocampus during and after pilocarpine-induced status epilepticus. *Exp. Brain Res.* 233 (5), 1529–1539. doi: 10.1007/s00221-015-4226-8

Wu, D., Li, B., Liu, H., Yuan, M., Yu, M., Tao, L., et al. (2018). In vitro inhibited effect of gap junction composed of Cx43 in the invasion and metastasis of testicular cancer resistanced to cisplatin. *BioMed. Pharmacother.* 98, 826–833. doi: 10.1016/j.biopha.2018.01.016

Xing, L., Yang, T., Cui, S., and Chen, G. (2019). Connexin Hemichannels in Astrocytes: Role in CNS Disorders. *Front. Mol. Neurosci.* 12, 23. doi: 10.3389/fnmol.2019.00023

Xu, X., Francis, R., Wei, C. J., Linask, K. L., and Lo, C. W. (2006). Connexin 43-mediated modulation of polarized cell movement and the directional migration of cardiac neural crest cells. *Development* 133 (18), 3629–3639. doi: 10.1242/dev.02543

Yi, C., Koulakoff, A., and Giaume, C. (2017). Astroglial connexins as a therapeutic target for Alzheimer's disease. *Curr. Pharm. Des.* 23 (33), 4958–4968. doi: 10.2174/1381612823666171004151215

Zhan, J. S., Gao, K., Chai, R. C., Jia, X. H., Luo, D. P., Ge, G., et al. (2017). Astrocytes in Migration. *Neurochem. Res.* 42 (1), 272–282. doi: 10.1007/s11064-016-2089-4

Zhang, A., Hitomi, M., Bar-Shain, N., Dalimov, Z., Ellis, L., Velpula, K. K., et al. (2015). Connexin 43 expression is associated with increased malignancy in prostate cancer cell lines and functions to promote migration. *Oncotarget* 6 (13), 11640–11651. doi: 10.18632/oncotarget.3449

Zhao, Z., Nelson, A. R., Betsholtz, C., and Zlokovic, B. V. (2015). Establishment and Dysfunction of the Blood-Brain Barrier. *Cell* 163 (5), 1064–1078. doi: 10.1016/j.cell.2015.10.067

Zhou, J. J., Cheng, C., Qiu, Z., Zhou, W. H., and Cheng, G. Q. (2015). Decreased connexin 43 in astrocytes inhibits the neuroinflammatory reaction in an acute mouse model of neonatal sepsis. *Neurosci. Bull.* 31 (6), 763–768. doi: 10.1007/s12264-015-1561-5

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

frontiers in Pharmacology

MINI REVIEW
published: 29 January 2020
doi: 10.3389/fphar.2019.01656

Altered Waste Disposal System in Aging and Alzheimer's Disease: Focus on Astrocytic Aquaporin-4

Marta Valenza[1,2*], *Roberta Facchinetti*[1], *Luca Steardo*[3] *and Caterina Scuderi*[1*]

[1] Department Physiology and Pharmacology "V. Erspamer", Sapienza University of Rome, Rome, Italy, [2] Epitech Group SpA, Saccolongo, Italy, [3] Università Telematica Giustino Fortunato, Benevento, Italy

Among the diverse cell types included in the general population named glia, astrocytes emerge as being the focus of a growing body of research aimed at characterizing their heterogeneous and complex functions. Alterations of both their morphology and activities have been linked to a variety of neurological diseases. One crucial physiological need satisfied by astrocytes is the cleansing of the cerebral tissue from waste molecules. Several data demonstrate that aquaporin-4 (AQP-4), a protein expressed by astrocytes, is crucially important for facilitating the removal of waste products from the brain. Aquaporins are water channels found in all district of the human organism and the most abundant isoform in the brain is AQP-4. This protein is involved in a myriad of astrocytic activities, including calcium signal transduction, potassium buffering, synaptic plasticity, astrocyte migration, glial scar formation and neuroinflammation. The highest density of AQP-4 is found at the astrocytic domains closest to blood vessels, the endfeet that envelop brain vessels, with low to zero expression in other astrocytic membrane regions. Increased AQP-4 expression and loss of polarization have recently been documented in altered physiological conditions. Here we review the latest findings related to aging and Alzheimer's disease (AD) on this topic, as well as the available knowledge on pharmacological tools to target AQP-4.

Keywords: aquaporin-4, aging, Alzheimer's disease, astrocytes, glymphatic system, brain clearance, perivascular space

OPEN ACCESS

Edited by:
Lorenzo Di Cesare Mannelli, University of Florence, Italy

Reviewed by:
Alexei Verkhratsky, University of Manchester, United Kingdom
Livio Luongo, Second University of Naples, Italy
Erika Gyengesi, Western Sydney University, Australia
Barbara Di Benedetto, University of Regensburg, Germany

***Correspondence:**
Marta Valenza
martavalenza@gmail.com
Caterina Scuderi
caterina.scuderi@uniroma1.it

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

Received: *30 October 2019*
Accepted: *17 December 2019*
Published: *29 January 2020*

Citation:
Valenza M, Facchinetti R, Steardo L and Scuderi C (2020) Altered Waste Disposal System in Aging and Alzheimer's Disease: Focus on Astrocytic Aquaporin-4. Front. Pharmacol. 10:1656. doi: 10.3389/fphar.2019.01656

INTRODUCTION

During the past 15 years, glial cells have gained noticeable attention, as their complex and heterogeneous functions were progressively getting discovered and understood. Glial cells have been recognized as essential supportive cells for neurons with a variety of specific and crucial homeostatic functions, including, but not limited to, uptake and release of chemical transmitters (Allen and Barres, 2009). For example, a growing body of literature demonstrates that synaptic function and plasticity require not just the presynaptic and postsynaptic neurons, but also the presence of glial cells, specifically astrocytes, Schwann cells, and microglia (Araque et al., 1999) with the contribution of the extracellular matrix too, forming a multi-partite structure referred as synaptic cradle (Dityatev and Rusakov, 2011; Verkhratsky and Nedergaard, 2014; Pekny et al., 2016; Verkhratsky and Nedergaard, 2018).

Among the diverse cell types included in the general population named glia, astrocytes emerge as being the focus of a growing body of research aimed at characterizing their heterogeneous and complex functions. Indeed, alterations of both their morphology and activities have been linked to a variety of neurological disorders and diseases (Scuderi et al., 2013; Scuderi et al., 2018b). Multiple and disparate changes occur in astrocytes (e.g., from hypertrophy to atrophy, from proliferation to cell death) in a highly heterogeneous and complex way, both context-dependent and disease-specific. Astroglial pathological modifications are driven by different signaling mechanisms and produce diverse responses from adaptive to maladaptive, and further they may change along the course of a disease (Sofroniew, 2014; Pekny et al., 2016; Verkhratsky et al., 2017).

One, out of many, crucial physiological need satisfied by astrocytes is the cleansing of the cerebral tissue from waste molecules. Indeed, without a waste disposal system, the brain would accumulate unwanted molecules that would interfere with its optimal functioning. Such cleansing system has been the topic of intense research and debates among scientists. In 2012 the original view of waste products disposed by diffusion was challenged by the publication of a research paper describing a water and solute clearance system regulated by astrocytes (Iliff et al., 2012). The authors indeed named it glymphatic system to underline the crucial role of glial cells. Experiments were carried out in living mice, injecting fluorescent tracers into the subarachnoid space of the brains, and then imaging their real-time movement using two-photon microscopy. Results suggested that the cerebrospinal fluid (CSF, mimicked by the tracers) moves by convective flow along the perivascular space between a vessel and the endfeet of astrocytes escheating the vasculature. The fluid penetrates the extracellular space of the parenchyma from the perivascular space as the artery branches into arterioles and capillaries. At this level, the CSF mixes with the interstitial fluid filling up of metabolic waste, moving by diffusion (Holter et al., 2017) toward the perivascular space of venules and capillaries to ultimately reach the lymphatic vessels (Louveau et al., 2015), which drain the molecules absorbed from the dural meninges to the cervical lymph nodes (Aspelund et al., 2015). This system was found dependent on aquaporin-4 (AQP-4), a bidirectional water channel highly expressed by astrocytes, since deletion of *Aqp-4* gene in mice severely reduced (nearly 70%) clearance from the brain (Iliff et al., 2012; Mestre et al., 2018). Authors then conclude that AQP-4 facilitates convective flow out of the periarterial space and into the interstitial space (Iliff et al., 2012; Nedergaard, 2013).

Thirteen aquaporins have been identified so far and, among them, the AQP-4, isolated from rat brain in 1994 (Hasegawa et al., 1994; Jung et al., 1994), is recognized as the most abundant water channel of the central nervous system (CNS). It is expressed by glial cells, specifically by astrocytes and ependymal cells, mostly in regions close to vessels throughout the CNS, including the spinal cord, and the cerebellum (Jung et al., 1994; Frigeri et al., 1995). Two isoforms have been identified in humans, that are AQP-4-M1 and AQP-4-M23 (Sorani et al., 2008a; Sorani et al., 2008b). Nielsen and collaborators were the firsts to describe that astrocytes express polarized AQP-4, such that the higher density of the channel is found at domains closest to blood vessels and the pia mater, with low to zero expression in other astrocytic membrane regions, except for some synapses (Nielsen et al., 1997).

The presence of the glymphatic disposal system in the human brain has not been fully demonstrated yet, although some evidence concurs to confirm it (Eide and Ringstad, 2015; Taoka et al., 2017; Rasmussen et al., 2018). Despite these, not all scientists believe that such glymphatic waste system actually exists, at least as presented by Iliff et al. (2012) because of some inconsistent findings suggesting that solute transport does not depend on the astrocytic AQP-4 (Smith et al., 2017; Iliff and Simon, 2019; Smith and Verkman, 2019). Debates are ongoing about the type of flow supporting the clearance system, as it is pressure-driven convective flow (generated by pulsation of arteries and collapse and inflation of veins) (Iliff et al., 2013; Ray et al., 2019), or diffusive down to gradient (Asgari et al., 2016; Smith et al., 2017; Smith and Verkman, 2018). Despite this, evidence demonstrates that AQP-4 deletion impairs blood-brain interface permeability to water (Papadopoulos and Verkman, 2005).

Despite the ongoing scientific debates, some new findings have been collected during the past 5 years valuing the notion that specific AQP-4 localization in astrocytes and its expression might be crucial aspects in physiological and pathological conditions (**Figure 1**). Here we review the latest findings related to aging and AD on this topic, as well as the available knowledge on pharmacological tools to target AQP-4. However, AQP-4 is involved in a myriad of astrocytic activities, including calcium signal transduction (Thrane et al., 2011), potassium buffering (Jin et al., 2013), synaptic plasticity (Fan et al., 2005; Ding et al., 2007; Zeng et al., 2007), astrocyte migration (Saadoun et al., 2005; Auguste et al., 2007), glial scar formation (Saadoun et al., 2005; Wu et al., 2014), and neuroinflammation (Li et al., 2011) (for extensive review refer to Xiao and Hu, 2014; Hubbard et al., 2018; Mader and Brimberg, 2019).

AQP-4 in Aging and Alzheimer's Disease

Aging is the greatest risk factor for developing dementia and Alzheimer's disease (AD). Aging is a process that involves the whole organism, including the clearance system of the brain. It is often associated with shorter duration of sleep time (Wolkove et al., 2007), which is the period of activity of the aforementioned cerebral waste disposal system (Xie et al., 2013). *Aqp-4* gene expression has been found increased in cerebral and cerebellar cortices of aged (17-month-old) mice compared to their adult counterpart (Gupta and Kanungo, 2013). Similarly, age-dependent raise in AQP-4 expression has been reported in the hippocampal CA1 region of 12-month-old compared to 6-month-old 3×Tg-AD mice, a triple transgenic model of AD, irrespective of genotype (Bronzuoli et al., 2019). In accordance,

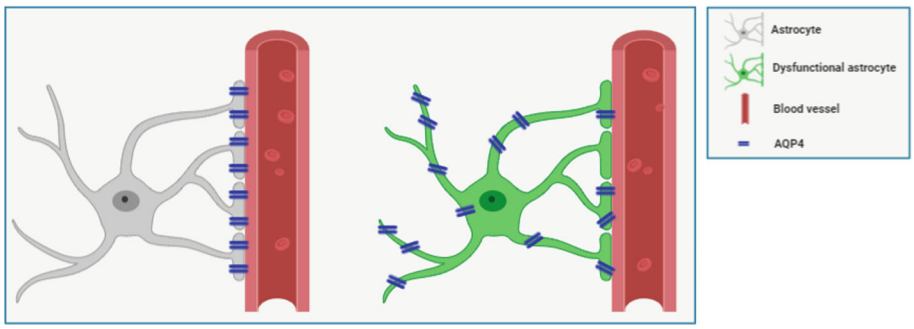

FIGURE 1 | Figure shows representative schemes for expression and polarization/localization of AQP-4 in healthy (*left*) and dysfunctional (*right*) perivascular astrocyte. Astrocytes processes wrap the vessel forming a sheath around it. Cerebrospinal fluid (CSF) flows in the perivascular space created around the vessel. The astrocytic water channel AQP-4 is polarized, as it is densely expressed by astrocytes almost exclusively at the endfeet, in direct contact with the perivascular space, where it facilitates the interchanges of water. In aging and some pathological conditions, such as Alzheimer's disease (AD), AQP-4 loses its polarization in reactive astrocytes and it is found diffusively expressed. Also, higher AQP-4 expression has been documented in Parkinson's disease, cerebral ischemia, amyotrophic lateral sclerosis, and other neurological diseases (for review see Xiao and Hu, 2014; Mader and Brimberg, 2019).

Zeppenfeld et al., reported in 2017 that altered AQP-4 immunostaining was associated with increasing age in *post-mortem* human cortices. Therefore, it can be hypothesized that the upregulation of astrocytic AQP-4 responds to a physiological need for compensating general astrocytes morphological or functional alterations known to occur both in rodents and human *post-mortem* aged brains (Hoozemans et al., 2011; Bronzuoli et al., 2019). However, this hypothesis needs further direct demonstrations.

Aged brains show also altered AQP-4 localization (Zeppenfeld et al., 2017). Indeed, a study from the Nedergaard group demonstrated increased perivascular GFAP in aged (18 months) compared to young (2–3 months) C57BL/6 mice, coupled with a significant, but modest, loss of perivascular localization (Kress et al., 2014). A loss of vascular localization of AQP-4 has been demonstrated in old (24-months) compared to young (6-months) TgSwDI mice, which develop age-dependent accumulation in amyloid, together with general reactive gliosis, as shown by increased number of GFAP-positive astrocytes and Iba 1-positive microglia (Duncombe et al., 2017). Preservation of perivascular localization of AQP-4 in aged human individuals was predictive of preserved cognitive abilities (Zeppenfeld et al., 2017). Additionally, the arterial pulsating force was lower as well as the rate of clearance of the tracer injected into the brains was slower in aged compared to young C57BL/6 mice (Kress et al., 2014).

Measurements of beta-amyloid (Aβ) deposition in human by positron emission tomography (PET) show that Aβ begins to abnormally deposit within the brain between age 40 and 50, thus far before clinical symptoms (Villemagne et al., 2013). This stage of the disease is termed preclinical or prodromal AD; it is characterized by patients having no symptoms of the disease yet, and only few molecular alterations have begun to appear (Hyman et al., 2012). Oxidative stress, as well as signs of neuroinflammation and reactive astrocytes, have been documented at early stages of the disease, before the appearance of massive Aβ deposition and tau hyper-phosphorylation (Zhu et al., 2004a; Zhu et al., 2004b; Jack et al., 2010; Rodriguez-Vieitez and Nordberg, 2018). In absence of neuronal atrophy, a premature presence of reactive astrogliosis can be detected in animal models of AD, as in 6-month-old 3×Tg-AD mice (age that corresponds to a mild stage of pathology). A study using a novel non-invasive magnetic resonance imaging protocol reports lower water influx into the CSF of mice expressing high senile plaque density (APP/PS1 mice) compared to their wild-type counterpart (Igarashi et al., 2014a), similar to what seen in AQP-4 knock-out mice (Igarashi et al., 2014b). AQP-4 knock-out mice show reduced (−50%) intracerebrally infused Aβ clearance compared with wild-type littermates (Iliff et al., 2012). The association of AQP-4 deletion in APP/PS1 mice brought to a significant increase of both soluble and insoluble Aβ in the brain, without affecting synthesis or degradation of the protein (Xu et al., 2015). Moreover, bidirectional relationship between sleep and AD has been reported, such that patients with AD experience sleep disturbances as well as poor sleep predisposes to AD (Ju et al., 2014). Indeed, brain waste products, such excessive Aβ and tau, are cleared during sleep time (Xie et al., 2013; Shokri-Kojori et al., 2018). Based on this, a recent report investigated the association of single-nucleotide polymorphisms (SNPs) in *Aqp-4* gene with sleep latency, duration, and amount of radiolabeled Aβ imaged through PET scans carried out in healthy volunteers >60 years old. They found one SNP associated with poor sleep quality, and two SNPs associated with short sleep duration and consequent higher Aβ burden. In contrast, one SNP, the rs2339214, was associated with higher Aβ and also longer sleep duration (Rainey-Smith et al., 2018). All these accumulating evidence suggests that deposits of Aβ and tau are consequences of impaired clearance, rather than of increased production (Benveniste et al., 2019).

Burfeind and collaborators identified five SNPs in the *Aqp-4* gene and analyzed their possible association with cognitive

decline exclusively in AD patients. Their results identified two *Aqp-4* SNPs associated with rapid, and two with slow, cognitive decline (Burfeind et al., 2017). Another report from the same group studied the association between perivascular AQP-4 localization and its expression levels with AD pathology in humans, showing for the first time that total AQP-4 expression was increased in the AD cortex compared to cognitively intact subjects, both young and aged. The raise was correlated with Aβ deposits. Additionally, loss of perivascular AQP-4 was associated with AD Braak stage and density of Aβ plaques (Zeppenfeld et al., 2017). Ten years before, increased expression of AQP-1, but not AQP-4, was reported in the frontal cortex of patients with early AD stage (Perez et al., 2007). AQP-4 was found highly diffused in the parenchyma of *post-mortem* human AD brains and of a mouse model of AD (5xFAD), with particular localization near Aβ plaques rather than near vasculature (Smith et al., 2019), supporting the hypothesis that a change in AQP-4 localization might be a crucial aspect in AD neuropathology. Interestingly, since 5xFAD mice showed increased neuronal Aβ, they propose that AQP-4 peri-plaques localization might be a defense mechanism to counteract Aβ deposition (Smith et al., 2019). However, further studies are needed to demonstrate this novel and intriguing hypothesis. Anyway, the cited evidence supports the idea that several alterations, including control of water, ions and solute clearance, occur in aging and early stages of AD.

Pharmacological Tools Targeting AQP-4

Despite the massive preclinical and clinical efforts, no effective treatments are currently available for patients with AD. Recent evidence concurs that the best time for intervention is when the disease is not fully overt. This preclinical phase of the disease is difficult to diagnose because, at present, there are no specific biomarkers able to reliably and timely detect it. Disappointing results of the latest clinical trials has prompted researchers to rethink possible pharmaceutical targets and therapeutic approaches, including targeting AQP-4. However, malfunction of the brain cleansing system because of aging brings to waste piling up, including proteins as Aβ and tau. Therefore, astrocytic AQP-4 seems to represent a possible pharmacological candidate to be targeted in AD at its earliest stage, before abnormal protein accumulation and neurodegeneration occur. So far, some molecules have been tested for activity to AQP-4, but none in *in vitro* or *in vivo* models of AD (Lan et al., 2016; Tradtrantip et al., 2017). Some phytocompounds with antioxidant properties have shown to be active on AQP-4. Among them, pinocembrin, a flavonoid contained in propolis, seems to be able to downregulate AQP-4 expression in a rodent model of focal cerebral ischemia (Gao et al., 2010); curcumin treatment reduced hypoxia-hypercapnia-induced brain edema by downregulating the messenger RNA (mRNA) expression levels of AQP-4 in rats (Yu et al., 2016) and dampening AQP-4 and GFAP overexpression in a rat model of acute spinal cord injury (Nesic et al., 2010). Similar results were published with epigallocatechin gallate treatment, an essential ingredient of green tea (Ge et al., 2013). Acute administration of carvacrol, a terpenoid, dose-dependently attenuates brain edema induced by cerebral hemorrhage in mice by downregulating brain *Aqp4* gene and protein expression, likely reducing astrocyte swelling (Nesic et al., 2010). Preliminary studies in our laboratory suggest that *in vivo* chronic treatment of 3×Tg-AD mice and their wild-type counterpart with the ALIAmide palmitoylethanolamide (PEA) is able to reduce the upregulated expression of hippocampal AQP-4 selectively in AD-like mice. Numerous evidence demonstrates the anti-inflammatory and neuroprotective properties of PEA (Scuderi et al., 2012; Scuderi et al., 2014; Skaper et al., 2015), and we have recently demonstrated *in vivo* the efficacy of a formulation of ultramicronized PEA (um-PEA) in reducing several AD-like molecular and behavioral signs in 3×Tg-AD mice (Bronzuoli et al., 2018; Scuderi et al., 2018a). However, further studies are needed to verify the effects of formulations containing PEA on AQP-4 expression and functions.

Interestingly, it has recently been reported that atorvastatin, already in use in the clinical setting as lipid-lowering drug, may prevent ischemic brain edema through downregulation of astrocytic AQP-4 expression in rats. Authors proposed a mechanism involving the attenuation of p38-MAPK signaling (Cheng et al., 2018). Similarly, 2-(nicotinamide)-1,3,4-thiadiazole (TGN-020) was shown to act as a potent AQP-4 inhibitor in a rodent model of ischemia (Pirici et al., 2018; Catalin et al., 2018). A Japanese herbal compound named Goreisan was able to reduce edema in an *in vivo* model of hypoxic-ischemic encephalopathy by reducing the lesion-induced upregulation of AQP-4 protein expression, and ameliorating the rat survival rate compared to the control group (Nakano et al., 2018). Similarly, in a rat model of traumatic brain injury (TBI), acute administration of the hormone ghrelin was able to prevent post-TBI upregulation of AQP-4 expression (Lopez et al., 2012). Chronic treatment with dabigatran etexilate, a thrombin inhibitor, showed an indirect effect on AQP-4, preventing its misplacement found in TgCRND8 mice, a mouse model of AD (Cortes-Canteli et al., 2019). Thus, converging evidence demonstrates that targeting AQP-4 seems to be a promising pharmacological approach in several brain pathologies. For example in major depressive disorder there is a clear reduction in the coverage of blood vessels by AQP-4-positive astrocyte endfeet (Rajkowska et al., 2013). Intriguingly, Di Benedetto and collaborators found that AQP-4 is necessary to mediate fluoxetine-induced growth of astrocytic processes in rats (Di Benedetto et al., 2016).

New AQP-4 partial antagonists have been discovered by library screening by Aeromics, Inc. (OH, USA). The drug AER-270, and its prodrug with enhanced solubility AER-271, have shown beneficial results on brain edema in two different model of cerebral injury in rats, reducing swelling and behavioral neurological damage (Farr et al., 2019). Since AQP-4 was found up-regulated in the aging brain, and mislocalized in AD, it would be interesting to test the hypothesis that treatment with AQP-4 modulator may slower brain senescence process and prevent

neurological deficit through a fine regulation of this water channel. However, the effect of therapeutic interventions targeting AQP-4 will depend on the balance between the beneficial increased water clearance and deleterious effects on astrocytic morphological changes. Since not all pathological conditions are associated with impaired blood brain barrier (BBB), AQP-4-targeting drug should be able to cross an intact BBB, as for example in prodromal stages of AD. However, reaching this perfect balance between maximum benefit and limited toxicity depends on future further understanding of the biology of AQP-4.

AUTHOR CONTRIBUTIONS

Conceptualization: MV, CS. Data curation: MV, RF, CS. Draft preparation: MV. Mini review final editing: MV, RF, LS, CS.

FUNDING

This work was supported by the Italian Ministry of Instruction, University and Research (MIUR) (PRIN 2015 grant n. 2015KP7T2Y_002) and the SAPIENZA University of Rome (grant n. MA116154CD981DAE) to CS.

REFERENCES

Allen, N. J., and Barres, B. A. (2009). Neuroscience: glia - more than just brain glue. *Nature* 457 (7230), 675–677. doi: 10.1038/457675a

Araque, A., Parpura, V., Sanzgiri, R. P., and Haydon, P. G. (1999). Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci.* 22 (5), 208–215. doi: 10.1016/s0166-2236(98)01349-6

Asgari, M., de Zelicourt, D., and Kurtcuoglu, V. (2016). Glymphatic solute transport does not require bulk flow. *Sci. Rep.* 6, 38635. doi: 10.1038/srep38635

Aspelund, A., Antila, S., Proulx, S. T., Karlsen, T. V., Karaman, S., Detmar, M., et al. (2015). A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J. Exp. Med.* 212 (7), 991–999. doi: 10.1084/jem.20142290

Auguste, K. I., Jin, S., Uchida, K., Yan, D., Manley, G. T., Papadopoulos, M. C., et al. (2007). Greatly impaired migration of implanted aquaporin-4-deficient astroglial cells in mouse brain toward a site of injury. *FASEB J.* 21 (1), 108–116. doi: 10.1096/fj.06-6848com

Benveniste, H., Liu, X., Koundal, S., Sanggaard, S., Lee, H., and Wardlaw, J. (2019). The glymphatic system and waste clearance with brain aging: a review. *Gerontology* 65 (2), 106–119. doi: 10.1159/000490349

Bronzuoli, M. R., Facchinetti, R., Steardo, L.Jr., Romano, A., Stecca, C., Passarella, S., et al. (2018). Palmitoylethanolamide dampens reactive astrogliosis and improves neuronal trophic support in a triple transgenic model of alzheimer's disease: in vitro and in vivo evidence. *Oxid. Med. Cell Longev.* 2018, 4720532. doi: 10.1155/2018/4720532

Bronzuoli, M. R., Facchinetti, R., Valenza, M., Cassano, T., Steardo, L., and Scuderi, C. (2019). Astrocyte function is affected by aging and not alzheimer's disease: a preliminary investigation in hippocampi of 3xtg-ad mice. *Front. Pharmacol.* 10, 644. doi: 10.3389/fphar.2019.00644

Burfeind, K. G., Murchison, C. F., Westaway, S. K., Simon, M. J., Erten-Lyons, D., Kaye, J. A., et al. (2017). The effects of noncoding aquaporin-4 single-nucleotide polymorphisms on cognition and functional progression of Alzheimer's disease. *Alzheimers Dement (N Y)* 3 (3), 348–359. doi: 10.1016/j.trci.2017.05.001

Catalin, B., Rogoveanu, O. C., Pirici, I., Balseanu, T. A., Stan, A., Tudorica, V., et al. (2018). Cerebrolysin and aquaporin 4 inhibition improve pathological and motor recovery after ischemic stroke. *CNS Neurol. Disord. Drug Targets* 17 (4), 299–308. doi: 10.2174/1871527317666180425124340

Cheng, Z. J., Dai, T. M., Shen, Y. Y., He, J. L., Li, J., and Tu, J. L. (2018). Atorvastatin pretreatment attenuates ischemic brain edema by suppressing aquaporin 4. *J. Stroke Cerebrovasc. Dis.* 27 (11), 3247–3255. doi: 10.1016/j.jstrokecerebrovasdis.2018.07.011

Cortes-Canteli, M., Kruyer, A., Fernandez-Nueda, I., Marcos-Diaz, A., Ceron, C., Richards, A. T., et al. (2019). Long-term dabigatran treatment delays alzheimer's disease pathogenesis in the tgcrnd8 mouse model. *J. Am. Coll. Cardiol.* 74 (15), 1910–1923. doi: 10.1016/j.jacc.2019.07.081

Di Benedetto, B., Malik, V. A., Begum, S., Jablonowski, L., Gomez-Gonzalez, G. B., Neumann, I. D., et al. (2016). Fluoxetine requires the endfeet protein aquaporin-4 to enhance plasticity of astrocyte processes. *Front. Cell Neurosci.* 10, 8. doi: 10.3389/fncel.2016.00008

Ding, J. H., Sha, L. L., Chang, J., Zhou, X. Q., Fan, Y., and Hu, G. (2007). Alterations of striatal neurotransmitter release in aquaporin-4 deficient mice: an *in vivo* microdialysis study. *Neurosci. Lett.* 422 (3), 175–180. doi: 10.1016/j.neulet.2007.06.018

Dityatev, A., and Rusakov, D. A. (2011). Molecular signals of plasticity at the tetrapartite synapse. *Curr. Opin. Neurobiol.* 21 (2), 353–359. doi: 10.1016/j.conb.2010.12.006

Duncombe, J., Lennen, R. J., Jansen, M. A., Marshall, I., Wardlaw, J. M., and Horsburgh, K. (2017). Ageing causes prominent neurovascular dysfunction associated with loss of astrocytic contacts and gliosis. *Neuropathol. Appl. Neurobiol.* 43 (6), 477–491. doi: 10.1111/nan.12375

Eide, P. K., and Ringstad, G. (2015). MRI with intrathecal MRI gadolinium contrast medium administration: a possible method to assess glymphatic function in human brain. *Acta Radiol. Open* 4 (11), 2058460115609635. doi: 10.1177/2058460115609635

Fan, Y., Zhang, J., Sun, X. L., Gao, L., Zeng, X. N., Ding, J. H., et al. (2005). Sex- and region-specific alterations of basal amino acid and monoamine metabolism in the brain of aquaporin-4 knockout mice. *J. Neurosci. Res.* 82 (4), 458–464. doi: 10.1002/jnr.20664

Farr, G. W., Hall, C. H., Farr, S. M., Wade, R., Detzel, J. M., Adams, A. G., et al. (2019). Functionalized Phenylbenzamides Inhibit Aquaporin-4 Reducing Cerebral Edema and Improving Outcome in Two Models of CNS Injury. *Neuroscience* 404, 484–498. doi: 10.1016/j.neuroscience.2019.01.034

Frigeri, A., Gropper, M. A., Turck, C. W., and Verkman, A. S. (1995). Immunolocalization of the mercurial-insensitive water channel and glycerol intrinsic protein in epithelial cell plasma membranes. *Proc. Natl. Acad. Sci. U.S.A.* 92 (10), 4328–4331. doi: 10.1073/pnas.92.10.4328

Gao, M., Zhu, S. Y., Tan, C. B., Xu, B., Zhang, W. C., and Du, G. H. (2010). Pinocembrin protects the neurovascular unit by reducing inflammation and extracellular proteolysis in MCAO rats. *J. Asian Nat. Prod. Res.* 12 (5), 407–418. doi: 10.1080/10286020.2010.485129

Ge, R., Zhu, Y., Diao, Y., Tao, L., Yuan, W., and Xiong, X. C. (2013). Anti-edema effect of epigallocatechin gallate on spinal cord injury in rats. *Brain Res.* 1527, 40–46. doi: 10.1016/j.brainres.2013.06.009

Gupta, R. K., and Kanungo, M. (2013). Glial molecular alterations with mouse brain development and aging: up-regulation of the Kir4.1 and aquaporin-4. *Age (Dordr)* 35 (1), 59–67. doi: 10.1007/s11357-011-9330-5

Hasegawa, H., Ma, T., Skach, W., Matthay, M. A., and Verkman, A. S. (1994). Molecular cloning of a mercurial-insensitive water channel expressed in selected water-transporting tissues. *J. Biol. Chem.* 269 (8), 5497, 5500.

Holter, K. E., Kehlet, B., Devor, A., Sejnowski, T. J., Dale, A. M., Omholt, S. W., et al. (2017). Interstitial solute transport in 3D reconstructed neuropil occurs by diffusion rather than bulk flow. *Proc. Natl. Acad. Sci. U.S.A.* 114 (37), 9894–9899. doi: 10.1073/pnas.1706942114

Hoozemans, J. J., Rozemuller, A. J., van Haastert, E. S., Eikelenboom, P., and van Gool, W. A. (2011). Neuroinflammation in Alzheimer's disease wanes with age. *J. Neuroinflammation* 8, 171. doi: 10.1186/1742-2094-8-171

Hubbard, J. A., Szu, J. I., and Binder, D. K. (2018). The role of aquaporin-4 in synaptic plasticity, memory and disease. *Brain Res. Bull.* 136, 118–129. doi: 10.1016/j.brainresbull.2017.02.011

Hyman, B. T., Phelps, C. H., Beach, T. G., Bigio, E. H., Cairns, N. J., Carrillo, M. C., et al. (2012). National institute on aging-alzheimer's association guidelines for the neuropathologic assessment of alzheimer's disease. *Alzheimers Dement* 8 (1), 1–13. doi: 10.1016/j.jalz.2011.10.007

Igarashi, H., Suzuki, Y., Kwee, I. L. , and Nakada, T. (2014a). Water influx into cerebrospinal fluid is significantly reduced in senile plaque bearing transgenic mice, supporting beta-amyloid clearance hypothesis of Alzheimer's disease. *Neurol. Res.* 36 (12), 1094–1098. doi: 10.1179/1743132814Y.0000000434

Igarashi, H., Tsujita, M., Kwee, I. L. , and Nakada, T. (2014b). Water influx into cerebrospinal fluid is primarily controlled by aquaporin-4, not by aquaporin-1: 17O JJVCPE MRI study in knockout mice. *Neuroreport* 25 (1), 39–43. doi: 10.1097/WNR.0000000000000042

Iliff, J., and Simon, M. (2019). CrossTalk proposal: the glymphatic system supports convective exchange of cerebrospinal fluid and brain interstitial fluid that is mediated by perivascular aquaporin-4. *J. Physiol.* 597 (17), 4417–4419. doi: 10.1113/JP277635

Iliff, J. J., Wang, M., Liao, Y., Plogg, B. A., Peng, W., Gundersen, G. A., et al. (2012). A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. *Sci. Transl. Med.* 4 (147), 147ra111. doi: 10.1126/scitranslmed.3003748

Iliff, J. J., Wang, M., Zeppenfeld, D. M., Venkataraman, A., Plog, B. A., Liao, Y., et al. (2013). Cerebral arterial pulsation drives paravascular CSF-interstitial fluid exchange in the murine brain. *J. Neurosci.* 33 (46), 18190–18199. doi: 10.1523/JNEUROSCI.1592-13.2013

Jack, C. R.Jr., Knopman, D. S., Jagust, W. J., Shaw, L. M., Aisen, P. S., Weiner, M. W., et al. (2010). Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol.* 9 (1), 119–128. doi: 10.1016/S1474-4422(09)70299-6

Jin, B. J., Zhang, H., Binder, D. K., and Verkman, A. S. (2013). Aquaporin-4-dependent K(+) and water transport modeled in brain extracellular space following neuroexcitation. *J. Gen. Physiol.* 141 (1), 119–132. doi: 10.1085/jgp.201210883

Ju, Y. E., Lucey, B. P., and Holtzman, D. M. (2014). Sleep and Alzheimer disease pathology–a bidirectional relationship. *Nat. Rev. Neurol.* 10 (2), 115–119. doi: 10.1038/nrneurol.2013.269

Jung, J. S., Bhat, R. V., Preston, G. M., Guggino, W. B., Baraban, J. M., and Agre, P. (1994). Molecular characterization of an aquaporin cDNA from brain: candidate osmoreceptor and regulator of water balance. *Proc. Natl. Acad. Sci. U.S.A.* 91 (26), 13052–13056. doi: 10.1073/pnas.91.26.13052

Kress, B. T., Iliff, J. J., Xia, M., Wang, M., Wei, H. S., Zeppenfeld, D., et al. (2014). Impairment of paravascular clearance pathways in the aging brain. *Ann. Neurol.* 76 (6), 845–861. doi: 10.1002/ana.24271

Lan, Y. L., Zhao, J., Ma, T., and Li, S. (2016). The potential roles of aquaporin 4 in alzheimer's disease. *Mol. Neurobiol.* 53 (8), 5300–5309. doi: 10.1007/s12035-015-9446-1

Li, L., Zhang, H., Varrin-Doyer, M., Zamvil, S. S., and Verkman, A. S. (2011). Proinflammatory role of aquaporin-4 in autoimmune neuroinflammation. *FASEB J.* 25 (5), 1556–1566. doi: 10.1096/fj.10-177279

Lopez, N. E., Krzyzaniak, M. J., Blow, C., Putnam, J., Ortiz-Pomales, Y., Hageny, A. M., et al. (2012). Ghrelin prevents disruption of the blood-brain barrier after traumatic brain injury. *J. Neurotrauma.* 29 (2), 385–393. doi: 10.1089/neu.2011.2053

Louveau, A., Smirnov, I., Keyes, T. J., Eccles, J. D., Rouhani, S. J., Peske, J. D., et al. (2015). Structural and functional features of central nervous system lymphatic vessels. *Nature* 523 (7560), 337–341. doi: 10.1038/nature14432

Mader, S., and Brimberg, L. (2019). Aquaporin-4 water channel in the brain and its implication for health and disease. *Cells* 8, 90.

Mestre, H., Hablitz, L. M., Xavier, A. L., Feng, W., Zou, W., Pu, T., et al. (2018). Aquaporin-4-dependent glymphatic solute transport in the rodent brain. *eLife* 7, e40070. doi: 10.7554/eLife.40070

Nakano, T., Nishigami, C., Irie, K., Shigemori, Y., Sano, K., Yamashita, Y., et al. (2018). Goreisan prevents brain edema after cerebral ischemic stroke by inhibiting aquaporin 4 upregulation in mice. *J. Stroke Cerebrovasc. Dis.* 27 (3), 758–763. doi: 10.1016/j.jstrokecerebrovasdis.2017.10.010

Nedergaard, M. (2013). Neuroscience. Garbage truck of the brain. *Science* 340 (6140), 1529–1530. doi: 10.1126/science.1240514

Nesic, O., Guest, J. D., Zivadinovic, D., Narayana, P. A., Herrera, J. J., Grill, R. J., et al. (2010). Aquaporins in spinal cord injury: the janus face of aquaporin 4. *Neuroscience* 168 (4), 1019–1035. doi: 10.1016/j.neuroscience.2010.01.037

Nielsen, S., Nagelhus, E. A., Amiry-Moghaddam, M., Bourque, C., Agre, P., and Ottersen, O. P. (1997). Specialized membrane domains for water transport in glial cells: high-resolution immunogold cytochemistry of aquaporin-4 in rat brain. *J. Neurosci.* 17 (1), 171–180. doi: 10.1523/JNEUROSCI.17-01-00171.1997

Papadopoulos, M. C., and Verkman, A. S. (2005). Aquaporin-4 gene disruption in mice reduces brain swelling and mortality in pneumococcal meningitis. *J. Biol. Chem.* 280 (14), 13906–13912. doi: 10.1074/jbc.M413627200

Pekny, M., Pekna, M., Messing, A., Steinhauser, C., Lee, J. M., Parpura, V., et al. (2016). Astrocytes: a central element in neurological diseases. *Acta Neuropathol.* 131 (3), 323–345. doi: 10.1007/s00401-015-1513-1

Perez, E., Barrachina, M., Rodriguez, A., Torrejon-Escribano, B., Boada, M., Hernandez, I., et al. (2007). Aquaporin expression in the cerebral cortex is increased at early stages of Alzheimer disease. *Brain Res.* 1128 (1), 164–174. doi: 10.1016/j.brainres.2006.09.109

Pirici, I., Balsanu, T. A., Bogdan, C., Margaritescu, C., Divan, T., Vitalie, V., et al. (2018). Inhibition of Aquaporin-4 improves the outcome of ischaemic stroke and modulates brain paravascular drainage pathways. *Int. J. Mol. Sci.* 19, 46

Rainey-Smith, S. R., Mazzucchelli, G. N., Villemagne, V. L., Brown, B. M., Porter, T., Weinborn, M., et al. (2018). Genetic variation in Aquaporin-4 moderates the relationship between sleep and brain Abeta-amyloid burden. *Transl. Psychiatry* 8 (1), 47. doi: 10.1038/s41398-018-0094-x

Rajkowska, G., Hughes, J., Stockmeier, C. A., Javier Miguel-Hidalgo, J., and Maciag, D. (2013). Coverage of blood vessels by astrocytic endfeet is reduced in major depressive disorder. *Biol. Psychiatry* 73 (7), 613–621. doi: 10.1016/j.biopsych.2012.09.024

Rasmussen, M. K., Mestre, H., and Nedergaard, M. (2018). The glymphatic pathway in neurological disorders. *Lancet Neurol.* 17 (11), 1016–1024. doi: 10.1016/S1474-4422(18)30318-1

Ray, L., Iliff, J. J., and Heys, J. J. (2019). Analysis of convective and diffusive transport in the brain interstitium. *Fluids Barriers CNS* 16 (1), 6. doi: 10.1186/s12987-019-0126-9

Rodriguez-Vieitez, E., and Nordberg, A. (2018). Imaging Neuroinflammation: Quantification of Astrocytosis in a Multitracer PET Approach. *Methods Mol. Biol.* 1750, 231–251. doi: 10.1007/978-1-4939-7704-8_16

Saadoun, S., Papadopoulos, M. C., Watanabe, H., Yan, D., Manley, G. T., and Verkman, A. S. (2005). Involvement of aquaporin-4 in astroglial cell migration and glial scar formation. *J. Cell Sci.* 118 (Pt 24), 5691–5698. doi: 10.1242/jcs.02680

Scuderi, C., Valenza, M., Stecca, C., Esposito, G., Carratu, M. R., and Steardo, L. (2012). Palmitoylethanolamide exerts neuroprotective effects in mixed neuroglial cultures and organotypic hippocampal slices *via* peroxisome proliferator-activated receptor-alpha. *J. Neuroinflammation* 9, 49. doi: 10.1186/1742-2094-9-2110.1186/1742-2094-9-49

Scuderi, C., Stecca, C., Iacomino, A., and Steardo, L. (2013). Role of astrocytes in major neurological disorders: the evidence and implications. *IUBMB Life* 65 (12), 957–961. doi: 10.1002/iub.1223

Scuderi, C., Stecca, C., Valenza, M., Ratano, P., Bronzuoli, M. R., Bartoli, S., et al. (2014). Palmitoylethanolamide controls reactive gliosis and exerts neuroprotective functions in a rat model of Alzheimer's disease. *Cell Death Dis.* 5, e1419. doi: 10.1038/cddis.2014.376

Scuderi, C., Bronzuoli, M. R., Facchinetti, R., Pace, L., Ferraro, L., Broad, K. D., et al. (2018a). Ultramicronized palmitoylethanolamide rescues learning and memory impairments in a triple transgenic mouse model of Alzheimer's disease by exerting anti-inflammatory and neuroprotective effects. *Transl. Psychiatry* 8 (1), 32. doi: 10.1038/s41398-017-0076-4

Scuderi, C., Noda, M., and Verkhratsky, A. (2018b). Editorial: Neuroglia Molecular Mechanisms in Psychiatric Disorders. *Front. Mol. Neurosci.* 11, 407. doi: 10.3389/fnmol.2018.0040

Shokri-Kojori, E., Wang, G. J., Wiers, C. E., Demiral, S. B., Guo, M., Kim, S. W., et al. (2018). beta-Amyloid accumulation in the human brain after one night of sleep deprivation. *Proc. Natl. Acad. Sci. U.S.A.* 115 (17), 4483–4488. doi: 10.1073/pnas.1721694115

Skaper, S. D., Facci, L., Barbierato, M., Zusso, M., Bruschetta, G., Impellizzeri, D., et al. (2015). N-palmitoylethanolamine and neuroinflammation: a novel

therapeutic strategy of resolution. *Mol. Neurobiol.* 52 (2), 1034–1042. doi: 10.1007/s12035-015-9253-8

Smith, A. J., and Verkman, A. S. (2018). The "glymphatic" mechanism for solute clearance in Alzheimer's disease: game changer or unproven speculation? *FASEB J.* 32 (2), 543–551. doi: 10.1096/fj.201700999

Smith, A. J., and Verkman, A. S. (2019). CrossTalk opposing view: Going against the flow: interstitial solute transport in brain is diffusive and aquaporin-4 independent. *J. Physiol.* 597 (17), 4421–4424. doi: 10.1113/JP277636

Smith, A. J., Yao, X., Dix, J. A., Jin, B. J., and Verkman, A. S. (2017). Test of the 'glymphatic' hypothesis demonstrates diffusive and aquaporin-4-independent solute transport in rodent brain parenchyma. *eLife* 6, e27679. doi: 10.7554/eLife.27679

Smith, A. J., Duan, T., and Verkman, A. S. (2019). Aquaporin-4 reduces neuropathology in a mouse model of Alzheimer's disease by remodeling peri-plaque astrocyte structure. *Acta Neuropathol. Commun.* 7 (1), 74. doi: 10.1186/s40478-019-0728-0

Sofroniew, M. V. (2014). Astrogliosis. *Cold Spring Harb. Perspect. Biol.* 7 (2), a020420. doi: 10.1101/cshperspect.a020420

Sorani, M. D., Manley, G. T. , and Giacomini, K. M. (2008a). Genetic variation in human aquaporins and effects on phenotypes of water homeostasis. *Hum. Mutat.* 29 (9), 1108–1117. doi: 10.1002/humu.20762

Sorani, M. D., Zador, Z., Hurowitz, E., Yan, D., Giacomini, K. M. , and Manley, G. T. (2008b). Novel variants in human Aquaporin-4 reduce cellular water permeability. *Hum. Mol. Genet.* 17 (15), 2379–2389. doi: 10.1093/hmg/ddn138

Taoka, T., Masutani, Y., Kawai, H., Nakane, T., Matsuoka, K., Yasuno, F., et al. (2017). Evaluation of glymphatic system activity with the diffusion MR technique: diffusion tensor image analysis along the perivascular space (DTI-ALPS) in Alzheimer's disease cases. *JPN J. Radiol.* 35 (4), 172–178. doi: 10.1007/s11604-017-0617-z

Thrane, A. S., Rappold, P. M., Fujita, T., Torres, A., Bekar, L. K., Takano, T., et al. (2011). Critical role of aquaporin-4 (AQP4) in astrocytic Ca2+ signaling events elicited by cerebral edema. *Proc. Natl. Acad. Sci. U.S.A.* 108 (2), 846–851. doi: 10.1073/pnas.1015217108

Tradtrantip, L., Jin, B. J., Yao, X., Anderson, M. O., and Verkman, A. S. (2017). Aquaporin-targeted therapeutics: state-of-the-field. *Adv. Exp. Med. Biol.* 969, 239–250. doi: 10.1007/978-94-024-1057-0_16

Verkhratsky, A., and Nedergaard, M. (2014). Astroglial cradle in the life of the synapse. *Philos. Trans. R. Soc Lond. B. Biol. Sci.* 369 (1654), 20130595. doi: 10.1098/rstb.2013.0595

Verkhratsky, A., and Nedergaard, M. (2018). Physiology of astroglia. *Physiol. Rev.* 98 (1), 239–389. doi: 10.1152/physrev.00042.2016

Verkhratsky, A., Zorec, R., and Parpura, V. (2017). Stratification of astrocytes in healthy and diseased brain. *Brain Pathol.* 27 (5), 629–644. doi: 10.1111/bpa.12537

Villemagne, V. L., Burnham, S., Bourgeat, P., Brown, B., Ellis, K. A., Salvado, O., et al. (2013). Amyloid beta deposition, neurodegeneration, and cognitive decline in sporadic Alzheimer's disease: a prospective cohort study. *Lancet Neurol.* 12 (4), 357–367. doi: 10.1016/S1474-4422(13)70044-9

Wolkove, N., Elkholy, O., Baltzan, M., and Palayew, M. (2007). Sleep and aging: 1. sleep disorders commonly found in older people. *CMAJ* 176 (9), 1299–1304. doi: 10.1503/cmaj.060792

Wu, Q., Zhang, Y. J., Gao, J. Y., Li, X. M., Kong, H., Zhang, Y. P., et al. (2014). Aquaporin-4 mitigates retrograde degeneration of rubrospinal neurons by facilitating edema clearance and glial scar formation after spinal cord injury in mice. *Mol. Neurobiol.* 49 (3), 1327–1337. doi: 10.1007/s12035-013-8607-3

Xiao, M., and Hu, G. (2014). Involvement of aquaporin 4 in astrocyte function and neuropsychiatric disorders. *CNS Neurosci. Ther.* 20 (5), 385–390. doi: 10.1111/cns.12267

Xie, L., Kang, H., Xu, Q., Chen, M. J., Liao, Y., Thiyagarajan, M., et al. (2013). Sleep drives metabolite clearance from the adult brain. *Science* 342 (6156), 373–377. doi: 10.1126/science.1241224

Xu, Z., Xiao, N., Chen, Y., Huang, H., Marshall, C., Gao, J., et al. (2015). Deletion of aquaporin-4 in APP/PS1 mice exacerbates brain Abeta accumulation and memory deficits. *Mol. Neurodegener.* 10, 58. doi: 10.1186/s13024-015-0056-1

Yu, L. S., Fan, Y. Y., Ye, G., Li, J., Feng, X. P., Lin, K., et al. (2016). Curcumin alleviates brain edema by lowering AQP4 expression levels in a rat model of hypoxia-hypercapnia-induced brain damage. *Exp. Ther. Med.* 11 (3), 709–716. doi: 10.3892/etm.2016.3022

Zeng, X. N., Sun, X. L., Gao, L., Fan, Y., Ding, J. H., and Hu, G. (2007). Aquaporin-4 deficiency down-regulates glutamate uptake and GLT-1 expression in astrocytes. *Mol. Cell Neurosci.* 34 (1), 34–39. doi: 10.1016/j.mcn.2006.09.008

Zeppenfeld, D. M., Simon, M., Haswell, J. D., D'Abreo, D., Murchison, C., Quinn, J. F., et al. (2017). Association of perivascular localization of aquaporin-4 with cognition and alzheimer disease in aging brains. *JAMA Neurol.* 74 (1), 91–99. doi: 10.1001/jamaneurol.2016.4370

Zhu, X., Raina, A. K., Lee, H. G., Casadesus, G., Smith, M. A., and Perry, G. (2004a). Oxidative stress signalling in Alzheimer's disease. *Brain Res.* 1000 (1-2), 32–39. doi: 10.1016/j.brainres.2004.01.012

Zhu, X., Raina, A. K., Perry, G., and Smith, M. A. (2004b). Alzheimer's disease: the two-hit hypothesis. *Lancet Neurol.* 3 (4), 219–226. doi: 10.1016/S1474-4422(04)00707-0

Conflict of Interest: MV discloses a collaboration with Epitech Group SpA, which had no role in the conceptualization and writing of the mini review, and in the decision to submit the paper for publication.

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

The reviewer AV declared a past co-authorship with several of the authors RF, CS, LS to the handling editor.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 21 February 2020
doi: 10.3389/fnins.2020.00061

Regionally Distinct Astrocytes Display Unique Transcription Factor Profiles in the Adult Brain

*Brittney Lozzi[1], Teng-Wei Huang[1], Debosmita Sardar[1], Anna Yu-Szu Huang[1,2] and Benjamin Deneen[1,2,3]**

[1] Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, United States, [2] Program in Developmental Biology, Baylor College of Medicine, Houston, TX, United States, [3] Department of Neurosurgery, Baylor College of Medicine, Houston, TX, United States

Astrocytes are the most abundant type of glial cell in the central nervous system and perform a myriad of vital functions, however, the nature of their diversity remains a longstanding question in neuroscience. Using transcription factor motif discovery analysis on region-specific gene signatures from astrocytes we uncovered universal and region-specific transcription factor expression profiles. This analysis revealed that motifs for Nuclear Factor-I (NFI) are present in genes enriched in astrocytes from all regions, with NFIB and NFIX exhibiting pan-astrocyte expression in the olfactory bulb, hippocampus, cortex, and brainstem. Further analysis into region-specific motif patterns, identified Nkx3-1, Stat4, Pgr, and Nkx6-1 as prospective region-specific transcription factors. Validation studies revealed that Nkx6-1 is exclusively expressed in astrocytes in the brainstem and associates with the promoters of several brainstem specific target genes. These studies illustrate the presence of multiple transcriptional layers in astrocytes across diverse brain regions and provide a new entry point for examining how astrocyte diversity is specified and maintained.

Keywords: astrocyte, transcription factor, brain, cellular diversity, bioinformatics

OPEN ACCESS

Edited by:
Juan Andrés Orellana, Pontifical Catholic University of Chile, Chile

Reviewed by:
Min-Yu Sun, Washington University in St. Louis, United States
Kevin Donald Broad, University College London, United Kingdom

***Correspondence:**
Benjamin Deneen
deneen@bcm.edu

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

Received: *01 October 2019*
Accepted: *15 January 2020*
Published: *21 February 2020*

Citation:
Lozzi B, Huang T-W, Sardar D, Huang AY-S and Deneen B (2020) Regionally Distinct Astrocytes Display Unique Transcription Factor Profiles in the Adult Brain. Front. Neurosci. 14:61. doi: 10.3389/fnins.2020.00061

INTRODUCTION

The brain is composed of an incredible array of diverse cell types, of which, glial cells account for approximately half of this mosaic (Fu et al., 2013; Herculano-Houzel, 2014). Astrocytes, a principal subtype of glial cell, were traditionally thought to be a homogenous population of cells that only function to provide support to neurons. However, astrocytes are now known to perform a multitude of essential functions, such as buffering neurotransmitters, regulating synaptogenesis, modulating synaptic transmission, and maintaining the blood-brain barrier (Abbott et al., 2006). In addition, calcium signaling within astrocytes has been shown to control neuronal physiology and animal behavior (Chai et al., 2017; Yu et al., 2018). Taken together, astrocytes are now known to contribute to nearly every aspect of brain physiology and function (Khakh and Deneen, 2019). Their ability to execute a wide array of functions challenges the notion that astrocytes are a homogenous population of cells.

Astrocytes are electrically silent cells, making it difficult to characterize their functional diversity based on electrophysiological activities. Methods for understanding neuronal cell diversity, such as whole cell electrophysiology (Anderson et al., 2001), morphological criteria, and imaging- based analysis reveals little information about astrocytes because they

are not excitable, exhibit grossly uniform (albeit complex) morphologies, and lack subtype-specific markers for imaging (Jobling and Gibbins, 1999; Khakh and Sofroniew, 2015). Critically, the lack of reliable, astrocyte-specific markers has severely hindered the development of tools to study astrocytes. The identification of Aldh1l1 as a marker that broadly and specifically labels astrocytes (Anthony and Heintz, 2007; Cahoy et al., 2008), and the subsequent development of transgenic mice (Anthony and Heintz, 2007) has enabled astrocytes to be isolated and further analyzed. These tools have also enabled further molecular probing of astrocyte transcriptomes, revealing extensive molecular heterogeneity (Bayraktar et al., 2015). Unique astrocytic gene signatures have been found across brain regions (Morel et al., 2017; Duran et al., 2019), and it has been demonstrated that region-specific astrocyte transcriptomes translate to neural-circuit based functional differences (Chai et al., 2017). In addition to regional diversity, five distinct astrocyte sub-populations have been identified across a host of brain regions, and characterization of these sub-populations revealed functional diversity amongst these subpopulations with respect to synapse formation (Lin et al., 2017). While our understanding of astrocyte heterogeneity has advanced considerably, many questions remain about how this intrinsic heterogeneity is encoded, and whether and how these regionally distinct signatures are converted to functional differences.

One important question remaining is what controls the unique expression profiles observed in astrocyte populations from distinct brain regions? In the spinal cord, positionally distinct subpopulations of astrocytes arise from the differential expression of transcription factors during development and this combinatorial transcription factor code results in three distinct astrocyte subpopulations in the developing spinal cord (Hochstim et al., 2008). Applying this rationale to the brain, we hypothesized that differential transcription factor expression contributes to the observed regional diversity of astrocytes in the mature brain. Toward this, we sought to decipher transcription factor expression profiles associated with astrocyte populations by surveying region specific molecular profiles. Bioinformatic analyses of astrocyte gene signatures from the olfactory bulb, hippocampus, cortex, and brainstem identified cohorts of transcription factors involved in modulating region-specific molecular signatures. We identified generalized astrocytic transcriptional regulators, as well as three region-specific transcription factors in adult astrocytes. Our findings suggest that differential expression of transcription factors influences astrocyte diversity in the mammalian brain.

MATERIALS AND METHODS

Animals, Tissue Dissociation, and FACS Analysis

All research and animal care procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee and housed in the Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility at Baylor College of Medicine. Both male and female BAC Aldh1l1–eGFP mice were used. All strains were maintained on C57BL6 background.

The olfactory bulb, hippocampus, cortex, and brainstem from 16-week old Aldh1l1–eGFP mice was dissected and dissociated using the protocol in Lin et al., 2017. Fluorescence activated cell sorting (FACS) was performed on a BD FACSAria III instrument (100-μm nozzle and 20-p.s.i. setting) with FACSDIVA software, and eGFP+ astrocytes were sorted into a 1.5-ml eppendorf tube containing RLT lysis buffer from the RNeasy Micro Kit (74004, QIAGEN) with 1% ß-Mercaptoethanol.

Total RNA Extraction, Library Preparation and Sequencing

Total RNA was extracted from Aldh1l1–eGFP+ FAC-sorted cells using the RNeasy Micro Kit (74004, QIAGEN) and quality controlled using the High Sensitivity RNA Analysis Kit (DNF-472-0500, Agilent formerly AATI) on a 12-Capillary Fragment Analyzer. cDNA synthesis, library construction and rRNA depletion was performed on 5 ng total of RNA using the Trio RNA-Seq System (0507-96, NuGEN). The resulting single index libraries were validated using the Standard Sensitivity NGS Fragment Analysis Kit (DNF-473-0500, Agilent formerly AATI) for size confirmation and quantified using the Quant-iT dsDNA Assay Kit, high sensitivity (Q33120, Thermo Fisher). Samples were diluted to equimolar concentrations (2 nM), pooled, and denatured according to the manufacturer's protocol. The final library dilution of 1.3 pM was sequenced on a NextSeq500 using the High Output v2 kit (FC-404-2002, Illumina) for paired-end (2 × 75) sequencing of approximately 40 million reads per sample.

Bioinformatics Analysis

Demultiplexed sequencing files were downloaded from BaseSpace and quality control was assessed using fastQC (v0.10.1) and MultiQC (v0.9) (Ewels et al., 2016). Reads were mapped to the mouse genome (10 mm) using STAR (v2.5.0a) (Love et al., 2014). Rsamtools (v2.0.0) and GenomicFeatures (v1.32.2) were used to build count matrices and gene models for expression quantification. UCSC transcripts were downloaded from Illumina iGenomes in GTF file format. We determined reads per million (RPM) using GenomicAlignments (v1.16.0). Principal component analysis (PCA) was performed using rlog transformed gene expression matrix of global gene expression >1 for each region. DESeq2 (v1.20.0) was used for both differential gene expression analysis and read count normalization. Expression heat maps were generated using ComplexHeatmap (v2.0.0).

Astrocyte Region-Specific Gene Signatures

To identify unique gene signatures, we compared global gene expression from one region to all three other regions using DESeq2. This process was repeated to determine region-specific gene expression patterns. We defined differentially expressed genes (DEGs) as those with normalized reads per million (RPM) >5 in at least two of the replicates and expression fold-change >1.5 at $p < 0.01$. Gene Ontologies associated with region-specific

DEGs were determined using Enrichr and visualized using GOplot (v1.0.2) and ggplot2.

Motif Analysis

To identify any transcription factor motifs that are enriched across region-specific gene signatures, the DEGs from each region were pooled to comprise one list of 3555 genes. These genes were analyzed using Hypergeometric Optimization of Motif EnRichment (HOMER) (v4.10) to identify transcription factor motifs enriched within 2 kb of the gene's promoter sequence. To be considered enriched across all regions the transcription factor motif had to be present in at least 50% of DEGs from each region. Transcription factors with enriched motifs were further analyzed to determine their expression patterns across regions. Those with an RPM >5 in at least two of the replicates were used for downstream analysis.

Applying the same parameters outlined above, DEGs from each region were individually subjected to motif analysis using HOMER to discover transcription factor motifs enriched within 2 kb of the gene's promoter sequence. The resulting list of enriched motifs was filtered based on expression data to identify regionally specific transcription factors. We considered a transcription factor regionally enriched only with a fold change >2 at $p < 0.01$ in the region of interest.

Immunocytochemistry

Perfusion and tissue collection were performed as described previously in Huang et al., 2016. Briefly, mice were deeply anesthetized by isoflurane and then fixed by transcardiac perfusion with PBS followed by 4% PFA in PBS. Tissues for histological analysis were harvested immediately after perfusion. The tissues were then fixed 6 h in 4% PFA in PBS and cryopreserved by overnight incubations in 20% sucrose. Tissues were embedded in OCT compound (Sakura) and sectioned. We collected 30 μm sections of brains with a cryostat and stained them as floating sections. Prewarmed solution of sodium citrate (pH 6.0) was added to immerse the sections, and the sections were incubated in 75°C water bath for 10 min. Sections were allowed to cool down to room temperature and then blocked for 20 min in a PBS solution containing 10% serum (matched to the host used for the secondary antibodies) and 0.3% Triton X-100. Primary antibody incubation was performed in the blocking solution overnight at 4°C for floating sections. Secondary antibody incubation was performed in the PBS solution with 0.1% Triton X-100 for floating sections at room temperature for 1 h. Sections were washed between incubations with PBS containing 0.1% Triton X-100. DAPI was included in the penultimate wash. We used these primary antibodies at the following dilutions: chicken anti-GFP 1:1000 (Abcam, ab13970), mouse anti-Nkx6.1 (DSHB, F55A10), rabbit anti-Pgr 1:200 (Invitrogen, MA5-14505), rabbit anti-NFIB (Millipore Sigma, HPA003956), and rabbit anti-NFIX (Abcam, ab101341). Secondary antibodies conjugated to DyLight 488, 549, or 649 were used at a dilution of 1:500 and raised in goat or donkey (Jackson ImmunoResearch Laboratories). Sections were mounted with antifade mounting medium (VECTASHIELD) and imaged via epifluorescent microscopy (Zeiss M1 with ApoTome2 and ZEN2 software) or Nikon A1-Rs confocal microscope.

Transcription Factor Target Identification

Potential transcription factor targets were predicted using HOMER's annotatePeaks.pl with the -m option. Each set of regional DEGs was interrogated for the presence of the associated transcription factor motif allowing zero mismatch. Only genes that had the appropriate motif sequence within 2 kb of the transcriptional start site were considered possible targets. The resulting list was then subset to include only genes with a fold change >2.5 for each region. Predicted target enrichment was visualized using ComplexHeatmap (v2.0.0) and circlize (v0.4.6). Gene Ontologies of predicted targets were determined using Enrichr (Chen et al., 2013; Kuleshov et al., 2016).

Chromatin Immunoprecipitation (ChIP)

Brainstems were collected from 16-week old mice for ChIP-PCR validation of Nkx6-1 targets. Tissue was coarsely chopped and washed with cold PBS before dissociation with a pellet homogenizer. The homogenate from 6 dissociated brainstems were pooled for subsequent sample preparation. Crosslinking was performed using freshly prepared 1.1% formaldehyde solution (11% formaldehyde, 100 mM NaCl, 1 mM EDTA, 50 mM HEPES pH 7.9) while rocking for 10 min and neutralized by adding glycine (125 mM). Samples were centrifuged at 3500 rpm for 5 min at 4°C, washed with PBS (containing 1 mM PMSF), and pellets were stored at −80°C or lysed immediately. All remaining buffers contain protease inhibitors (Roche Cat. 04693132001). To release nuclei cell pellets were resuspended in PBS/PMSF containing 0.5% Igepal and washed with cold ChIP-Buffer 1 (0.25% Triton-X100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES pH 6.5) and rotated for 10 min at 4°C followed by centrifugation at 1200 rpm for 5 min at 4°C and washing with ChIP-Buffer 2 (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES pH 6.5) while rotating at room temperature. Cells were collected via centrifugation and incubated in ChIP lysis buffer (0.5% SDS, 5 mM EDTA, 25 mM Tris–HCl pH 8) for 15–20 min at room temperature to lyse nuclei. Lysates were sonicated to approximately 200–500 bp length fragments using a Bioruptor (Diagenode, model XL). Fragment lengths of chromatin were confirmed using the Standard Sensitivity NGS Fragment Analysis Kit (DNF-473-0500, Agilent formerly AATI) on a 12-Capillary Fragment Analyzer and quantified using Quant-it dsDNA assay kit (Cat. Q33120). An input of 100 μg of sonicated chromatin was used for each experiment, and 1 ug was saved as input chromatin. Samples were diluted 5-fold with ChIP-dilution buffer (1% Triton-X100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl pH 8) and immunoprecipitation was performed overnight at 4°C with 10 μg of Nkx6-1 antibody (F55410, DSHB) and mouse IgG (Santa Cruz Biotechnology, sc-2025) while rotating. Samples were then incubated with Dynabeads (Invitrogen) for 6 h and purified through a series of washes with TSE1 buffer (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris–HCl pH 8, 150 mM NaCl), TSE2 buffer (0.1% SDS, 1%

Triton-X100, 2 mM EDTA, 20 mM Tris–HCl, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8) and TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA). Samples with beads were then incubated at 65°C for 20 min in ChIP Elution buffer (1% SDS, 0.1 M NaHCO3). ChIP samples and input control were incubated at 55°C with proteinase K (0.2 mg/ml) and NaCl (125 mM) for 3 h followed by overnight incubation at 65°C to reverse crosslinking. Immunoprecipitated DNA was purified using the Qiagen PCR purification kit and analyzed using primers specific to the Hoxc4 and Hoxb3 promoters (Hoxc4 -forward: 5′-GGC CAAGAGGGTTGG, reverse: 5′-GCAGTCTGTGTAGGTCA CAG, Hoxb3-forward: 5′-GCCATTCTGTGTAGACAAGAGC, reverse: 5′-CGGAGAGACGGCTAACAC).

RESULTS

Astrocytes Display Brain Region-Specific Gene Expression Signatures

Aldh1l1 is a validated marker of astrocytes (Anthony and Heintz, 2007; Cahoy et al., 2008) and Aldh1l1-eGFP mice have been generated as a tool that broadly, yet specifically, labels astrocytes throughout the brain (**Figures 1A–E**). To query region specific gene signatures from astrocytes, Aldh1l1-eGFP mice were used to FACS isolate astrocytes from four brain regions (olfactory bulb, hippocampus, cortex, and brainstem) for mRNA-Seq analysis (**Figure 1F**). We performed whole transcriptome RNA-Sequencing, and to verify that the resultant molecular profiles reflect astrocyte-specific signatures, we compared our data set with existing gene signatures linked to neurons and astrocytes (Lin et al., 2017), finding that our astrocyte expression profiles are consistent with astrocytic-signatures (**Figure 1G** and **Supplementary Datasheet 1**). To further confirm that these cells exhibit molecular features exclusive to astrocytes, we examined expression of established markers of astrocytes, neurons, oligodendrocytes, and microglia from our sequencing data (**Figure 1H**). Together, these data indicate that we have successfully isolated Aldh1l1-eGFP astrocytes and profiled their transcriptomes from distinct brain regions.

To evaluate the regional diversity of these astrocyte populations, we probed the transcriptome of the four brain regions using various bioinformatics approaches. First, we used principal component analysis (PCA) as an unbiased approach to analyze global gene expression patterns in each region. The PCA revealed distinct gene expression patterns that were unique for each region (**Figure 2A**). The olfactory bulb (OB) and brainstem (BS) displayed the greatest expression pattern variation, suggesting that astrocytes in the OB and BS are transcriptomically different from astrocytes in other regions. Additionally, the hippocampus (HC) and cortex (CX) exhibited only 19% variability, indicating that astrocytes in the HC and CX share the most similar molecular expression patterns. A similar relationship was also observed between cortical and hippocampal samples in the study from Morel et al., 2017. These results indicate that our independently derived datasets are consistent with previous studies and further support the notion that astrocytes maintain region specific molecular signatures.

Next, we sought to identify region-specific gene signatures. Toward this, we performed differential gene expression analysis by comparing one region to all three other regions to determine region-specific differentially expressed genes (DEGs). The identified DEGs are unique to the region of interest, such that a DEG is significantly up or down regulated only in the respective region when compared to all other regions and exhibits a fold change >1.5 at $p < 0.01$. We found 1360 DEGs in the OB, 398 DEGs in the HC, 505 DEGs in the CX, and 1292 DEGs in the BS (**Supplementary Table 1**). Visualized in **Figures 2B–E** we show the expression of each set of DEGs across regions to highlight the enrichment of these DEGs in their respective region. This transcriptome analysis supports observations from the PCA, showing that astrocytes in each of the four regions demonstrate unique, region-specific expression profiles and that the OB and BS are more molecularly distinct than astrocytes from other brain regions. Interestingly, the HC displayed fewer DEGs than any other region, and of those DEGs only 15% were upregulated, while >80% of hippocampal DEGs are downregulated compared to other regions.

Finally, to gain insight into the cellular pathways regulated by these DEGs, we performed gene ontology (GO) analysis on the DEGs from each brain region (**Figure 2F**). We found that DEGs from each region are involved in an array of diverse biological processes. For instance, the HC DEGs were enriched for ligand-gated cation channel activity, while the DEGs from the OB were associated with cell-cell junction assembly and adhesion. Critically, we also found some enriched biological processes that are conserved across regions including synaptic transmission and glutamate receptor activity. These results indicate that astrocytes from these distinct brain regions exhibit two broad layers of molecular features: conserved and unique.

Region-Specific Gene Signatures Display Universal Transcription Factor Expression

To investigate how transcriptional regulation maintains conserved gene ontologies across the brain, we pooled all region-specific DEGs, hypothesizing that since these distinct DEGs are associated with some functionally redundant gene ontologies across the brain, they are likely to be regulated by universally conserved transcription factors. We analyzed the pool of 3555 DEGs from all regions for transcription factor motif enrichment, querying transcription factor binding sites within 2 kb of the 5′ promoter regions of these genes. We found seven transcription factor motifs (RPM >5) that were significantly enriched in DEGs from all four brain regions (**Figure 3A**), and the top three most significantly enriched motifs were that of Nkx2-2, Maz and NFI-family members NFIA, NFIB, NFIX (**Figures 3B–D**). Interestingly, these transcription factors have previously been implicated in developmental oligodendrogenesis and gliogenesis (Deneen et al., 2006; Cai et al., 2010; Liu et al., 2016) but have not been studied in adult astrocytes. To ensure equal representation of these transcription factor motifs across DEGs from each

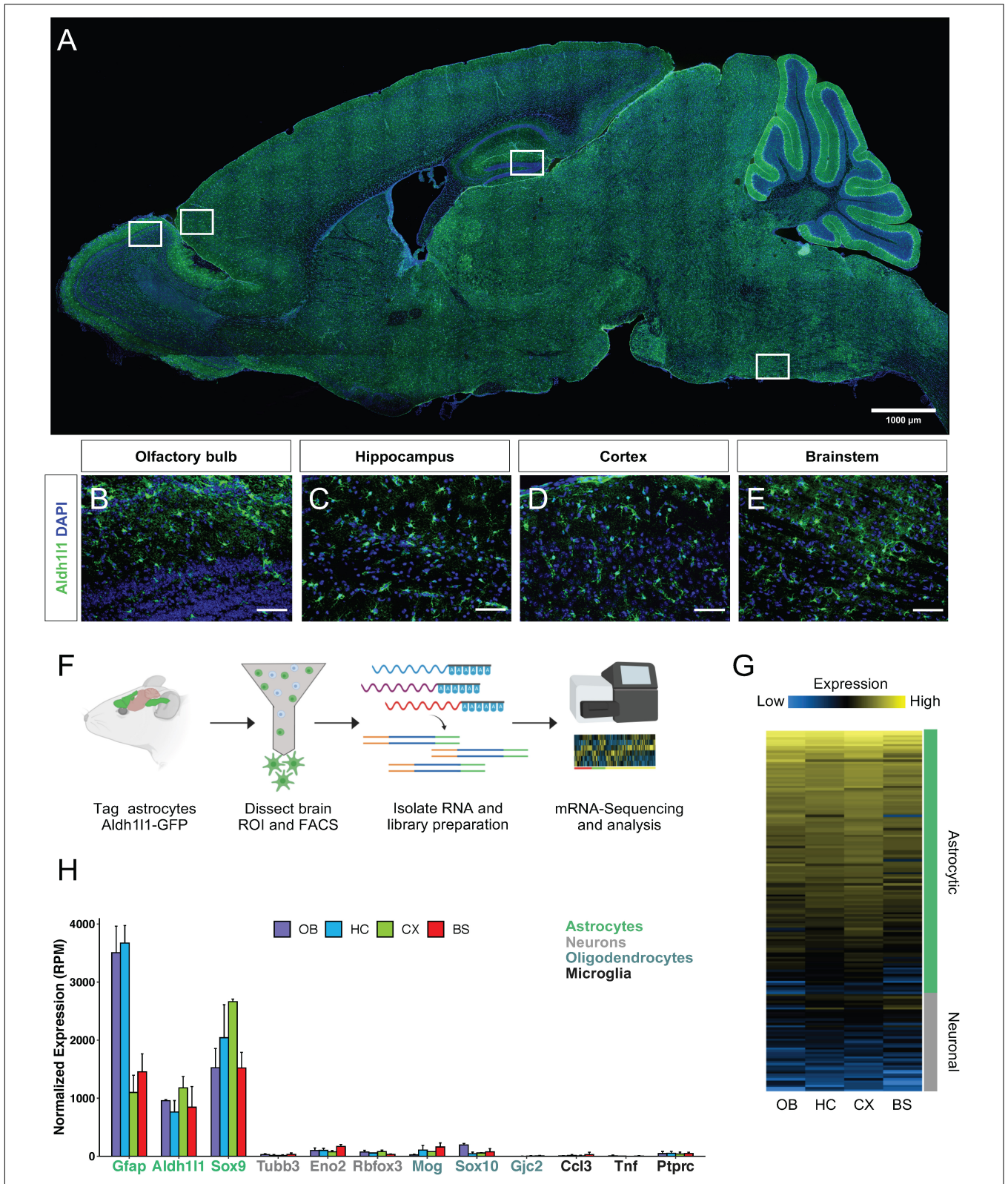

FIGURE 1 | Isolation and verification of Aldh1l1-eGFP astrocytes from selected regions. **(A–E)** Validation of Aldh1l1-eGFP cell specificity through immunofluorescence in, **(A)** whole brain, **(B)** olfactory bulb, **(C)** hippocampus, **(D)** cortex, and **(E)** brainstem. **(F)** Schematic of the approach used to investigate astrocyte regional diversity in the adult mouse brain. **(G)** Validation of cell identity through normalized expression of astrocyte and neuron specific genes. **(H)** Gene expression levels (in reads per million; RPM) of markers for astrocytes, neurons, oligodendrocytes, and microglia. Scale bar = 100 µm. OB, olfactory bulb; HC, hippocampus; CX, cortex; BS, brainstem.

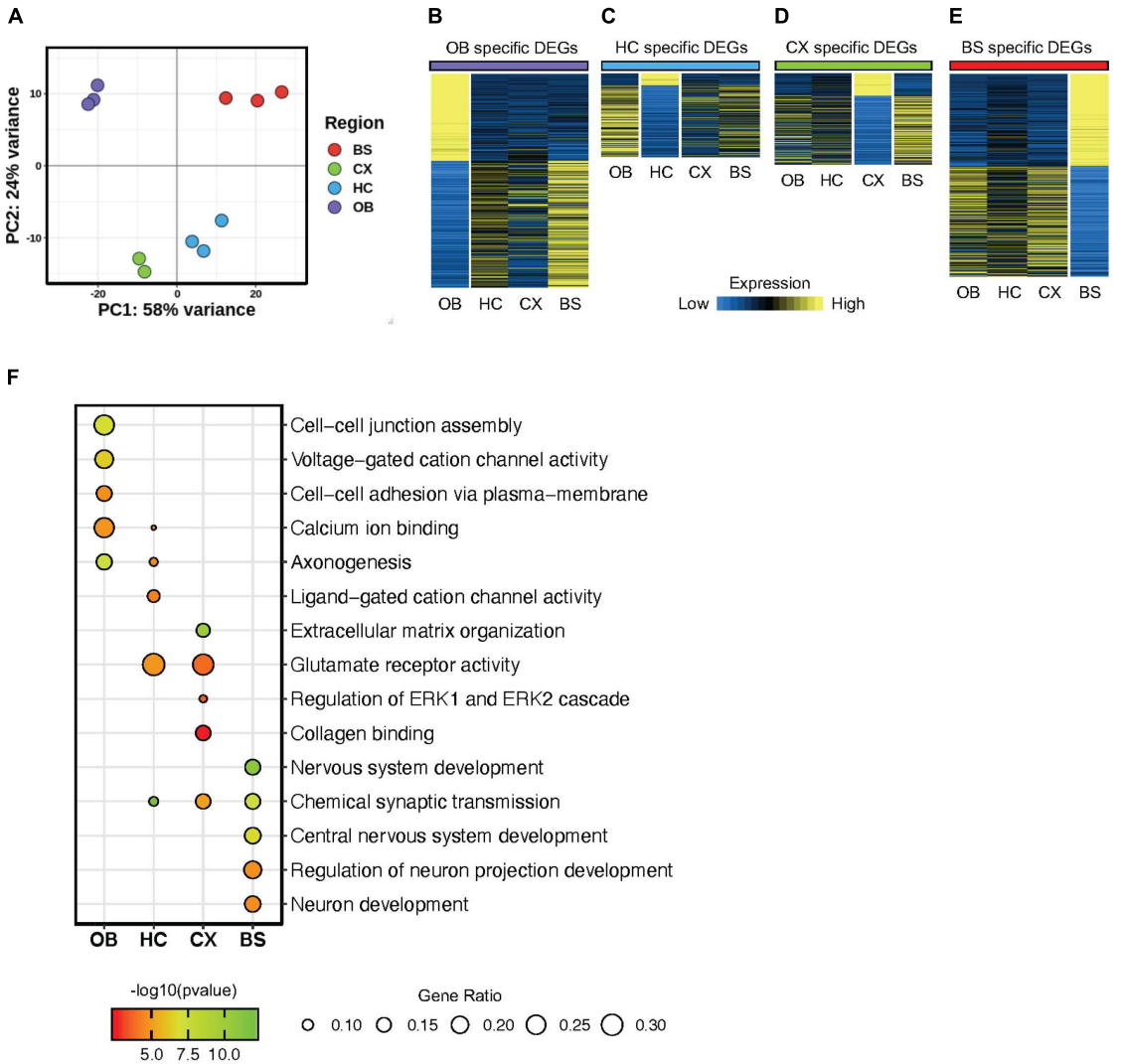

FIGURE 2 | Astrocytes display regionally distinct gene signatures. **(A)** Principal component analysis plot from RNA-Seq results of four brain regions. **(B–E)** Heatmap showing differentially expressed genes from each region **(B)** OB DEGs; purple, **(C)** HC DEGs; blue, **(D)** CX DEGs; green, **(E)** BS DEGs; red p-value < 0.01 for all regions. **(F)** Gene Ontology analysis plot of regional differentially expressed genes.

region we determined how many genes from each regional signature had an Nkx2-2, Maz, and NFI binding site within 10 kb of the transcriptional start site. The Nkx2-2 motif was identified in 67% of DEGs from each region. The Maz motif was most enriched in the HC DEGs but was still present in 77% of DEGs from each of the other three regions. The NFI motif was the most frequently identified of the three transcription factors in all the regions with its binding sequence appearing in at least 96% of DEGs from each region (**Figure 3E**). We also determined GO categories associated with the genes containing these universally conserved transcription factor binding sites to gain insight into the pathways or biological processes through which they may act. As expected, genes containing these motifs are found in ontology categories centralized around brain development and astrocyte function (**Figure 3F**).

Our *in silico* analysis suggests that NFI transcription factors operate in mature astrocytes to regulate the expression of key genes associated with synaptic physiology. Moreover, NFI transcription factors exhibit a significantly higher motif frequency in all regional DEGs; therefore, we set out to validate expression of the NFI family members in adult astrocytes from all four regions. Since NFIA is known to be expressed in adult astrocytes (Laug et al., 2019), we sought to confirm expression of the other NFI family members: NFIB and NFIX. Using immunohistochemistry, we found that NFIB and NFIX co-localize with Aldh1l1-eGFP astrocytes in all four brain regions (**Figure 3G**). We find that NFIB and NFIX expression is equally widespread across all four brain regions and can be found in approximately 86% of Aldh1l1-eGFP expressing astrocytes throughout the brain (**Figure 3H**). Furthermore, consistent with

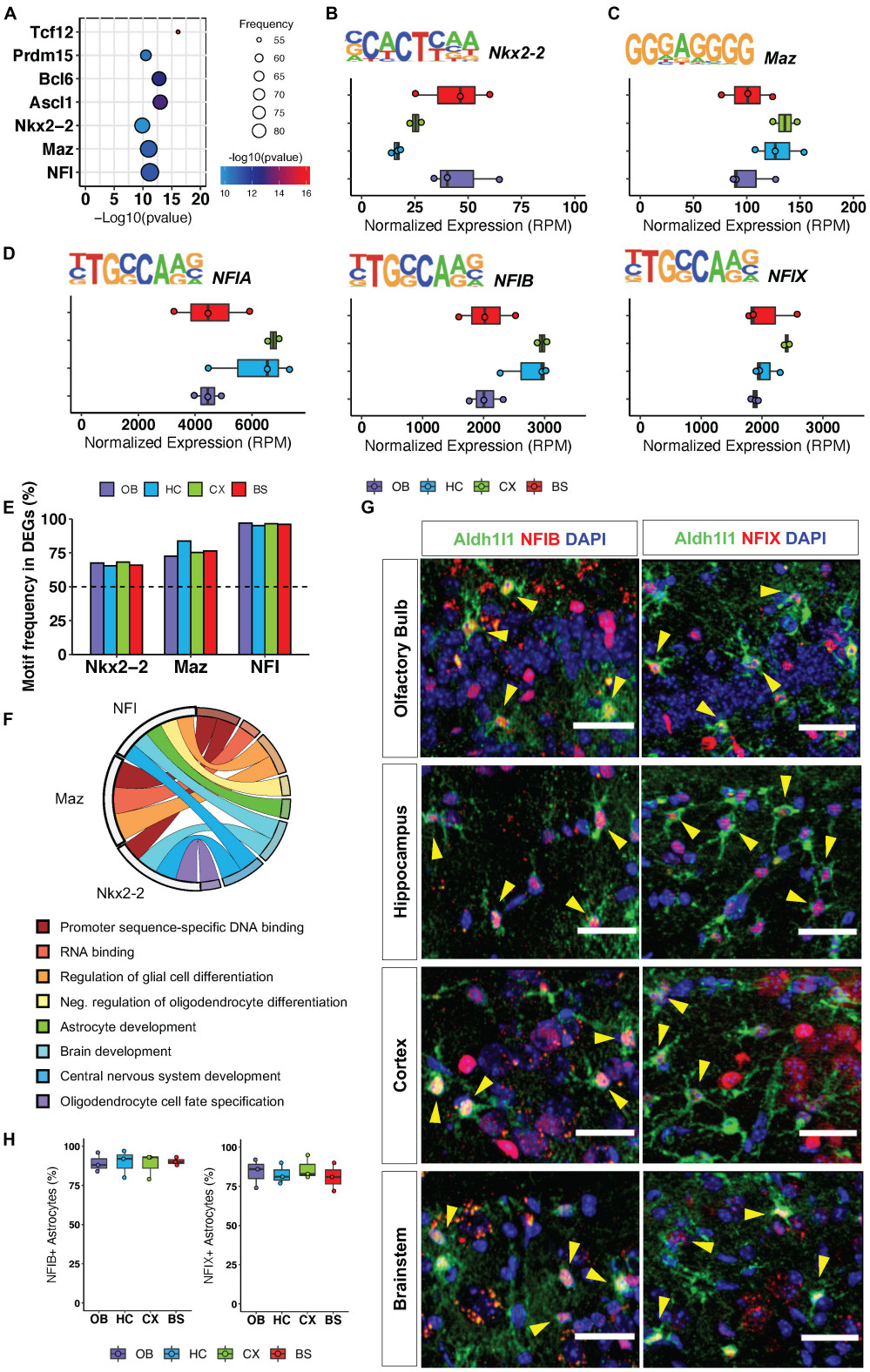

FIGURE 3 | Regional gene signatures exhibit universally conserved transcription factor motif enrichment. **(A)** Significant transcription factor motifs enriched in all regional DEGs. **(B–D)** Motif sequence and normalized expression of top 3 most significantly enriched motifs across surveyed brain regions **(B)** Nkx2-2, **(C)** Maz, **(D)** NFIA/NFIB/NFIX. **(E)** Frequency of motif occurrence in each region DEGs **(F)** Statistically significant Gene Ontologies associated with each transcription factor. p-value < 0.05. **(G)** Representative images of immunofluorescence staining of Aldh1l1-eGFP (green) and NFIB or NFIX (red). Yellow arrows indicate double positive cells. **(H)** Quantification of immunofluorescence staining. $n = 3$ scale bar = 50 μm.

previous findings (Chen et al., 2017), we also observed expression of NFIB and NFIX in neurons, though not to the same extent as their astrocytic expression. Taken together, these analyses suggest the presence of conserved transcription factor programs across diverse brain regions that function to maintain expression of genes that regulate core astrocytic functions.

Astrocytes Exhibit Region-Specific Transcription Factor Signatures

Thus far, our observations indicate the existence of transcription factors that are universally expressed in nearly all adult astrocytes, across a host of diverse brain regions. Given that transcription factor patterning has been shown to define distinct regional expression profiles in the spinal cord (Liu et al., 2003; Hochstim et al., 2008) and because our cross-region comparisons identified unique molecular profiles for each brain region (**Figures 2B–E**), we next sought to determine whether astrocytes from these distinct regions also exhibit unique transcription factor expression profiles. Toward this, we first analyzed the DEGs from each region individually for transcription factor motif enrichment. After compiling a list of known motif sequences enriched in each region, we filtered the list so that only significantly upregulated transcription factors were considered to determine which, if any, were enriched in only one region (**Figure 4A**). Using a fold change threshold of 2 at $p < 0.01$ we were able to identify uniquely enriched transcription factors in the OB, CX, and BS (**Figures 4B–E**). The HC did not show any significant transcription factor enrichment owing to the fact that it did not exhibit a robust DEG profile (**Figure 4C**). In the OB and BS, several transcription factors were significantly upregulated, but only the most highly upregulated transcription factor was used for downstream networking analysis and validation. We found Nkx3-1 in the OB (**Figure 4B**) and Nkx6-1 in the BS (**Figure 4E**), both of which were almost exclusively expressed in the region of interest. In the CX, we found that Pgr was detectable in all four regions but was expressed more than 4-fold in the CX compared to other regions (**Figure 4D**). These

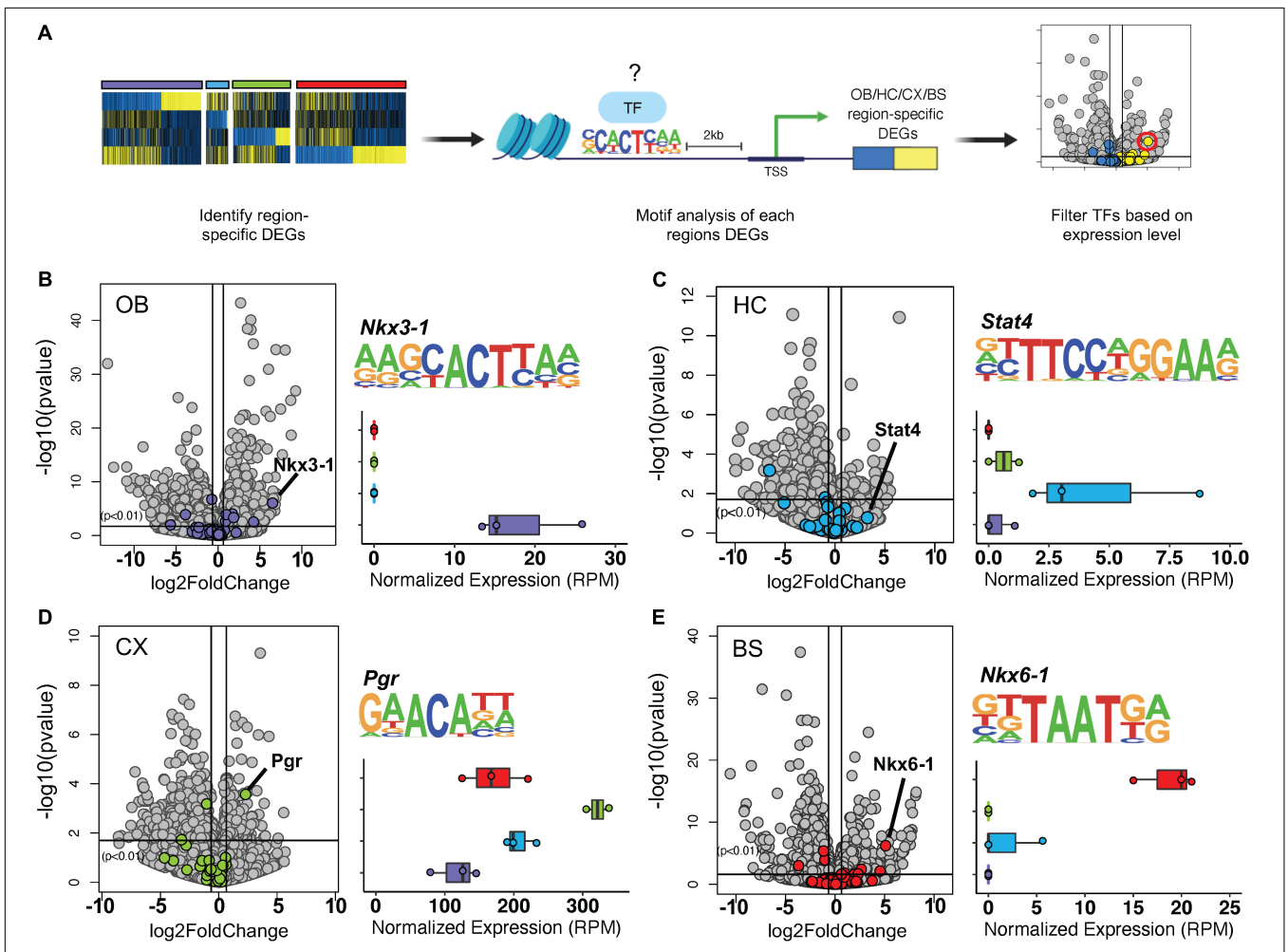

FIGURE 4 | Transcription factor motif enrichment is regionally distinct. **(A)** Schematic of bioinformatic approach to identify region-specific transcription factors. **(B–E)** Volcano plot showing significance versus log2 fold change. Transcription factor motifs enriched in each region's DEGs colored. Most upregulated statistically significant transcription factor motif sequence and normalized expression shown. **(B)** Nkx3-1 in OB, **(C)** Stat4 in HC, **(D)** Pgr in CX, **(E)** Nkx6-1 in BS. p-value < 0.01.

results suggest that astrocytes exhibit region-specific expression of transcription factors.

Next, we sought to validate our bioinformatics analyses by performing immunolabeling with antibodies to candidate transcription factors on brains from 16-week old Aldh1l1-eGFP mice. We found that the CX-specific transcription factor, Pgr, was expressed in neurons throughout the brain. However, Pgr co-localized with 51% (**Figure 5Q**) of Aldh1l1-eGFP expressing astrocytes only in the CX (**Figures 5A–H**), supporting our hypothesis that Pgr may regulate astrocytic molecular profiles in the CX. Antibody staining of Nkx6-1 revealed that it is expressed exclusively in the BS, where it co-localizes with 74% of Aldh1l1-eGFP astrocytes (**Figures 5I–P,R**). Interestingly, not all astrocytes expressed Pgr or Nkx6-1 in their respective regions, likely due to additional layers of local diversity (Lin et al., 2017). These results, in conjunction with our validation studies on NFI-family

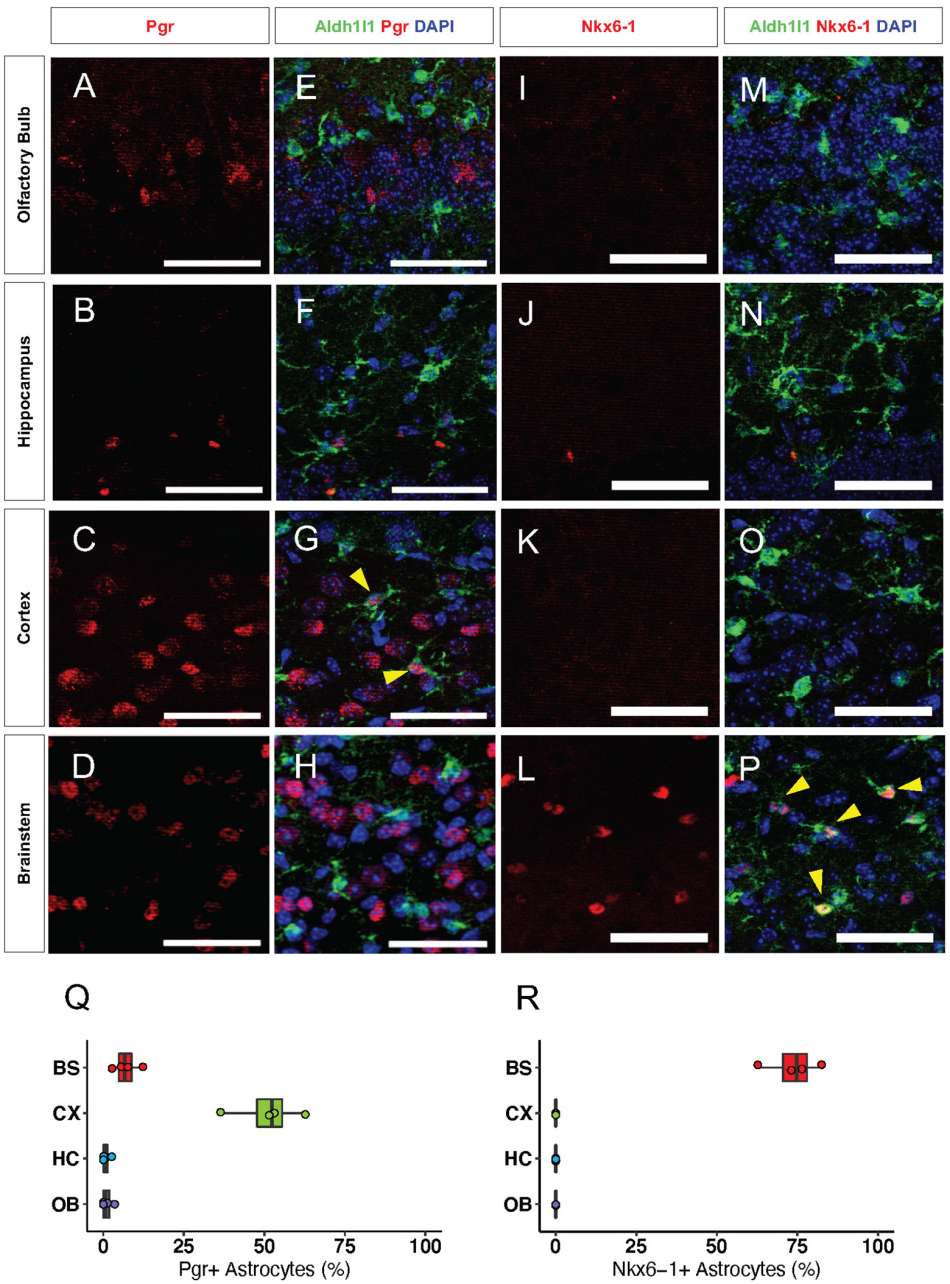

FIGURE 5 | Transcription factors are regionally specific *in vivo*. **(A–H)** Representative images of Immunofluorescence staining of Pgr (red) in the **(A)** OB, **(B)** HC, **(C)** CX, and **(D)** BS. **(I–P)** Antibody staining of Nkx6-1 (red) in the **(I)** OB, **(J)** HC, **(K)** CX, and **(L)** BS. **(E–H)** and **(M–P)** are the same panels as in **(A–D)** and **(I–L)**, respectively, but include Aldh1l1-eGFP expression (green). Yellow arrows indicate double positive cells. **(Q–R)** Quantification of immunofluorescence staining. **(Q)** Pgr and **(R)** Nkx6-1. $n = 4$ Scale bar = 50 μm.

members, indicate that astrocytes exhibit both universal and region-specific transcription factor expression profiles.

Predicted Targets of Regional Transcription Factors

It has been suggested that astrocytes maintain regional heterogeneity to afford them specialized functions for interacting with neurons in their specific regional circuitry units (Chung et al., 2015; Hasel et al., 2017). Region-specific transcription factor profiles may be a means to orchestrate these specialized profiles, therefore, we set out to investigate the region-specific transcriptional networks controlled by the above-mentioned transcription factors. Toward this, we sought to identify potential targets of the region-specific transcription factors identified above. First, we further curated the region-specific signatures, focusing on the location of the region-specific transcription factor motif sequence using HOMER, and relative expression of a given DEG (**Figure 6A**). A gene was considered a target if it had an instance of the motif sequence within 2 kb of the transcription start site and was significantly upregulated by at least a 2.5-fold at $p < 0.01$. Using this approach, we identified 66 predicted targets of Nkx3-1 in the OB, 45 for Nkx6-1 in the BS, and only 5 for Pgr in the CX. As there were no significantly enriched transcription factors identified in the HC it was not considered in subsequent analysis. The log2 transformed RPM of each transcription factor's targets are visualized in a heatmap (**Figure 6B**).

Next, to determine what biological pathways these potential targets were associated with we performed GO analysis (**Figure 6C**). Targets of each transcription factor were associated with distinct GO categories such as sensory perception of smell in the OB and pattern specification process in the BS. To determine if these regional transcription factors are actively regulating potential targets, we chose the top 3 most likely candidates from each region shown in **Figure 6D**. Since Nkx6-1 showed the most specific expression pattern in mouse brain (**Figures 5L,P**),

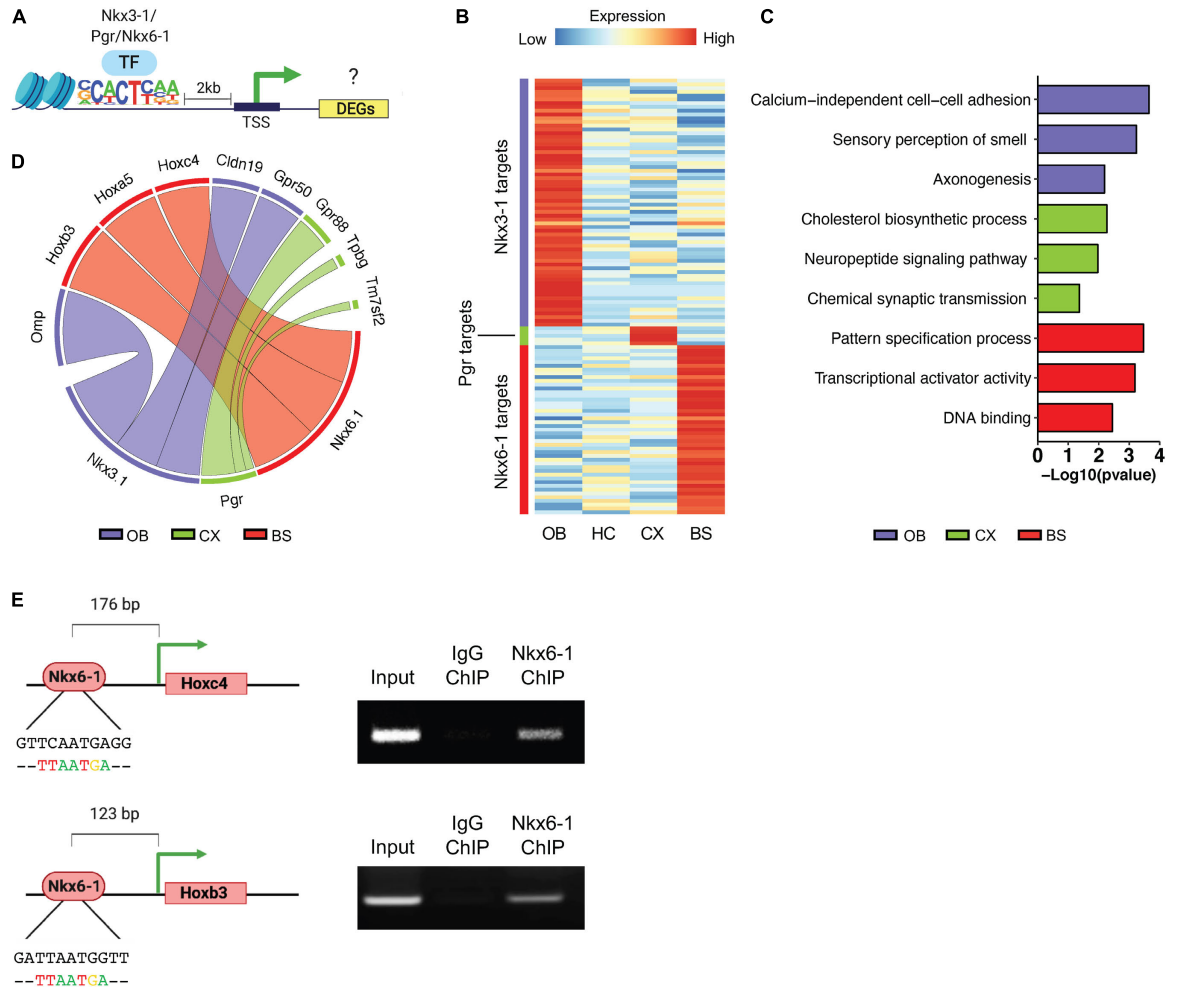

FIGURE 6 | Transcriptional regulatory network profiling. **(A)** Schematic of methodology for predicting transcription factor targets. **(B)** Heatmap showing log2 transformed expression of potential transcription factor targets. **(C)** Gene ontology analysis of predicted targets. **(D)** Circos plot to visualize top predicted targets by transcription factor, line width represents fold change. **(E)** ChIP-PCR Schematic and ChIP-PCR result of Nkx6-1 binding at Hoxc4 and Hoxb3 promoters in the brainstem.

we sought to validate the top targets of Nkx6-1 in the BS by ChIP-PCR. Among the top 3 most likely candidates, Hoxc4 and Hoxb3 were found to have a Nkx6-1 binding motif at their proximal promoters. We collected the BS from 16-week old mice, performed chromatin immunoprecipitation (ChIP) with Nkx6-1, and PCR amplified regions with the Nkx6-1 motif (**Figure 6E**). We confirmed binding of Nkx6-1 at the promoters of Hoxc4 and Hoxb3 in the adult BS (**Figure 6E**). Taken together, these data provide additional support that our bioinformatic pipeline can identify region-specific transcription factors that are active in manipulating the regional molecular landscape of astrocytes.

DISCUSSION

A Bioinformatic Approach to Study Astrocyte Heterogeneity

The molecular heterogeneity of astrocytes across diverse brain regions has been profiled extensively in recent years, but a clear understanding of the mechanisms that give rise to their vast diversity has long eluded us. In the present study we hypothesized that differential expression of transcription factors controls the region-specific molecular signatures observed in astrocytes across the brain. To test this, we analyzed the astrocyte transcriptomes across four brain regions to first establish DEGs in each region of interest that constitute region-specific gene signatures. Further analysis of region-specific DEGs revealed functionally redundant gene ontologies are associated with the unique gene profiles from each region. Together, these observations suggest that core astrocyte functions are achieved through distinct molecular mechanisms. This prompted us to search these signatures for transcription factors whose expression is conserved across all region-specific DEGs, as these transcription factors may regulate functionally redundant gene ontology pathways. Critically, we find the motif sequence of the NFI family of transcription factors enriched in DEGs and ubiquitous expression of NFIB/NFIX in astrocytes from all four regions (**Figure 3**). Finally, we identified transcription factors in the OB, CX, and BS whose expression is enriched in only the region of interest to regulate region-specific pathways in astrocytes. These results present a new bioinformatic approach to study astrocyte diversity through the lens of transcription factors and the essential regulatory mechanisms they offer.

Unique and Conserved Transcription Factors Regulate the Astrocyte Transcriptome

Transcriptomic analyses have indeed revealed unique region-specific astrocyte signatures that translate to spatially distinct functional differences (Chai et al., 2017; Morel et al., 2017). However, an important question remains regarding how regionally distinct astrocytes in the brain are endowed with these unique molecular and functional features. One explanation is that a homogenous population of astrocytes migrate throughout the brain during development, and after reaching their final location they develop region-specific molecular and function distinctions. Toward this, it has been suggested that astrocytes undergo molecular reorganization upon terminal migration to become specialized for interacting with neighboring neurons in their specific region (Molofsky et al., 2014; Chai et al., 2017). Indeed, studies have shown that astrocytes from different regions uniquely modify their molecular signatures upon loss of neuronal glutamatergic signaling (Morel et al., 2014) or activation of sonic hedgehog signaling from neighboring neurons (Farmer et al., 2016).

Another possibility is that these diverse features of astrocytes are developmentally pre-ordained, where molecularly distinct subpopulations of astrocytes are specified, and each subtype migrates to different locations where they maintain region-specific heterogeneity into adulthood. In support of this mechanism, it has been shown that astrocyte spatial identity (Tsai et al., 2012) and heterogeneity (Morel et al., 2017) is intrinsically defined by early embryonic dorsoventral axis patterning. Additionally, a combinatorial code involving differential expression of transcription factors during development was shown to specify astrocyte positional identity which results in distinct populations of astrocytes in the spinal cord (Hochstim et al., 2008). Whether astrocyte heterogeneity is specified in conjunction with developmental patterning or cultivated later according to regional circuitry requirements remains to be determined, but undoubtably transcriptional regulation plays a role in the complex quandary of astrocyte diversity.

Here, we ask if a transcription factor code can be defined for maintaining regionally distinct astrocyte populations. By interrogating region-specific DEGs our analysis revealed Nkx2-2, Maz and NFI family members as transcription factors that are universally conserved across the brain and regulate functionally redundant gene ontologies. Nkx2-2 is known to repress neurogenesis to promote oligodendrocyte precursor cell differentiation (Zhou et al., 2001). Nkx2-2 does not co-localize with the astrocytic marker GFAP (Qi et al., 2001), and enrichment of the its motif in adult astrocytes suggests that it likely represses these astrocytic genes during oligodendrocyte development. The second conserved regulator, Maz, has been shown to stimulate gliogenesis *in vitro* by regulating Notch signaling (Liu et al., 2016). Here, we chose to focus on the third regulator, the NFI family of transcription factors because its conserved motif sequence occurs most frequently in all regional gene profiles. Previously, NFIA and NFIB have been shown as necessary and sufficient to initiate gliogenesis (Deneen et al., 2006). Less is known about the role of NFIX in glia, although it has been suggested that NFIB can activate NFIX after the gliogenic switch to regulate terminal glial differentiation in the spinal cord (Matuzelski et al., 2017). Despite being characterized during developmental gliogenesis we find expression of all NFI family members in adult astrocytes. These data suggest a continued importance of gliogenic fate determinants in adult astrocytes, and it warrants further investigation.

Additionally, our data suggests that Nkx3-1, Pgr, and Nkx6-1 may act as region-specific transcription factors to regulate the unique molecular profiles observed in astrocytes across regions. The HC had the fewest DEGs compared to the other regions, making it more difficult to identify enriched transcription factors. However, the identification of region-specific transcription factors in the OB, CX, and BS suggests that the unique molecular signatures identified in each region may be maintained by Nkx3-1, Pgr, and Nkx6-1, respectively. Nkx3-1 expression was reported in the OB, but it has not been studied in astrocyte function or maintenance (Tanaka et al., 1999). Previous studies have shown that Pgr regulates Nrg1 to modulate synaptic activity and synaptogenesis in astrocytes (Lacroix-Fralish et al., 2006). It is widely accepted that Nkx6-1 is involved in specification (Zhao et al., 2014), patterning (Liu et al., 2003) and astrocyte positional identity (Hochstim et al., 2008) in the developing spinal cord. A role for Nkx6-1 in development has been well defined, but it has not been studied in adult astrocytes. Thus, we further validated the expression of Pgr and Nkx6-1 *in vivo* and found that Pgr is expressed in neurons throughout the brain, but colocalizes with astrocytes only in the cortex while Nkx6-1 expression is exclusive to the BS where it labels most astrocytes. These data supported our hypothesis that astrocyte regional heterogeneity is maintained by region-specific transcription factors. Furthermore, the region-specific expression of a developmental patterning factor suggests astrocyte diversity may be intrinsically specified during development and it merits investigation in future studies.

The Transcriptional Networks That Modulate Astrocyte Diversity

While understanding how astrocyte diversity is specified and maintained is important it is also critical to determine why they display such broad diversity. One likely explanation is that astrocyte diversity enables specialized interactions between astrocytes and the neuronal circuits of their spatial domain (Tsai et al., 2012). Indeed, when comparing astrocyte involvement in neural circuits from the hippocampus and striatum differences were observed between the two regions in potassium buffering, glutamate recycling, and calcium signaling, among others (Chai et al., 2017). These data highlight the importance of defining the transcriptional networks regulated by region specific transcription factors and translating molecular data into functional profiles of astrocyte heterogeneity.

To investigate the regulatory networks that may be controlled by these region-specific transcription factors we determined which genes they are regulating by predicted targets of Nkx3-1, Pgr, and Nkx6-1 in their respective brain regions. Nkx3-1 and Nkx6-1 had the largest number of potential targets and because Nkx6-1 showed the most exclusive region-specific expression in the BS, we confirmed direct regulation of one of its targets, Hoxc4 and Hoxb3, using ChIP-PCR. It is well documented that Hox genes follow distinct regional expression patterns and have been implicated in dorso-ventral patterning of the spinal cord and hindbrain (Gaufo et al., 2004; Di Bonito et al., 2013a). The role of Hox genes in the adult brain is not well understood, although studies show their expression can be detected in various regions throughout the adult brain (Hutlet et al., 2016). Given the specific expression patterns of Hox genes observed both during development (Di Bonito et al., 2013b) and in adulthood (Hutlet et al., 2016) these targets of Nkx6-1 hint at multilayered transcription factor regulation to control regional astrocyte diversity.

Our discovery of Nkx6-1 as a region-specific transcription factor in the adult brain, coupled with its established role in developmental patterning of astrocytes in the spinal cord, suggests that astrocytes in the brain are also subject to transcriptionally regulated patterning. These prospective patterning mechanisms could contribute to molecularly and functionally diverse populations of astrocytes throughout the brain. In summary, our study not only provides evidence of a region-specific transcription factor code through the identification of Nkx6-1, but also opens the door to identifying and characterizing astrocytic patterning transcription factors in the brain. In addition, our study defines a new approach to study astrocyte diversity by interrogating transcription factor profiles to provide insights into region-specific gene regulatory networks across the brain.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GEO repository, accession GSE143282.

ETHICS STATEMENT

The animal study was reviewed and approved by the Baylor College of Medicine IACUC.

AUTHOR CONTRIBUTIONS

BL and BD conceived the project. T-WH performed all the immunostaining. DS assisted with the writing and ChIP assay. AH bred the mice and assisted with FACS. BL performed all bioinformatics analysis.

FUNDING

This work was funded by the National Institutes of Health (grant numbers NIH NS071153 and NIH 1RF-AG054111) and National Multiple Sclerosis Society.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00061/full#supplementary-material

REFERENCES

Abbott, N. J., Rönnbäck, L., and Hansson, E. (2006). Astrocyte–endothelial interactions at the blood–brain barrier. *Nat. Rev. Neurosci.* 7:41. doi: 10.1038/nrn1824

Anderson, R. L., Jobling, P., and Gibbins, I. L. (2001). Development of electrophysiological and morphological diversity in autonomic neurons. *J. Neurophysiol.* 86, 1237–1251. doi: 10.1152/jn.2001.86.3.1237

Anthony, T. E., and Heintz, N. (2007). The folate metabolic enzyme ALDH1L1is restricted to the midline of the early CNS, suggesting a role in human neural tube defects. *J. Compar. Neurol.* 500, 368–383. doi: 10.1002/cne.21179

Bayraktar, O. A., Fuentealba, L. C., Alvarez-Buylla, A., and Rowitch, D. H. (2015). Astrocyte development and heterogeneity. *Cold Spring Harb. Perspect. Biol.* 7:a020362. doi: 10.1101/cshperspect.a020362

Cahoy, J. D., Emery, B., Kaushal, A., Foo, L. C., Zamanian, J. L., Christopherson, K. S., et al. (2008). A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J. Neurosci.* 28, 264–278. doi: 10.1523/JNEUROSCI.4178-07.2008

Cai, J., Zhu, Q., Zheng, K., Li, H., Qi, Y., Cao, Q., et al. (2010). Co-localization of Nkx6. 2 and Nkx2. 2 homeodomain proteins in differentiated myelinating oligodendrocytes. *Glia* 58, 458–468. doi: 10.1002/glia.20937

Chai, H., Diaz-Castro, B., Shigetomi, E., Monte, E., Octeau, J. C., Yu, X., et al. (2017). Neural circuit-specialized astrocytes: transcriptomic, proteomic, morphological, and functional evidence. *Neuron* 95, 531–549. doi: 10.1016/j.neuron.2017.06.029

Chen, E. Y., Tan, C. M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G. V., et al. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinform.* 14:128. doi: 10.1186/1471-2105-14-128

Chen, K.-S., Harris, L., Lim, J. W., Harvey, T. J., Piper, M., Gronostajski, R. M., et al. (2017). Differential neuronal and glial expression of nuclear factor I proteins in the cerebral cortex of adult mice. *J. Compar. Neurol.* 525, 2465–2483. doi: 10.1002/cne.24206

Chung, W.-S., Allen, N. J., and Eroglu, C. (2015). Astrocytes control synapse formation, function, and elimination. *Cold Spring Harb. Perspect. Biol.* 7:a020370. doi: 10.1101/cshperspect.a020370

Deneen, B., Ho, R., Lukaszewicz, A., Hochstim, C. J., Gronostajski, R. M., and Ander-Son, D. J. (2006). The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. *Neuron* 52, 953–968. doi: 10.1016/j.neuron.2006.11.019

Di Bonito, M., Glover, J. C., and Studer, M. (2013a). Hox genes and region-specific sensorimotor circuit formation in the hindbrain and spinal cord. *Dev. Dyn.* 242, 1348–1368. doi: 10.1002/dvdy.24055

Di Bonito, M., Narita, Y., Avallone, B., Sequino, L., Mancuso, M., Andolfi, G., et al. (2013b). Assembly of the auditory circuitry by a Hox genetic network in the mouse brainstem. *PLoS Genet.* 9:e1003249. doi: 10.1371/journal.pgen.1003249

Duran, R. C.-D., Wang, C.-Y., Zheng, H., Deneen, B., and Wu, J. Q. (2019). Brain region-specific gene signatures revealed by distinct astrocyte subpopulations unveil links to glioma and neurodegenerative diseases. *eNeuro* 6:e00288-19. doi: 10.1523/ENEURO.0288-18.2019

Ewels, P., Magnusson, M., Lundin, S., and Käller, M. (2016). Multiqc: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* 32, 3047–3048. doi: 10.1093/bioinformatics/btw354

Farmer, W. T., Abrahamsson, T., Chierzi, S., Lui, C., Zaelzer, C., Jones, E. V., et al. (2016). Neurons diversify astrocytes in the adult brain through sonic hedgehog signaling. *Science* 351, 849–854. doi: 10.1126/science.aab3103

Fu, Y., Rusznák, Z., Herculano-Houzel, S., Watson, C., and Paxinos, G. (2013). Cellular composition characterizing postnatal development and maturation of the mouse brain and spinal cord. *Brain Struct. Funct.* 218, 1337–1354. doi: 10.1007/s00429-012-0462-x

Gaufo, G. O., Wu, S., and Capecchi, M. R. (2004). Contribution of Hox genes to the diversity of the hindbrain sensory system. *Development* 131, 1259–1266. doi: 10.1242/dev.01029

Hasel, P., Dando, O., Jiwaji, Z., Baxter, P., Todd, A. C., Heron, S., et al. (2017). Neurons and neuronal activity control gene expression in astrocytes to regulate their development and metabolism. *Nat. Commun.* 8:15132. doi: 10.1038/ncomms15132

Herculano-Houzel, S. (2014). The glia/neuron ratio: how it varies uniformly across brain structures and species and what that means for brain physiology and evolution. *Glia* 62, 1377–1391. doi: 10.1002/glia.22683

Hochstim, C., Deneen, B., Lukaszewicz, A., Zhou, Q., and Anderson, D. J. (2008). Identification of positionally distinct astrocyte subtypes whose identities are specified by a homeodomain code. *Cell* 133, 510–522. doi: 10.1016/j.cell.2008.02.046

Huang, T.-W., Kochukov, M. Y., Ward, C. S., Merritt, J., Thomas, K., Nguyen, T., et al. (2016). Progressive changes in a distributed neural circuit underlie breathing abnormalities in mice lacking Mecp2. *Journal of Neuroscience* 36, 5572–5586. doi: 10.1523/JNEUROSCI.2330-15.2016

Hutlet, B., Theys, N., Coste, C., Ahn, M.-T., Doshishti-Agolli, K., Lizen, B., et al. (2016). Systematic expression analysis of Hox genes at adulthood reveals novel patterns in the central nervous system. *Brain Structure and Function* 221, 1223–1243. doi: 10.1007/s00429-014-0965-8

Jobling, P., and Gibbins, I. L. (1999). Electrophysiological and morphological diversity of mouse sympathetic neurons. *J. Neurophysiol.* 82, 2747–2764. doi: 10.1152/jn.1999.82.5.2747

Khakh, B. S., and Deneen, B. (2019). The emerging nature of astrocyte diversity. *Annu. Rev. Neurosci.* 42, 187–207. doi: 10.1146/annurev-neuro-070918-050443

Khakh, B. S., and Sofroniew, M. V. (2015). Diversity of astrocyte functions and phenotypes in neural circuits. *Nat. Neurosci.* 18:942. doi: 10.1038/nn.4043

Kuleshov, M. V., Jones, M. R., Rouillard, A. D., Fernandez, N. F., Duan, Q., Wang, Z., et al. (2016). Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res.* 44, W90–W97. doi: 10.1093/nar/gkw377

Lacroix-Fralish, M. L., Tawfik, V. L., Nutile-Mcmenemy, N., Harris, B. T., and Deleo, J. A. (2006). Differential regulation of Neuregulin 1 expression by progesterone in astrocytes and neurons. *Neuron Glia Biol.* 2, 227–234. doi: 10.1017/S1740925X07000385

Laug, D., Huang, T.-W., Huerta, N. A. B., Huang, A. Y.-S., Sardar, D., Ortiz-Guzman, J., et al. (2019). Nuclear factor I-A regulates diverse reactive astrocyte responses after CNS injury. *J. Clin. Invest.* 129, 4408–4418. doi: 10.1172/JCI127492

Lin, C.-C. J., Yu, K., Hatcher, A., Huang, T.-W., Lee, H. K., Carlson, J., et al. (2017). Identification of diverse astrocyte populations and their malignant analogs. *Nat. Neurosci.* 20:396. doi: 10.1038/nn.4493

Liu, B., Ma, A., Zhang, F., Wang, Y., Li, Z., Li, Q., et al. (2016). MAZ mediates the cross-talk between CT-1 and NOTCH1 signaling during gliogenesis. *Sci. Rep.* 6:21534. doi: 10.1038/srep21534

Liu, R., Cai, J., Hu, X., Tan, M., Qi, Y., German, M., et al. (2003). Region-specific and stage-dependent regulation of Olig gene expression and oligodendrogenesis by Nkx6. 1 homeodomain transcription factor. *Development* 130, 6221–6231. doi: 10.1242/dev.00868

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA -seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Matuzelski, E., Bunt, J., Harkins, D., Lim, J. W., Gronostajski, R. M., Richards, L. J., et al. (2017). Transcriptional regulation of Nfix by NFIB drives astrocytic maturation within the developing spinal cord. *Dev. Biol.* 432, 286–297. doi: 10.1016/j.ydbio.2017.10.019

Molofsky, A. V., Kelley, K. W., Tsai, H.-H., Redmond, S. A., Chang, S. M., Madireddy, L., et al. (2014). Astrocyte-encoded positional cues maintain sensorimotor circuit integrity. *Nature* 509:189. doi: 10.1038/nature13161

Morel, L., Chiang, M. S. R., Higashimori, H., Shoneye, T., Iyer, L. K., Yelick, J., et al. (2017). Molecular and functional properties of regional astrocytes in the adult brain. *J. Neurosci.* 37, 8706–8717. doi: 10.1523/JNEUROSCI.3956-16.2017

Morel, L., Higashimori, H., Tolman, M., and Yang, Y. (2014). Vglut1+ neuronal glutamatergic signaling regulates postnatal developmental maturation of cortical protoplasmic astroglia. *J. Neurosci.* 34, 10950–10962. doi: 10.1523/JNEUROSCI.1167-14.2014

Qi, Y., Cai, J., Wu, Y., Wu, R., Lee, J., Fu, H., et al. (2001). Control of oligodendrocyte differentiation by the Nkx2. 2 homeodomain transcription factor. *Development* 128, 2723–2733.

Tanaka, M., Lyons, G. E., and Izumo, S. (1999). Expression of the Nkx3. 1 homobox gene during pre and postnatal development. *Mech. Dev.* 85, 179–182.

Tsai, H.-H., Li, H., Fuentealba, L. C., Molofsky, A. V., Taveira-Marques, R., Zhuang, H., et al. (2012). Regional astrocyte allocation regulates CNS synaptogenesis and repair. *Science* 337, 358–362. doi: 10.1126/science.1222381

Yu, X., Taylor, A. M., Nagai, J., Golshani, P., Evans, C. J., Coppola, G., et al. (2018). Reducing astrocyte calcium signaling in vivo alters striatal microcircuits and causes repetitive behavior. *Neuron* 99, 1170–1187. doi: 10.1016/j.neuron.2018.08.015

Zhao, X., Chen, Y., Zhu, Q., Huang, H., Teng, P., Zheng, K., et al. (2014). Control of astrocyte progenitor specification, migration and maturation by Nkx6.1 *homeodomain* transcription factor. *PLoS One* 9:e109171. doi: 10.1371/journal.pone.0109171

Zhou, Q., Choi, G., and Anderson, D. J. (2001). The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2. 2. *Neuron* 31, 791–807. doi: 10.1016/S0896-6273(01)00414-7

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

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 02 April 2020
doi: 10.3389/fnins.2020.00223

Vinpocetine Protects Against Cerebral Ischemia-Reperfusion Injury by Targeting Astrocytic Connexin43 via the PI3K/AKT Signaling Pathway

Mingming Zhao[1], Shuai Hou[1], Liangshu Feng[1], Pingping Shen[1], Di Nan[1], Yunhai Zhang[2,3], Famin Wang[2,3], Di Ma[1] and Jiachun Feng[1]**

[1] Department of Neurology and Neuroscience Center, The First Hospital of Jilin University, Changchun, China, [2] Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences, Suzhou, China, [3] Jiangsu Key Laboratory of Medical Optics, Suzhou, China

Vinpocetine (Vinp) is known for its neuroprotective properties. However, the protective mechanism of Vinp against cerebral ischemia/reperfusion (I/R) injury should be further explored. This study was designed to investigate the neuroprotective effects of Vinp against oxygen-glucose deprivation/reoxygenation (OGD/R) injury *in vitro* and cerebral I/R injury *in vivo* and explore whether this mechanism would involve enhancement of astrocytic connexin 43 (Cx43) expression via the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway. *In vitro*, we detected astrocytic viability and extracellular nitric oxide by an assay kit, intracellular reactive oxygen species by a DCFH-DA probe, inflammation and apoptosis-related protein expression by immunofluorescence staining, and the astrocytic apoptosis rate by flow cytometry. *In vivo*, we measured the cerebral infarction volume, superoxide dismutase activity, malondialdehyde content, and the expression of inflammation and apoptosis-related proteins. The results indicated that Vinp ameliorated the detrimental outcome of I/R injury. Vinp attenuated astrocytic injury induced by OGD/R and reduced cerebral infarction volume and cerebral edema in rats with cerebral I/R injury. Moreover, Vinp reduced oxidative stress, inflammation, and apoptosis induced by cerebral I/R injury in brain tissues. Meanwhile, Vinp increased p-Cx43 and p-AKT expression, and the p-Cx43/Cx43 and p-AKT/AKT ratio, which was decreased by cerebral I/R injury. Coadministration of PI3K inhibitors LY294002 and BKM120 blunted the effects of Vinp. This study suggests that Vinp protects against cerebral I/R injury via Cx43 phosphorylation by activating the PI3K/AKT pathway.

Keywords: stroke, cerebral ischemia/reperfusion, oxygen-glucose deprivation/reoxygenation, vinpocetine, astrocyte, connexin 43, PI3K/AKT

OPEN ACCESS

Edited by:
Stefania Ceruti,
Università degli Studi di Milano, Italy

Reviewed by:
Benjamin Deneen,
Baylor College of Medicine,
United States
Pavel Honsa,
Nebion AG, Czechia

***Correspondence:**
Di Ma
april8316@hotmail.com
Jiachun Feng
fengjcfrank@hotmail.com

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

Received: *18 October 2019*
Accepted: *28 February 2020*
Published: *02 April 2020*

Citation:
Zhao M, Hou S, Feng L, Shen P, Nan D, Zhang Y, Wang F, Ma D and Feng J (2020) Vinpocetine Protects Against Cerebral Ischemia-Reperfusion Injury by Targeting Astrocytic Connexin43 via the PI3K/AKT Signaling Pathway. Front. Neurosci. 14:223. doi: 10.3389/fnins.2020.00223

INTRODUCTION

Ischemic stroke has high morbidity and mortality and seriously affects patient quality of life (Ribeiro et al., 2015). Timely recovery of blood and oxygen supply to the ischemic brain tissue is essential for ischemic penumbra survival. Thrombolytic therapy is the best treatment option for ischemic stroke (Sheth et al., 2015). However, reperfusion aggravates the damage and provokes dysfunction through a cascade of events such as calcium overload, excitotoxicity, oxidative stress, inflammatory responses, and apoptosis, which are collectively termed "ischemia-reperfusion injury" (I/R injury) (Dirnagl et al., 1999). Therefore, effectively blocking the cascade of cerebral I/R injury and exploring effective drugs for the treatment of ischemic stroke are very important.

Astrocytes are abundant in the central nervous system, and they play essential roles in maintaining brain function under physiologic conditions and in influencing neuronal survival under pathologic conditions, such as cerebral I/R injury and other brain insults (Garman, 2011; Falkowska et al., 2015; Verkhratsky et al., 2017). During ischemic stroke, astrocytes may be activated and produce and release reactive oxygen species (ROS), pro-inflammatory cytokines, and other factors that may negatively influence the survival of neurons in the penumbra (Swanson et al., 2004). Thus, preventing astrocytic inflammatory and apoptotic effects may be a promising strategy for neuroprotection in ischemic stroke (Cekanaviciute and Buckwalter, 2016; Choudhury and Ding, 2016; Liu and Chopp, 2016).

The PI3K/AKT signaling pathway regulates a wide range of cellular functions, including cellular differentiation, proliferation, inflammation, and apoptosis (Cantley, 2002). Studies have shown that phosphorylation of AKT (Ser473) reduces neuronal apoptosis caused by cerebral I/R injury (Fukunaga and Kawano, 2003; Zhao et al., 2006), and LY294002-mediated inhibition of the PI3K/AKT pathway blocked the cardioprotective effect of atorvastatin against I/R injury in cardiocytes by downregulating Connexin 43 (Cx43) (Bian et al., 2015). Moreover, activated AKT can phosphorylate the C-terminal Ser373 residue of Cx43 (Solan and Lampe, 2014). Since Cx43 is the most commonly expressed gap junction protein in astrocytes (Orellana et al., 2011), and increased Cx43 expression can reduce neuronal damage after cerebral I/R (Nakase et al., 2003), we speculate that Cx43 is involved in the PI3K/AKT pathway's protective effects against cerebral I/R injury.

Vinpocetine (Vinp) is a semi-synthetic alkaloid derivative isolated from the leaves of *Phyllostachys pubescens*. Its anti-inflammatory and anti-platelet aggregation effects on improving cerebral blood flow, brain metabolism, and cognition have been confirmed by various studies (Zhang et al., 2018; Zhang et al., 2018). Vinp has been widely used in the treatment of stroke, cerebral arteriosclerosis, and chronic cerebral insufficiency, and it exhibits unique advantages in the treatment of dementia and epilepsy. A previous study showed that Vinp similarly decreased the inflammatory response by inhibiting NF-κB and TNF-α expression after cerebral I/R injury (Wang et al., 2014); however, its specific mechanism remains unknown. Cerebral I/R injury can activate both astrocytes and microglia, which may produce inflammatory cytokines and other toxic mediators (Kim et al., 2016; Duris et al., 2018). Microglial TLR4/MyD88/NF-κB has been shown to be one of the mechanisms by which Vinp protects against cerebral I/R injury (Wu et al., 2017). However, so far, no study has focused on whether Vinp's protective effects against cerebral I/R injury is related to astrocytes. Hence, we hypothesized that Vinp may affect astrocytic Cx43 via the PI3K/AKT pathway and thereby provide neuroprotection.

In this study, we explored the neuroprotective roles of vinpocetine against oxygen-glucose deprivation/reoxygenation (OGD/R) injury *in vitro* and cerebral I/R injury *in vivo* and explore whether this mechanism would involve enhancement of astrocytic connexin 43 (Cx43) expression via the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway.

MATERIALS AND METHODS

Animal Care

The experiments adhered to the ethical standards of the Institutional Animal Care Committee and were approved by the Animals Ethics Committee of Jilin University. Male Wistar rats (250–280 g) and newborn rats were obtained from the Experimental Animal Center of Jilin University. Animals were maintained in a specific pathogen-free animal breeding room at 24°C under a 12 h day/night cycle with free access to water and food. All possible measures were taken to avoid animals suffering at each stage of the experiment.

Primary Rat Astrocytic Culture

Astrocytes were obtained from the cerebral cortex of newborn rats as previously described (Schildge et al., 2013). Newborn Wistar rats were decapitated, and the cerebral cortices were isolated in cold Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (DMEM/F12) medium. Then, the meninges were carefully removed, and the tissues were treated with 0.125% trypsin solution for 15 min at 37°C. DMEM/F12 containing 10% fetal bovine serum (FBS) was added, and the mixture was centrifuged at 1300 rpm for 5 min. The sediment was resuspended with DMEM/F12 containing 10% FBS. At a concentration of 10^5/ml, cells were planted onto 75 cm^2 flasks in 15 ml DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin and placed in an incubator (Thermo Scientific, Waltham, MA, United States) at 37°C with 95% air and 5% CO_2. After 24 h, the medium was changed in the flasks, and then half of the medium was changed every 3 days. After approximately 12 days, the astrocytic cultures reached confluency. Oligodendrocytes and microglia were deprived from astrocytic cultures by shaking on an orbital shaker for 6 h at 37°C (Schildge et al., 2013). The astrocytic cultures were treated with 0.25% trypsin solution for 3 min at 37°C. Then, the cells were harvested, and they were adjusted to a density of 2×10^5 cells/ml and planted on flasks. The third generation of primary cultured astrocytes were used in our study. The purity of astrocytes was higher than 95%, as confirmed by immunofluorescence staining with a specific marker, the glial fibrillary acidic protein (GFAP)

(ab7260, Abcam, United States). A representative result is shown in **Supplementary Figure S1A**.

Oxygen-Glucose Deprivation/Reoxygenation (OGD/R) *in vitro* Model

As described previously (Ferrer-Acosta et al., 2017), oxygen-glucose deprivation/reoxygenation (OGD/R) is a classic *in vitro* model of I/R injury. Briefly, astrocytes were washed three times with glucose-free DMEM and cultured in the same medium in a hypoxia chamber with a mixture of 95% N_2 and 5% CO_2 for 12 h. Then, the astrocytes were cultured in normal DMEM medium and re-oxygenated under normoxic conditions (95% air, 5% CO_2) for 6 h.

The astrocytic cultures were divided into five groups: (1) a control group, stimulated with DMSO; (2) an OGD/R group, stimulated with DMSO during OGD/R injury; (3) an OGD/R + Vinp group, stimulated with Vinp (30 μM) (Gedeon Richter Pharmaceutical Co., Ltd., Budapest, Hungary) during OGD/R injury; and (4) an OGD/R + Vinp + LY group, stimulated with LY294002 (20 μM) (ab120243, Abcam, Cambridge, MA, United States) and Vinp during OGD/R injury; (5) OGD/R + Vinp + BKM group, stimulated with BKM120 (2 μM) (S2247, Selleck, Houston, TX, United States). LY and Vinp were dissolved in DMSO at a final concentration of 100 mM (Hong et al., 2013; Takac et al., 2013; Nivison-Smith et al., 2015), and BKM was dissolved in DMSO at a final concentration of 10 mM. As described above, all groups were stimulated with the same volume of DMSO, and for the control group 0.33% DMSO proved to have no obvious toxicity on astrocytes (**Supplementary Figure S1B**).

Cell Viability and Cytotoxicity Assay

Commercial cell counting Kit-8 (CCK-8) (Do-jindo, Kumamoto, Japan) was used to detect cell viability (Ishiyama et al., 1997). Primary astrocytes cultured to the third generation were seeded in 96-well plates at a density of 10^4/well. The 96-well plates were placed in a cell culture incubator for 24 h before being subjected to OGD/R. Thereafter, 10 μL CCK-8 reagent was added to each well. The 96-well plates were then placed in the cell culture incubator for 2 h, and the absorbance at 450 nm was measured by a microplate reader (Multiskan, Thermo Scientific, Waltham, MA, United States).

Cytotoxicity was determined by measuring the lactate dehydrogenase (LDH) of the cell culture supernatant using the Cytotoxicity Detection Kit (C0016, Beyotime, Shanghai, China) according to the manufacturer's instructions (Lobner, 2000). Briefly, the sample maximum enzyme activity control wells were set according to the instructions. Astrocytic supernatants from each group were centrifuged. In each well of the 96-well plate was added 120 μL supernatant and 60 μL reagent. Then, the 96-well plate was incubated at room temperature for 30 min in the dark, and the absorbance at 490 nm was measured by a microplate reader. Experiments were repeated five times, and each experiment contained five duplicate wells for each astrocyte group.

Detection of Intracellular ROS and Extracellular NO

The ROS Assay kit (S0033, Beyotime) was used to detect ROS in astrocytes (Eruslanov and Kusmartsev, 2010). Briefly, astrocytes were seeded at a density of 104 cells/well in 96-well plates. After exposure to OGD/R injury, 10 μM of DCFH-DA in serum-free DMEM medium was added to each well. After incubation for 30 min in the cell culture incubator, each well was washed three times with serum-free DMEM and examined by a microplate reader using excitation/emission wavelengths of 488/525 nm.

Astrocytic Nitric oxide (NO) release was detected using the NO Assay Kit (S0021, Beyotime) (Weissman and Gross, 2001). Astrocytes were seeded in 96-well plates. A total of 50 μL/well of Griess Reagent I and 50 μL/well of Griess Reagent II were added into each well immediately after the astrocytes were exposed to OGD/R injury. The standard curve was constructed according to the instructions. The absorbance at 540 nm was measured by a microplate reader.

Astrocytic Immunofluorescence Analysis

Astrocytes were fixed with 4% paraformaldehyde at room temperature for 30 min and washed three times with PBS. After permeabilization with 0.2% Triton X-100 for 10 min and blocking with 10% goat serum in PBS for 1 h, the cells were incubated with rabbit anti-IL-1β (ab9722, Abcam; 1: 100), anti-TNF-α (ab66579, Abcam; 1: 100), anti-Bcl-2 (ab194583, Abcam; 1: 50), and anti-caspase-3 antibodies (ab13847, Abcam; 1:50) overnight at 4°C, followed by incubation with goat anti-rabbit IgG Fc (Alexa Fluor 647, ab150091, Abcam; 1:200) for 2 h at 25°C. The cells were then incubated with DAPI for 5 min and examined under a fluorescence microscope (OLYMPUS BX51, Tokyo, Japan).

Astrocytic Apoptosis Assay

Apoptosis was assessed by flow cytometry using an FITC Annexin V Apoptosis Detection Kit I (556547, Becton Dickinson, Franklin Lakes, NJ, United States) according to the manufacturer's instructions (Frey, 1997). Briefly, cells were rinsed with ice-cold PBS and then resuspended in 100 μL binding buffer (10^5 cells). A total of 5 μL Annexin V and 5 μL PI were added to each sample, and they were incubated for 15 min at 25°C in the dark. Then, 400 μL binding buffer was added to each tube and cells were immediately analyzed using a FACSC-LSR (Becton Dickinson) and evaluated with the Flow Jo 7.6 software.

Middle Cerebral Artery Occlusion (MCAO) Model and Animal Grouping

The MCAO model, a classic *in vivo* model of I/R injury, was prepared as previously described (Longa et al., 1989). Briefly, Wistar rats were anesthetized with chloral hydrate (350 mg/kg, i.p.). Then, a midline incision in the neck was made to expose the left external and internal arteries (ECA and ICA). The ECA was cut between two ligations, and a 0.26 mm silicone-tipped filament (2636, Xinlong Inc., Beijing, China) was inserted into the ICA via the ECA at approximately 20 mm until a resistance was felt, which ensured the occlusion of the MCA. Then, the suture was tightened around the ECA stump and the incision was closed.

After surgery, rats remained in the cage for 2 h. Then, the animals were anesthetized again, and the filament was removed. During surgery, the rats' body temperature was maintained at a normal level by heating pads. After awakening, the rats were maintained in cages with free access to food and water for 12 h.

A total of 64 male Wistar rats were randomly divided into four groups: (1) a sham group: the rats were injected with 0.9% normal saline and were not subjected to MCAO; (2) an I/R group: the rats were injected with 0.9% normal saline and subjected to MCAO; (3) a Vinp + I/R group: the rats were injected with Vinp (10 mg/kg) and subjected to MCAO; and (4) a Vinp + I/R + LY group: the rats were initially injected with LY294002 (0.3 mg/kg) and then with Vinp 15 min later and subjected to MCAO. All injections were administered intraperitoneally 30 min prior to MCAO.

Neurological Evaluation

Neurological evaluation was performed after 2 h of ischemia and 12 h of perfusion by a researcher blinded to the experimental groups. Evaluation was performed using a modified form (Longa et al., 1989) as follows: (0) no deficits; (1) difficulty to fully extend the contralateral forelimb; (2) inability to extend the contralateral forelimb; (3) mild circling to the contralateral side; (4) severe circling; and (5) falling to the contralateral side. Finally, the rats were anesthetized and decapitated for the brain water content assay, TTC staining, western blot, immunofluorescence, SOD activity, and MDA content analyses.

TTC Staining

2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, MI, United States) staining was used to visualize the ischemic infarction (Bederson et al., 1986). After decapitation, the brains were sliced into 2 mm sections, and each slice was incubated in a 2% solution of TTC at room temperature for 20 min and fixed in 4% paraformaldehyde. The brain sections were photographed using a high-resolution digital camera (Olympus). The infarct size was measured using the Image J software (NIH Image, National Institutes of Health, Bethesda, MD, United States). The percentage of the infarction size was calculated as described previously (Jackman et al., 2011).

Brain Water Content Assay

The classic wet-dry method was used to measure brain water content (Agrawal et al., 1968). Immediately after the rats were sacrificed, the brains were taken and weighed to obtain the wet weight. The samples were then dried in an oven at 100°C for 48 h. They were then weighed again to obtain the dry weight. Water content = (wet weight−dry weight)/wet weight × 100%.

Measurement of SOD Activity and MDA Content

Commercially available detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to detect SOD activity and MDA content according to the manufacturer's instructions as previously described (Hou et al., 2016). Briefly, SOD activity was assessed using the xanthine oxidase method, and MDA content was measured with the thiobarbituric acid method. The samples were analyzed with a spectrophotometer (BioRad, San Diego, CA, United States).

Immunofluorescent Analysis of Brain Sections

After decapitation, the brains were harvested immediately, immersed into pre-chilled isopentane (Beijing Chemical Factory, Beijing, China), and placed inside a −80°C refrigerator for 10 min for snap-freezing. Then, the brains were embedded in optimum cutting temperature compound (Sakura Finetek Inc., Torance, CA, United States) and stored in the −80°C refrigerator. Subsequently, 10-μM section of the brain were obtained using a cryomicrotome (Leica, Nussloch, Germany). The sections were fixed with 4% paraformaldehyde at room temperature for 15 min and washed three times with PBS. After permeabilization with 1% Triton X-100 for 10 min and subsequent blocking with 10% goat serum in PBS for 1 h, the sections were incubated with mouse anti-GFAP (ab10062, Abcam, 1:500) and rabbit anti-TNF-α (ab66579, Abcam; 1: 200) overnight at 4°C, followed by incubation with Alexa Fluor 647-conjugated goat anti-mouse IgG (ab150115, Abcam; 1:200) and Alexa Fluor488-conjugated goat anti-rabbit IgG (ab150077, Abcam; 1:200) for 1 h at 25°C. Experimental negative control was a section without any primary antibody treatment. The slices were then incubated with DAPI for 5 min and examined under a confocal microscope (Leica TCS SP5, Nussloch, Germany).

Western Blot Analysis

The western blot analysis was conducted as previously described (Hou et al., 2016). The cortex in the same set of rats or the cultured astrocytes was crumbled and homogenized with ice-cold lysis buffer (RIPA: NaVO3: PMSF: NaF = 92:5:2:1). Proteins were extracted from the cerebral cortex tissue, and the protein concentrations were assayed. Each sample (50 μg) was loaded on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis apparatus and electrophoresis was carried out until the bromophenol blue dye reached the bottom of the gel. Then, the proteins were electro-transferred to polyvinylidene fluoride membranes, and the membranes were placed in 5% skim milk powder dissolved in TBS with 0.1% Tween-20 for 1 h. The membranes were incubated with anti-Cx43 (ab11370, Abcam; 1:1000), anti-p-Cx43 (PA5-37584, Thermo Fisher Scientific; 1:1000), anti-AKT (4691, Cell Signaling Technology, Danvers, MA, United States; 1:1000), anti-p-AKT (13038, Cell Signaling Technology; 1:1000), anti-IL-1β (ab9722, Abcam; 1: 1000), anti-TNF-α (ab66579, Abcam; 1: 1000), anti-IL-10 (ab9969, Abcam; 1:2000), anti-Bcl-2 (ab194583, Abcam; 1:500), anti-caspase-3 (ab13847, Abcam; 1:500), and anti-β-actin (ab13847, Abcam; 1:2000) antibodies diluted in 5% skim milk powder dissolved in TBST overnight at 4°C. The membranes were then washed with PBST and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h. The protein bands were quantified with the Quantitation One software (Bio-Rad Laboratories, Hercules, CA, United States).

Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM) from at least three independent experiments using Graphpad Prism 6 (Inc., San Diego, CA, United States). Analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* tests. $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ denoted the significance thresholds.

RESULTS

Vinp Increased Astrocytic Viability and Attenuated Astrocytic Injury Induced by OGD/R

To investigate the neuroprotective effects of Vinp in primary cultured astrocytes *in vitro*, we assessed cell viability in each group via the CCK-8 assay (**Figure 1A**). The results showed that approximately half of the astrocytes survived in OGD/R group compared with the control group (56.86 ± 2.62%, $P < 0.001$). Compared with OGD/R group, viability was significantly improved in astrocytes treated with OGD/R + Vinp (78.94 ± 2.78%, $P < 0.001$). However, this elevation was reversed in the OGD/R + Vinp + LY group compared with the OGD/R + Vinp group (61.77 ± 2.09%, $P < 0.001$). We also measured astrocytic injury by testing the amount of LDH released into the supernatant. This test showed that OGD/R injury significantly increased the release of LDH compared with that in the control group (35.77 ± 2.60% vs. 8.62 ± 0.75%, $P < 0.001$). Treatment with OGD/R + Vinp remarkably decreased the release of LDH compared with that in the OGD/R group (20.14 ± 1.99%, $P < 0.001$). In comparison with OGD/R + Vinp group, coadministration of LY294002, a PI3K inhibitor, resulted in an apparent increased release of LDH (38.63 ± 1.81%, $P < 0.001$). These findings indicated that Vinp could promote cell survival and reduce cell damage in astrocytes subjected to OGD/R, and the inhibition of the PI3K/AKT pathway could abolish the protection of Vinp.

Vinp Attenuated Oxidative Stress in Astrocytes Induced by OGD/R Injury

Increased ROS production is considered an initial step in OGD/R injury (Dirnagl et al., 1999). To examine the effect of Vinp on OGD/R injury-induced oxidative stress in astrocytes, we detected intracellular ROS and NO released into the extracellular supernatant (**Figures 1C,D**). The results showed that intracellular ROS and extracellular NO was significantly increased after OGD/R injury compared with the control group ($P < 0.001$), but this elevation was reversed in the OGD/R + Vinp group compared with the OGD/R group ($P < 0.01$). Compared with the OGD/R + Vinp group, intracellular ROS and extracellular NO in the OGD/R + Vinp + LY group was markedly increased ($P < 0.01$). The data revealed that Vinp decreased oxidative stress in astrocytes induced by OGD/R injury, which was attenuated by a PI3K/AKT pathway inhibitor (LY294002).

Vinp Alleviated Inflammatory Cytokine Expression in Astrocytes After OGD/R Injury

As previously mentioned, the large amounts of ROS generated during OGD/R can induce inflammation by activating astrocytes (Chan, 2001; Duris et al., 2018). To investigate the anti-inflammatory effects of Vinp, we used immunofluorescence staining to observe the expression of TNF-α and IL-1β (**Figures 1E,F**). OGD/R injury resulted in significant increase in astrocytic TNF-α and IL-1β expression compared with control group, and this increase was blocked by treatment with Vinp during OGD/R injury. Moreover, LY reversed the effect of Vinp on astrocytes subjected to OGD/R, significantly increasing TNF-α and IL-1β expression. The results indicated that the PI3K/AKT pathway is involved in the anti-inflammatory effects of Vinp against OGD/R injury.

Vinp Altered Apoptosis-Related Protein Expression and Reduced the Apoptotic Rate in Astrocytes After OGD/R Injury

As a cascade event, increased ROS may induce excessive inflammatory responses which could activate pro-apoptotic pathways (Duris et al., 2018), and therefore we evaluated the anti-apoptotic effects of Vinp. Annexin V FITC/PI staining and flow cytometry were used to detect the astrocytic apoptotic rate (**Figure 2A**), and immunofluorescence staining was processed to observe the expression of caspase-3 and Bcl-2 (**Figures 2B,C**). We found that OGD/R injury resulted in a significant increase in astrocytic caspase-3 expression, a significant reduction in Bcl-2 expression, and an increase in apoptotic rate compared to the control group ($P < 0.001$). Compared with the OGD/R group, caspase-3 expression and astrocytic apoptotic rate effectively decreased while Bcl-2 expression increased in the OGD/R + Vinp group ($P < 0.001$). Furthermore, results showed LY reversed the effect of Vinp on OGD/R-treated astrocytes, significantly increasing astrocytic caspase-3 expression, reducing Bcl-2 expression, and increasing apoptotic rate ($P < 0.001$). These results indicated that the PI3K/AKT pathway is involved in the anti-apoptotic effects of Vinp against OGD/R injury.

Vinp Decreased Brain Water Content and Infarction Size in Rats After Cerebral I/R Injury

Next, we prepared the classic MCAO models to evaluate the early effects of Vinp against cerebral I/R injury *in vivo*. As previously described, neurological deficit scores, brain water content, and infarction size were measured to evaluate the effects of Vinp against cerebral I/R injury after the rats were subjected to ischemia for 2 h and reperfusion for 12 h. In the I/R group, there were obvious symptoms of neurological deficits including deviation to the right, circling, and inability to fully extend the right upper limb. Treatment with Vinp did not significantly reduce the neurological deficit scores (**Figure 3A**, $P = 0.18$). The cerebral infarct size after MCAO is shown in **Figures 3B,C**. In the I/R group, significant cerebral infarction was observed

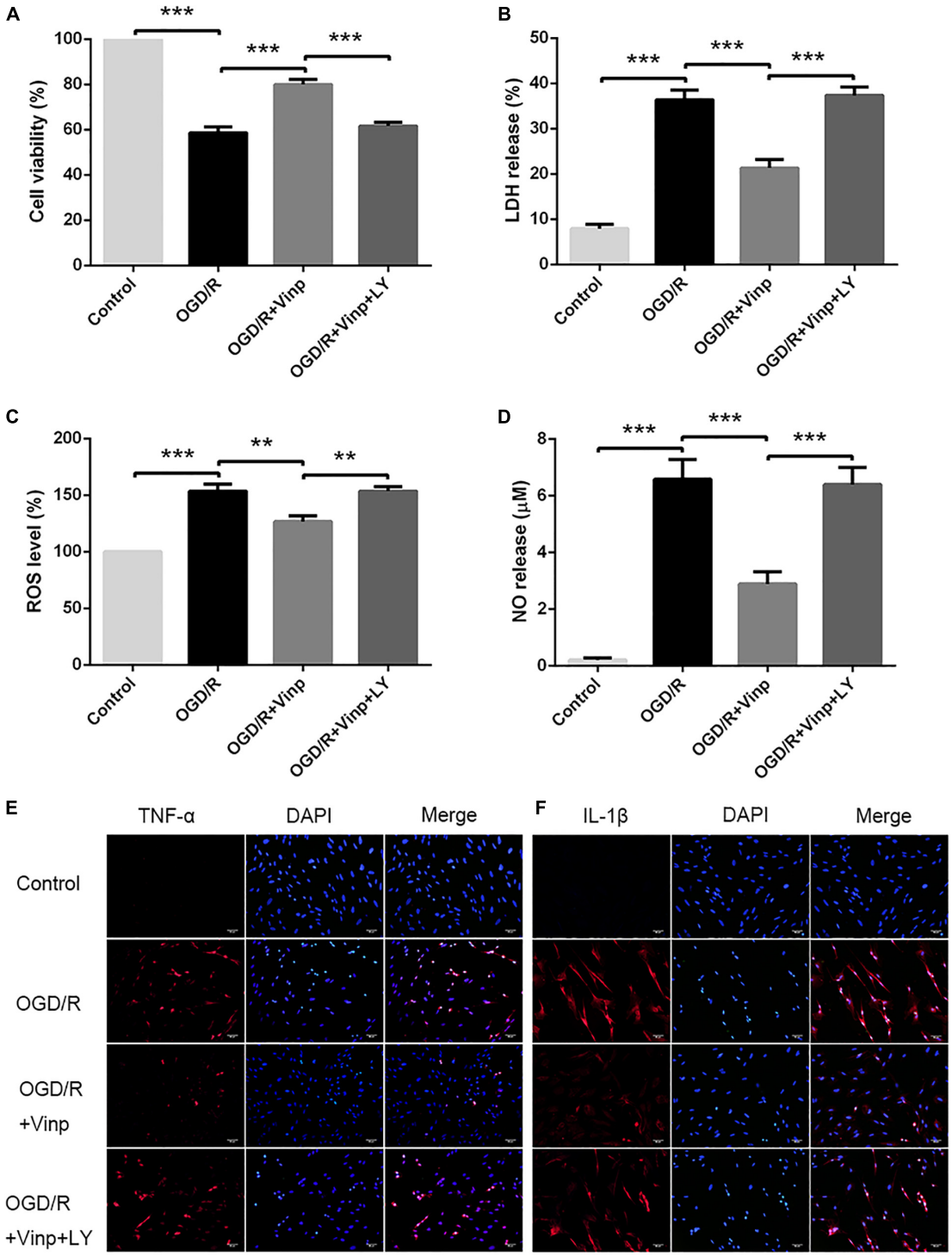

FIGURE 1 | Effects of Vinp on astrocytic viability, cytotoxicity, oxidative stress, and inflammation after OGD/R. **(A)** Astrocytic viability calculated as a percentage relative to the control group ($n = 5$ in each group). **(B)** LDH release calculated as a percentage relative to maximum enzyme activity control well ($n = 5$ in each group). **(C)** ROS levels calculated as a percentage relative to the control group ($n = 5$ in each group). **(D)** NO released into the supernatant evaluated by NO assay kit ($n = 5$ in each group). **(E)** Immunostaining showed the expression of TNF-α in astrocytes after OGD/R. **(F)** Immunostaining showed the expression of IL-1β in astrocytes after OGD/R, and the nuclei were counterstained with DAPI ($n = 3$ in each group). Scale bars = 50 μm. Data are shown as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ using one-way ANOVA followed by Tukey's *post hoc* tests. Vinp: Vinpocetine; LY: LY294002; OGD/R: oxygen-glucose deprivation/reoxygenation.

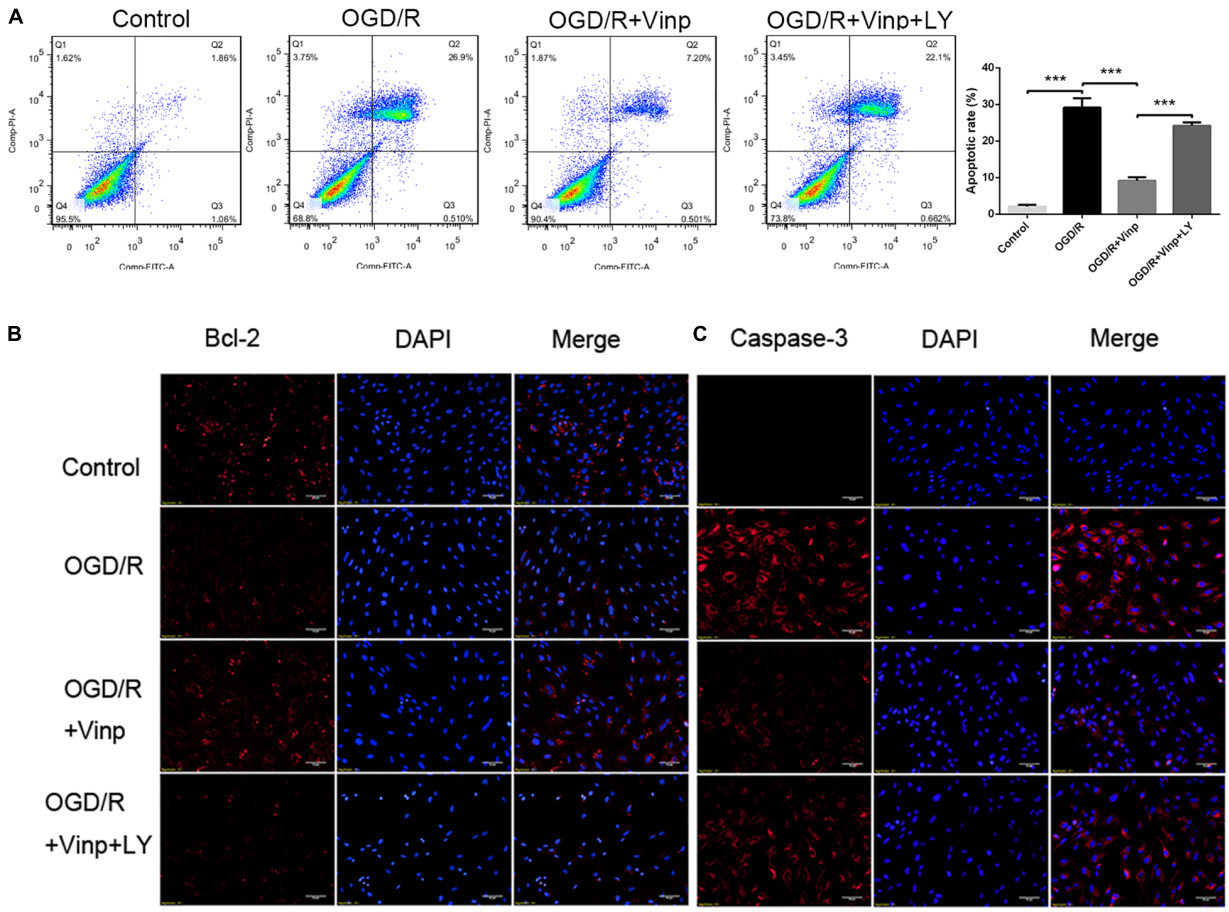

FIGURE 2 | Effects of Vinp on astrocytic apoptosis after OGD/R. **(A)** Immunostaining showed the expression of Bcl-2 and caspase-3 in astrocytes after OGD/R. **(B)** Immunostaining showed the expression of Bcl-2 and caspase-3 in astrocytes after OGD/R. The nuclei were counterstained with DAPI. Scale bars = 50 μm. **(C)** Representative flow cytometry images of apoptosis observed with Annexin V FITC/PI staining. **(D)** Apoptosis analysis calculated as a percentage relative to total cells. Data are shown as the mean ± SEM (n = 3 in each group). ***$P < 0.001$ using one-way ANOVA followed by Tukey's *post hoc* tests. Vinp: Vinpocetine; LY: LY294002; OGD/R: oxygen-glucose deprivation/reoxygenation.

compared with the sham group (32.92 ± 1.63%, $P < 0.001$), and this phenotypic alteration was mostly abrogated in the I/R + Vinp group compared with the I/R group (8.06 ± 1.10%, $P < 0.001$). Compared to the I/R + Vinp group, infarct size in the I/R + Vinp + LY group was significantly enlarged (28.04 ± 1.05%, $P < 0.001$). The results of the water content of brain tissues were consistent with the trend observed for infarction size (**Figure 3D**). The results revealed that Vinp decreased the infarction size and brain edema, while inhibition of PI3K/AKT reversed the protection of Vinp.

Vinp Attenuated Oxidative Stress in the Rat Cerebral Cortex After Cerebral I/R Injury

We further examined oxidative stress in ischemic cerebral cortices as the *in vitro* study, which is considered the initial step of cerebral I/R injury. SOD activity is an important antioxidant enzyme, and MDA content reflects oxidative damage (Chan, 2001), therefore, we examined SOD activity and MDA content (**Figures 3E,F**). Compared to the sham group, SOD activity significantly decreased, while MDA content increased in the I/R group ($P < 0.001$). Treatment with Vinp effectively increased SOD activity and decreased MDA content compared with the I/R group ($P < 0.001$), whereas LY reversed the effects of Vinp by decreasing SOD activity and increasing MDA content compared with the I/R + Vinp group ($P < 0.01$). The above findings suggested that Vinp attenuated oxidative stress induced by cerebral I/R injury, which is related to the PI3K/AKT pathway.

Vinp Reduced Inflammation and Apoptosis in the Rat Cerebral Cortex After Cerebral I/R Injury

To validate if the response cascade was caused by cerebral I/R injury, we examined the inflammation and apoptosis *in vivo*. First, we performed immunofluorescent analysis to observe the reactive astrocytes and inflammatory cytokine by double immunostaining the brain cryosections with anti-GFAP and anti-TNF-α antibodies (**Figure 4**). Cerebral I/R injury resulted

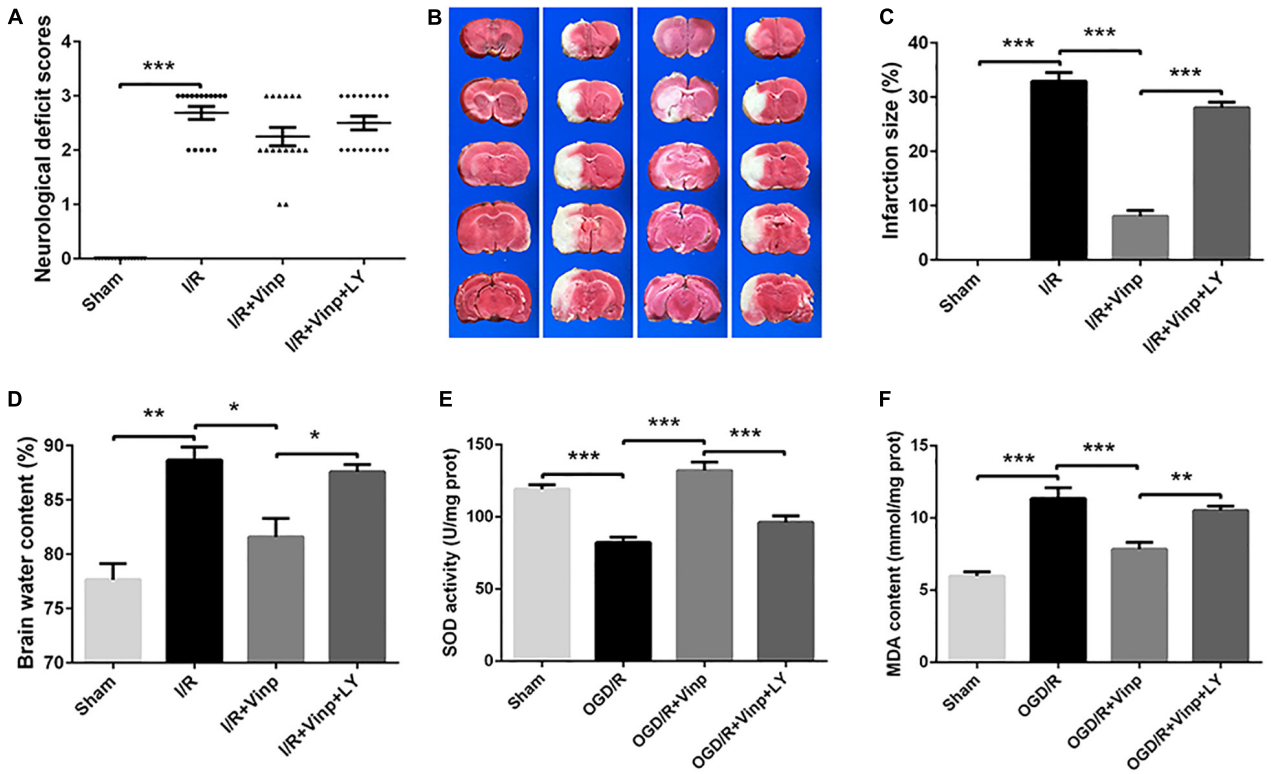

FIGURE 3 | Effects of Vinp on infarction size, neurological deficits, brain water content, and oxidative stress in rats following MCAO. **(A)** Neurological deficit analysis (n = 16 in each group). **(B)** Brain water content analysis (n = 3 in each group). **(C)** Representative images of cerebral infarction after ischemia for 2 h and reperfusion for 12 h in rat brains by TTC staining. **(D)** Analysis of infarct size calculated as a percent relative to total cerebral volume (n = 3 in each group). **(E)** Analysis of SOD activity (n = 5 in each group). **(F)** Analysis of MDA content (n = 5 in each group). *P < 0.05, **P < 0.01, ***P < 0.001 using one-way ANOVA followed by Tukey's *post hoc* tests. Vinp: Vinpocetine; LY: LY294002; I/R: ischemia/reperfusion; MCAO: middle cerebral artery occlusion; TTC: 2,3,5-triphenyltetrazolium chloride.

in a significant increase in the expression of GFAP and TNF-α, and the co-localization of GFAP and TNF-α was compared with that in the sham group. However, this increase was blocked by Vinp treatment. Furthermore, LY reversed the effect of Vinp in reactive astrocytes, significantly increasing the expression of GFAP and TNF-α, and consequently, the co-localization of GFAP and TNF-α. The results showed that Vinp treatment significantly decreased cerebral I/R injury-induced inflammation by reducing astrocyte activation. Then, western blot analysis was used to detect the expression of inflammation-associated proteins, IL-1β, TNF-α, and IL-10, and apoptosis-related proteins, caspase-3 and Bcl-2 (**Figures 5A,B**). IL-1β, TNF-α, and caspase-3 expression significantly increased while Bcl-2 expression significantly decreased in the I/R group compared with the sham group (P < 0.001). IL-1β, TNF-α, and caspase-3 expression decreased while IL-10 and Bcl-2 expression increased in the I/R + Vinp group compared to the I/R group (P < 0.001). Conversely, LY blocked the above effects of Vinp, significantly increasing IL-1β, TNF-α, and caspase-3 expression while decreasing IL-10 and Bcl-2 expression (P < 0.01). Overall, these results indicated that the PI3K/AKT pathway is involved in the anti-inflammatory and anti-apoptotic effects of Vinp against cerebral I/R injury.

Vinp Activated p-Cx43 via the PI3K/AKT Pathway in the Rat Cerebral Cortex After Cerebral I/R Injury

In order to explore whether the above protective effects of Vinp are exerted by targeting Cx43 via the PI3K/AKT pathway, we examined the expression of Cx43, p-Cx43, AKT, and p-AKT. Compared to the sham group, I/R injury significantly repressed the expression of Cx43 and p-Cx43 and the p-Cx43/Cx43 ratio (**Figure 5C**, P < 0.01). Interestingly, Vinp could increase Cx43 and p-Cx43 expression and the p-Cx43/Cx43 ratio compared with the I/R group, indicating the activation of the Cx43 (P < 0.001). However, significant reduction of p-Cx43 expression and the p-Cx43/Cx43 ratio was observed in the I/R + Vinp + LY group compared with I/R + Vinp group (P < 0.01). The expression of p-AKT and the ratio of p-AKT/AKT were similar to those of p-Cx43 and p-Cx43/Cx43, while the AKT levels did not significantly differ among the groups (**Figure 5D**).

Vinp Activated p-Cx43 via the PI3K/AKT Pathway in Astrocytes After OGD/R Injury

To further explore whether the abovementioned protective mechanism of Vinp are exerted by targeting the astrocytes, we

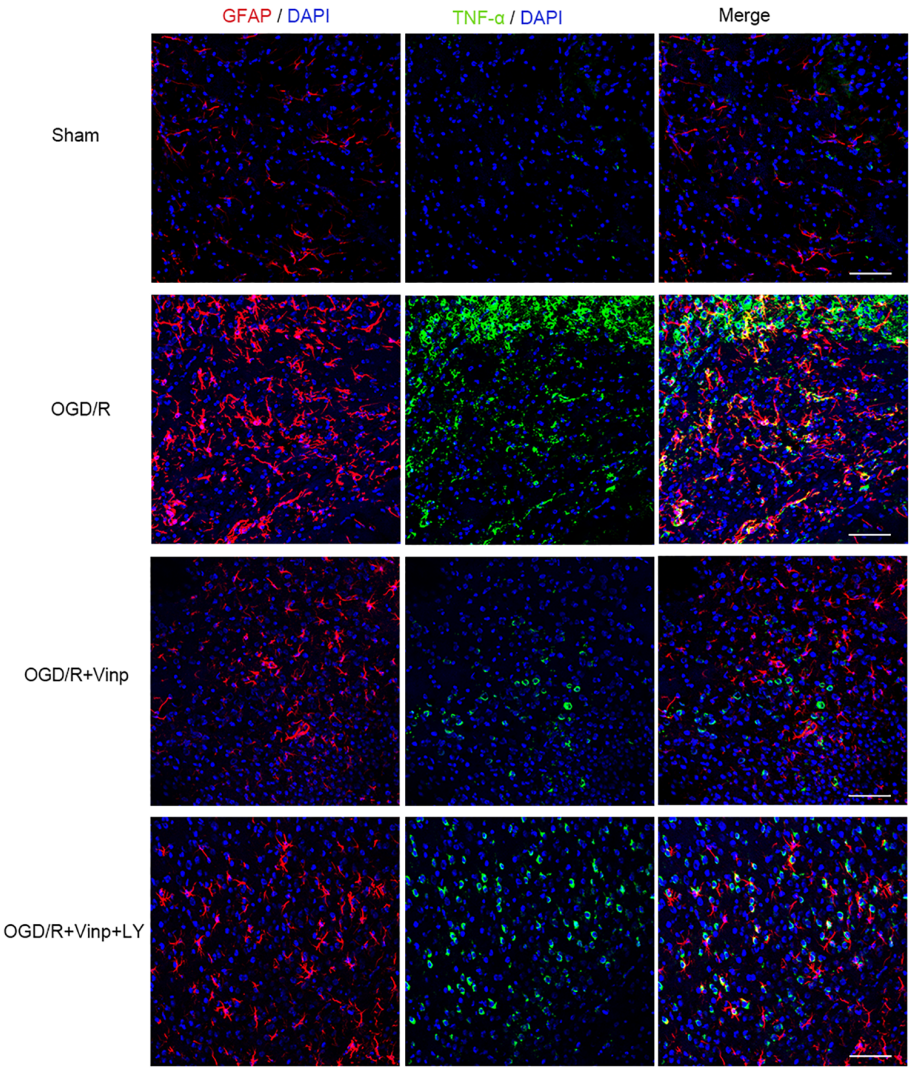

FIGURE 4 | Effects of Vinp on reactive astrocytes and TNF-α in the rat cerebral cortex after I/R injury. Brain slices were analyzed with double immunostaining using GFAP (red, a reactive astrocytic marker) and TNF-α (green, a pro-inflammatory cytokine). The nuclei were stained with DAPI (n = 3 in each group). Scale bars = 50 μm. Vinp: Vinpocetine; LY: LY294002; I/R: ischemia/reperfusion;

examined the expression of Cx43, p-Cx43, AKT, and p-AKT *in vitro* astrocyte cultures treated with BKM120 (a specific class I PI3K inhibitor) (**Figure 6**). The results of Cx43, p-Cx43, AKT, and p-AKT expression, and the ratio of p-Cx43/Cx43 and p-AKT/AKT in each group of *in vitro* cultured astrocytes were consistent with those observed *in vivo*. Moreover, there was no significant difference between the OGD/R + Vinp + LY and OGD/R + Vinp + BKM groups for the abovementioned proteins and their phosphorylation. These results provide more evidence that Vinp protects against cerebral I/R injury by targeting astrocytic Cx43 via the PI3K/AKT pathway.

DISCUSSION

Ischemic stroke triggers a complex cascade of events, such as excitotoxicity, calcium overload, oxidative stress, inflammation, and apoptosis, which finally leads to dysfunction. For decades, studies on ischemic stroke had mainly focused on neurons. It is a rather recent concept that astrocytes could be a promising therapeutic target for neuroprotection in ischemic stroke (Cekanaviciute and Buckwalter, 2016; Choudhury and Ding, 2016; Liu and Chopp, 2016). One of the mechanisms is that astrocytes could transmit chemical signals or small molecule metabolites through their gap junctions, thereby affecting neuronal survival (Liu and Chopp, 2016).

Vinp was originally invented as a drug for the treatment of diseases caused by cerebrovascular disorders, such as stroke and vascular dementia. Vinp has been shown to be a cyclic nucleotide phosphodiesterase 1 inhibitor (Souness et al., 1989) that also inhibits voltage-dependent Na^+ channels (Sitges et al., 2005) and IκB kinase (IKK) to exert its anti-inflammatory effects (Jeon et al., 2010). It also exerts significant antioxidant

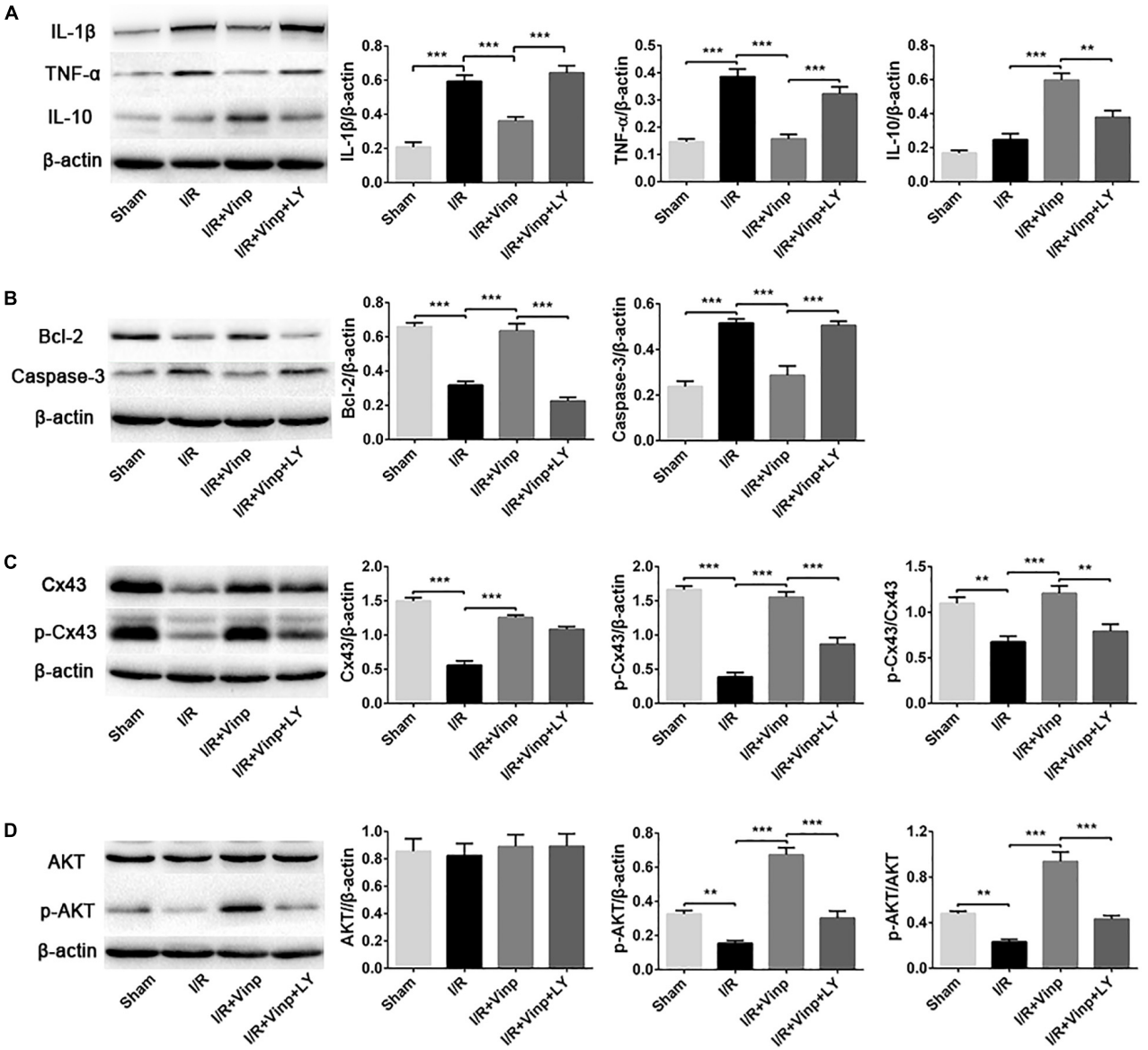

FIGURE 5 | Effects of Vinp on inflammation and apoptosis-related proteins, and the expression of Cx43, p-Cx43, AKT, and p-AKT in the rat cerebral cortex after I/R injury. **(A)** Representative western blot images and quantification of proteins related to inflammation. **(B)** Representative western blot images and quantification of proteins related to apoptosis. **(C)** Representative western blot images of Cx43 and p-Cx43, and quantification of Cx43, p-Cx43, and p-Cx43/Cx43. **(D)** Representative western blot images of AKT and p-AKT, and quantification of AKT, p-AKT and p-AKT/AKT. Data are shown as the mean ± SEM (n = 5 in each group). $^{**}P < 0.01$, $^{***}P < 0.001$ using one-way ANOVA followed by Tukey's *post hoc* tests. Vinp: Vinpocetine; LY: LY294002; I/R: ischemia/reperfusion.

activity by scavenging hydroxyl radicals (Pereira et al., 2000). Although a previous study showed that Vinp can inhibit the inflammatory response caused by cerebral I/R injury and reduce the cerebral infarction volume (Wang et al., 2014), the protective mechanism remains unclear. A study that focused on the role of microglia in the neuroprotection of Vinp proved that microglial TLR4/MyD88/NF-κB is one of the mechanisms by which Vinp protects against cerebral I/R injury (Wu et al., 2017). However, the role of astrocytes in the effect of Vinp against the cascade injury caused by cerebral I/R is unclear.

In this study, we investigated the early protective effects of Vinp *in vivo* and *in vitro* in ischemic stroke models and revealed previously unknown related mechanisms. We found that in cerebral I/R injury rats, Vinp significantly protected against I/R injury by reducing cerebral infarction volume and brain edema. Interestingly, Vinp didn't significantly reduce the neurological deficit scores, which is contrary to the previous study (Wu et al., 2017). This discrepancy may be a result of the differences in time points and the number of animals used in the two studies. Since the time point of our study *in vivo* was cerebral ischemia for 2 h and reperfusion for

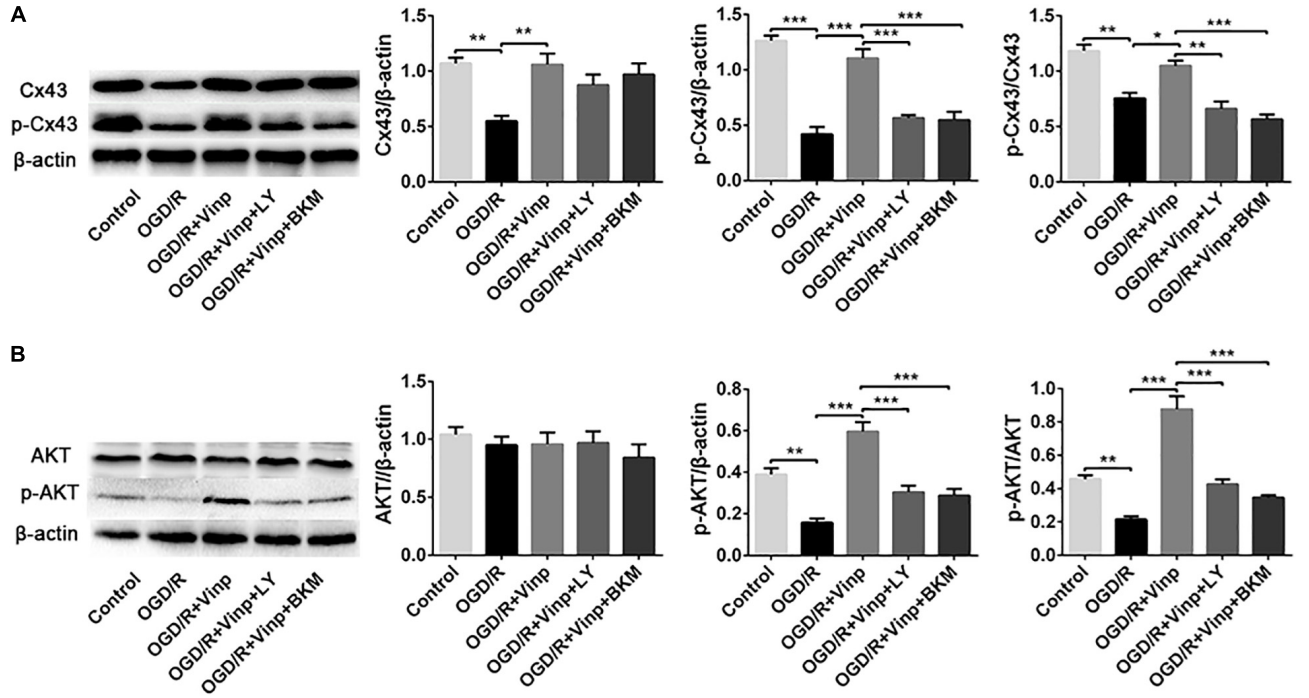

FIGURE 6 | Effects of Vinp on the expression of Cx43, p-Cx43, AKT, and p-AKT in astrocytes after OGD/R. (A) Representative western blot images of Cx43 and p-Cx43, and quantification of Cx43, p-Cx43, and p-Cx43/Cx43. (B) Representative western blot images of AKT and p-AKT, and quantification of AKT, p-AKT and p-AKT/AKT. Data are shown as the mean ± SEM ($n = 3$ in each group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ using one-way ANOVA followed by Tukey's *post hoc* tests. Vinp: Vinpocetine; LY: LY294002; OGD/R: oxygen-glucose deprivation/reoxygenation; BKM: BKM120.

12 h, the degree of neurological deficit may be different at the time point of ischemia for 1 h and reperfusion for 24 h used in the previous study. Furthermore, the number of animals per group was 16 in this study, while it was 7 in the previous study, which may lead to different statistical results. Similarly, findings indicated that Vinp could promote cell survival and reduce cell damage (reduced LDH release) in OGD/R astrocytes. We also explored the neuroprotection of Vinp against the cascade events caused by cerebral I/R injury. Since it is widely considered that the initial step of cerebral I/R is the generation of large amounts of ROS (Dirnagl et al., 1999), we tested the activity of SOD and the content of MDA in the ischemic cortices *in vivo*, and intracellular ROS and NO released into the supernatant *in vitro*. The results revealed that Vinp had an antioxidant effect. It is known that increased ROS may activate astrocytes and microglia, which may produce pro-inflammatory mediators (Chan, 2001; Duris et al., 2018). Blocking the production of pro-inflammatory cytokines would be an important strategy to protect against I/R injury. Thus, we examined the pro-inflammatory cytokines IL-1β and TNF-α by immunofluorescence staining in astrocytes subjected to OGD/R, and the results were consistent with the *in vivo* immunofluorescence double immunostaining with the astrocytic marker GFAP and TNF-α and with the immunoblotting results of pro-inflammatory and anti-inflammatory cytokines. The above *in vivo* and *in vitro* results suggest that Vinp could exert anti-inflammatory effects through astrocytes. Oxidative stress and excessive inflammatory responses could activate pro-apoptotic pathways (Duris et al., 2018), and the activation of caspase-3 is the central part of apoptosis. Thus, we examined the expression of caspase-3 and anti-apoptotic protein Bcl-2 by immunofluorescence staining *in vitro* and immunoblotting *in vivo*. The above results revealed that Vinp attenuated oxidative stress damage, inflammatory responses, and apoptosis both *in vivo* and *in vitro*.

Next, we explored the mechanisms involved in the protection of Vinp. *In vitro* experiments, LY294002 was found to block Vinp's effects on intracellular ROS and extracellular NO, TNF-α and IL-1β expression, caspase-3 and Bcl-2 expression, astrocytic apoptotic rate. *In vivo* experiments, LY294002 was found to block the effects of Vinp on SOD activity, MDA content, IL-1β, TNF-α, IL-10, BCL-2, and caspase-3 expression. Moreover, *in vivo* immunofluorescence experiments, LY294002 reversed the effect of Vinp on reactive astrocytes, significantly increasing the expression of GFAP and TNF-α and the co-localization of GFAP and TNF-α. Overall, these results indicated that the PI3K/AKT pathway is involved in the anti-oxidative, anti-inflammatory, and anti-apoptotic effects of Vinp against cerebral I/R injury. Additionally, we found that I/R injury resulted in decreased Cx43 expression and enhanced Cx43 dephosphorylation. However, all these changes were inhibited by Vinp, suggesting that Cx43 may play an important role in Vinp's neuroprotection. More importantly, the inhibition of the PI3K/AKT pathway by LY294002 blocked the above neuroprotective effects of Vinp

and reversed the p-Cx43 and p-Cx43/Cx43 changes *in vivo*. We further confirmed the above findings by examining the PI3K/AKT pathway and Cx43 *in vitro* cultured astrocytes with the addition of BKM120 (a specific class I PI3K inhibitor, Burger et al., 2011; Maira et al., 2012). In conclusion, this study showed that Vinp regulates Cx43 in cerebral I/R injury through the PI3K/AKT signaling pathway and provided evidence for its clinical application.

Previous studies have reported that Cx43 is highly phosphorylated under physiological conditions, and ischemia will lead to Cx43 dephosphorylation. Cx43 dephosphorylation is accompanied by the opening of the Cx43 hemichannel, leading to increased influx of several harmful substances and enlargement of the infarct size (Chew et al., 2010). Increased Cx43 expression can reduce neuronal damage after cerebral I/R (Nakase et al., 2003). Our previous studies showed that OGD/R injury can cause Cx43 hemichannel opening and increase in the release of ATP, which could activate the microglia to release numerous inflammatory factors causing neuronal death (Yin et al., 2018). In addition, the inflammatory response of astrocytes increases after ischemic stroke, leading to increased release of extracellular inflammatory factors that affect neuronal survival (Kawabori and Yenari, 2015; Anrather and Iadecola, 2016). Consistent with previous results, we found that cerebral I/R injury downregulated Cx43 and p-Cx43, decreased SOD activity, increased MDA content, decreased the expression of anti-apoptotic protein Bcl-2, and enhanced the expression of apoptotic protein caspase-3 and pro-inflammatory cytokines TNF-α and IL-1β. Effectively, Vinp enhanced Cx43 and p-Cx43 expression and attenuated the aforementioned detrimental effects caused by cerebral I/R, indicating that Vinp likely exerts neuroprotection by targeting Cx43. Studies have shown that Cx43 affects the activation of the inflammasome and the progression of acute kidney injury by regulating the intracellular oxidative status (Huang et al., 2019). Thus, we hypothesized that the protective mechanism of Vinp may be involved in the inhibition of Cx43 internalization and dephosphorylation, accompanied by the closure of the Cx43 hemichannel to reduce intracellular reactive oxygen species, thereby reducing the inflammatory cascade and apoptosis.

Last, we explore the upstream mechanism of Cx43. Cx43 has multiple phosphorylation sites that can be activated by different kinases (including PKA, AKT, and PKC) (Solan and Lampe, 2009), where the C-terminal Ser373 site of the Cx43 can be phosphorylated by AKT (Solan and Lampe, 2014). The PI3K/AKT is an important anti-apoptotic pathway within the cell, and it can induce the formation of IKK by influencing NF-κB and Bcl-2 by phosphorylating GSK-3β, which play a protective role with anti-inflammatory and anti-apoptotic effects (Park et al., 2006; Mullonkal and Toledo-Pereyra, 2007). A previous study showed that PI3K/AKT plays a crucial role in modulating Cx43 expression (Bhattacharjee et al., 2009), conveying mechanical signals to the Cx43 hemichannel and mediating its opening in osteocytes (Batra et al., 2014). Besides, Cx43 has been shown to decrease expression in the heart of *AKT1*$^{-/-}$*/iAKT2* knockout mice, revealing that AKT plays an important role in maintaining systolic function and Cx43 protein stability (Ock et al., 2018). Inhibition of the PI3K/AKT pathway by LY294002 can reduce Cx43 expression and block the cardioprotective effect of atorvastatin (Bian et al., 2015). Our results indicate that Vinp activates the PI3K/AKT pathway by enhancing the expression of p-AKT (Ser473) to exert anti-oxidative stress and anti-inflammatory effects, thereby exerting anti-apoptotic effects. However, there was no significant change in the expression of AKT, indicating that AKT exerts the above effects through phosphorylation rather than protein expression. Treatment with Vinp and the PI3K/AKT pathway inhibitor LY294002 abolished the upregulation of p-Cx43(Ser373) caused by Vinp after cerebral I/R injury, but not the significant downregulation of Cx43 expression, strongly suggesting that phosphorylation is the manifestation of Cx43 activity. These results were also confirmed *in vitro* using a more than 95% pure primary astrocyte culture. Meanwhile, the expression of AKT and Cx43 induced by the treatment of Vinp and BKM120 (a specific class I PI3K inhibitor,Burger et al., 2011; Maira et al., 2012) was not significantly different than that induced by the treatment of Vinp and LY294002 *in vitro*, which provided further evidence that Vinp targets the PI3K/AKT pathway and regulates the phosphorylation of Cx43. Taken together, our study suggests that the C-terminal Ser373 site of Cx43 can be phosphorylated by AKT activity and plays an important role in the neuroprotection of Vinp.

Our study showed that apoptosis is consistent with changes in proinflammatory factors and oxidative stress, whether in cerebral I/R injury or through the addition of Vinp or LY294002. Previous studies have shown that cerebral I/R injury could induce increased oxidative stress which may induce excessive inflammatory responses that finally activate pro-apoptotic pathways (Dirnagl et al., 1999). Activation of either the Fas, TNF, and TRAIL receptor-mediated extrinsic pathways or direct activation of intrinsic pathways ultimately leads to activation of caspase-3 (Duris et al., 2018). Therefore, we speculate that the anti-apoptotic effect of Vinp may be partly due to its antioxidant and anti-inflammatory effects. Unfortunately, our study could not elucidate the molecular mechanism by which inflammation interacts with apoptosis in the protective effects of Vinp against cerebral I/R. Despite this limitation, this study clearly showed the protective mechanism of Vinp against ischemic stroke.

This study has some other limitations that must be acknowledged. First, we analyzed the early effect of Vinp at cerebral ischemia for 2 h and reperfusion for 12 h based on the previous results of our research group, however, analyzing the effects of Vinp at a longer time point is required. Second, knocking-out the PI3K signaling pathway at the gene level, and not just via PI3K inhibitors, could provide more precise results. Furthermore, further studies are needed to test the effects of Vinp on additional cerebral ischemic models.

CONCLUSION

Our study provides new insights into the treatment of ischemic stroke and indicates that Vinp provided neuroprotection against oxidative stress, inflammatory responses, and apoptosis caused by cerebral I/R injury and that this protection may involve astrocytic

Cx43 regulation via the PI3K/AKT signaling pathway. Therefore, Vinp could be potentially used to develop a promising drug for the treatment of ischemic stroke.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of China's Guidelines for Care and Use of Laboratory Animals, the Animals Ethics Committee of Jilin University. The protocol was reviewed and approved by the Animals Ethics Committee of Jilin University.

AUTHOR CONTRIBUTIONS

JF, DM, and MZ conceived and designed the experiments. MZ, SH, and LF performed the experiments. MZ, PS, and FW analyzed the data. DN, YZ, and DM contributed the reagents, materials, and analysis tools. MZ, DM, and JF wrote the manuscript.

FUNDING

This project was supported by the National Key R&D Program of China (2017YFC0110304); Hungarian-Chinese Scientific Cooperation Foundation (HCSCF-2016-4 and HCSCF-2017-4); National Natural Science Foundation of China (81771257, 81701158, 81801145, 31600820, 31600251, and 81801207); The Science and Technology planning project of Jilin Province (20180101161JC and 20180520134JH); The National Key R&D Program of China (2017YFC1308401), and Innovation Team Foundation of Jilin University (2017TD-30).

ACKNOWLEDGMENTS

We thank Gedeon Richter Pharmaceutical Co., Ltd., in Hungary for providing the vinpocetine and Editage for language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00223/full#supplementary-material

REFERENCES

Agrawal, H. C., Davis, J. M., and Himwich, W. A. (1968). Developmental changes in mouse brain: weight, water content and free amino acids. *J. Neurochem.* 15, 917–923. doi: 10.1111/j.1471-4159.1968.tb11633.x

Anrather, J., and Iadecola, C. (2016). Inflammation and stroke: an overview. *Neurotherapeutics* 13, 661–670. doi: 10.1007/s13311-016-0483-x

Batra, N., Riquelme, M. A., Burra, S., Kar, R., Gu, S., and Jiang, J. X. (2014). Direct regulation of osteocytic connexin 43 hemichannels through AKT kinase activated by mechanical stimulation. *J. Biol. Chem.* 289, 10582–10591. doi: 10.1074/jbc.M114.550608

Bederson, J. B., Pitts, L. H., Germano, S. M., Nishimura, M. C., Davis, R. L., and Bartkowski, H. M. (1986). Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke* 17, 1304–1308. doi: 10.1161/01.str.17.6.1304

Bhattacharjee, R., Kaneda, M., Nakahama, K., and Morita, I. (2009). The steady-state expression of connexin43 is maintained by the PI3K/Akt in osteoblasts. *Biochem. Biophys. Res. Commun.* 382, 440–444. doi: 10.1016/j.bbrc.2009.03.044

Bian, B., Yu, X., Wang, Q., Teng, T., and Nie, J. (2015). Atorvastatin protects myocardium against ischemia-reperfusion arrhythmia by increasing Connexin 43 expression: a rat model. *Eur. J. Pharmacol.* 768, 13–20. doi: 10.1016/j.ejphar.2015.09.023

Burger, M. T., Pecchi, S., Wagman, A., Ni, Z. J., Knapp, M., Hendrickson, T., et al. (2011). Identification of NVP-BKM120 as a potent, selective, orally bioavailable class I PI3 Kinase inhibitor for treating cancer. ACS. *Med. Chem. Lett.* 2, 774–779. doi: 10.1021/ml200156t

Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science* 296, 1655–1657. doi: 10.1126/science.296.5573.1655

Cekanaviciute, E., and Buckwalter, M. S. (2016). Astrocytes: integrative regulators of neuroinflammation in stroke and other neurological diseases. *Neurotherapeutics* 13, 685–701. doi: 10.1007/s13311-016-0477-8

Chan, P. H. (2001). Reactive oxygen radicals in signaling and damage in the ischemic brain. *J. Cereb. Blood Flow Metab.* 21, 2–14. doi: 10.1097/00004647-200101000-00002

Chew, S. S., Johnson, C. S., Green, C. R., and Danesh-Meyer, H. V. (2010). Role of connexin43 in central nervous system injury. *Exp. Neurol.* 225, 250–261. doi: 10.1016/j.expneurol.2010.07.014

Choudhury, G. R., and Ding, S. (2016). Reactive astrocytes and therapeutic potential in focal ischemic stroke. *Neurobiol. Dis.* 85, 234–244. doi: 10.1016/j.nbd.2015.05.003

Dirnagl, U., Iadecola, C., and Moskowitz, M. A. (1999). Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci.* 22, 391–397. doi: 10.1016/s0166-2236(99)01401-0

Duris, K., Splichal, Z., and Jurajda, M. (2018). The role of inflammatory response in stroke associated programmed cell death. *Curr. Neuropharmacol.* 16, 1365–1374. doi: 10.2174/1570159x16666180222155833

Eruslanov, E., and Kusmartsev, S. (2010). Identification of ROS using oxidized DCFDA and flow-cytometry. *Methods Mol. Biol.* 594, 57–72. doi: 10.1007/978-1-60761-411-1_4

Falkowska, A., Gutowska, I., Goschorska, M., Nowacki, P., Chlubek, D., and Baranowska-Bosiacka, I. (2015). Energy metabolism of the brain, including the cooperation between astrocytes and neurons, especially in the context of glycogen metabolism. *Int. J. Mol. Sci.* 16, 25959–25981. doi: 10.3390/ijms161125939

Ferrer-Acosta, Y., Gonzalez-Vega, M. N., Rivera-Aponte, D. E., Martinez-Jimenez, S. M., and Martins, A. H. (2017). Monitoring astrocyte reactivity and proliferation in vitro under ischemic-like conditions. *J. Vis. Exp.* 128. doi: 10.3791/55108

Frey, T. (1997). Correlated flow cytometric analysis of terminal events in apoptosis reveals the absence of some changes in some model systems. *Cytometry* 28, 253–263. doi: 10.1002/(sici)1097-0320(19970701)28:3<253::aid-cyto10>3.0.co;2-o

Fukunaga, K., and Kawano, T. (2003). Akt is a molecular target for signal transduction therapy in brain ischemic insult. *J. Pharmacol. Sci.* 92, 317–327. doi: 10.1254/jphs.92.317

Garman, R. H. (2011). Histology of the central nervous system. *Toxicol. Pathol.* 39, 22–35. doi: 10.1177/0192623310389621

Hong, D. H., Choi, I. W., Son, Y. K., Kim, D. J., Na, S. H., Jung, W. K., et al. (2013). The effect of PI3 kinase inhibitor LY294002 on voltage-dependent K(+)

channels in rabbit coronary arterial smooth muscle cells. *Life Sci.* 92, 916–922. doi: 10.1016/j.lfs.2013.03.006

Hou, S., Shen, P. P., Zhao, M. M., Liu, X. P., Xie, H. Y., Deng, F., et al. (2016). Mechanism of Mitochondrial Connexin43's Protection of the Neurovascular Unit under Acute Cerebral Ischemia-Reperfusion Injury. *Int. J. Mol. Sci.* 17:E679. doi: 10.3390/ijms17050679

Huang, Y., Mao, Z., Zhang, Z., Obata, F., Yang, X., Zhang, X., et al. (2019). Connexin43 contributes to inflammasome activation and lipopolysaccharide-initiated acute renal injury via modulation of intracellular oxidative status. *Antioxid Redox Signal.* 31, 1194–1212. doi: 10.1089/ars.2018.7636

Ishiyama, M., Miyazono, Y., Sasamoto, K., Ohkura, Y., and Ueno, K. (1997). A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. *Talanta* 44, 1299–1305. doi: 10.1016/s0039-9140(97)00017-9

Jackman, K., Kunz, A., and Iadecola, C. (2011). Modeling focal cerebral ischemia in vivo. *Methods Mol. Biol.* 793, 195–209. doi: 10.1007/978-1-61779-328-8_13

Jeon, K. I., Xu, X., Aizawa, T., Lim, J. H., Jono, H., Kwon, D. S., et al. (2010). Vinpocetine inhibits NF-kappaB-dependent inflammation via an IKK-dependent but PDE-independent mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9795–9800. doi: 10.1073/pnas.0914414107

Kawabori, M., and Yenari, M. A. (2015). Inflammatory responses in brain ischemia. *Curr. Med. Chem.* 22, 1258–1277. doi: 10.2174/0929867322666150209154036

Kim, J. Y., Park, J., Chang, J. Y., Kim, S. H., and Lee, J. E. (2016). Inflammation after ischemic stroke: the role of leukocytes and glial cells. *Exp. Neurobiol.* 25, 241–251. doi: 10.5607/en.2016.25.5.241

Liu, Z., and Chopp, M. (2016). Astrocytes, therapeutic targets for neuroprotection and neurorestoration in ischemic stroke. *Prog. Neurobiol.* 144, 103–120. doi: 10.1016/j.pneurobio.2015.09.008

Lobner, D. (2000). Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis? *J. Neurosci. Methods* 96, 147–152. doi: 10.1016/s0165-0270(99)00193-4

Longa, E. Z., Weinstein, P. R., Carlson, S., and Cummins, R. (1989). Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 20, 84–91. doi: 10.1161/01.str.20.1.84

Maira, S. M., Pecchi, S., Huang, A., Burger, M., Knapp, M., Sterker, D., et al. (2012). Identification and characterization of NVP-BKM120, an orally available pan-class I PI3-kinase inhibitor. *Mol. Cancer Ther.* 11, 317–328. doi: 10.1158/1535-7163.mct-11-0474

Mullonkal, C. J., and Toledo-Pereyra, L. H. (2007). Akt in ischemia and reperfusion. *J. Invest. Surg.* 20, 195–203. doi: 10.1080/08941930701366471

Nakase, T., Fushiki, S., and Naus, C. C. (2003). Astrocytic gap junctions composed of connexin 43 reduce apoptotic neuronal damage in cerebral ischemia. *Stroke* 34, 1987–1993. doi: 10.1161/01.str.0000079814.72027.34

Nivison-Smith, L., O'Brien, B. J., Truong, M., Guo, C. X., Kalloniatis, M., and Acosta, M. L. (2015). Vinpocetine modulates metabolic activity and function during retinal ischemia. *Am. J. Physiol. Cell Physiol.* 308, C737–C749. doi: 10.1152/ajpcell.00291.2014

Ock, S., Lee, W. S., Kim, H. M., Park, K. S., Kim, Y. K., Kook, H., et al. (2018). Connexin43 and zonula occludens-1 are targets of Akt in cardiomyocytes that correlate with cardiac contractile dysfunction in Akt deficient hearts. *Biochim. Biophys. Acta Mol. Basis Dis.* 1864(4 Pt A), 1183–1191. doi: 10.1016/j.bbadis.2018.01.022

Orellana, J. A., Froger, N., Ezan, P., Jiang, J. X., Bennett, M. V., Naus, C. C., et al. (2011). ATP and glutamate released via astroglial connexin 43 hemichannels mediate neuronal death through activation of pannexin 1 hemichannels. *J. Neurochem.* 118, 826–840. doi: 10.1111/j.1471-4159.2011.07210.x

Park, S. S., Zhao, H., Mueller, R. A., and Xu, Z. (2006). Bradykinin prevents reperfusion injury by targeting mitochondrial permeability transition pore through glycogen synthase kinase 3β. *J. Mo.l Cell Cardiol.* 40, 708–716. doi: 10.1016/j.yjmcc.2006.01.024

Pereira, C., Agostinho, P., and Oliveira, C. R. (2000). Vinpocetine attenuates the metabolic dysfunction induced by amyloid beta-peptides in PC12 cells. *Free Radic. Res.* 33, 497–506. doi: 10.1080/10715760000301041

Ribeiro, P. W., Cola, P. C., Gatto, A. R., da Silva, R. G., Luvizutto, G. J., Braga, G. P., et al. (2015). Relationship between dysphagia, national institutes of health stroke scale score, and predictors of pneumonia after ischemic stroke. *J. Stroke Cerebrovasc. Dis.* 24, 2088–2094. doi: 10.1016/j.jstrokecerebrovasdis.2015.05.009

Schildge, S., Bohrer, C., Beck, K., and Schachtrup, C. (2013). Isolation and culture of mouse cortical astrocytes. *J. Vis. Exp.* 19:50079. doi: 10.3791/50079

Sheth, K. N., Smith, E. E., Grau-Sepulveda, M. V., Kleindorfer, D., Fonarow, G. C., and Schwamm, L. H. (2015). Drip and ship thrombolytic therapy for acute ischemic stroke: use, temporal trends, and outcomes. *Stroke* 46, 732–739. doi: 10.1161/strokeaha.114.007506

Sitges, M., Galvan, E., and Nekrassov, V. (2005). Vinpocetine blockade of sodium channels inhibits the rise in sodium and calcium induced by 4-aminopyridine in synaptosomes. *Neurochem. Int.* 46, 533–540. doi: 10.1016/j.neuint.2005.02.001

Solan, J. L., and Lampe, P. D. (2009). Connexin43 phosphorylation: structural changes and biological effects. *Biochem. J.* 419, 261–272. doi: 10.1042/bj20082319

Solan, J. L., and Lampe, P. D. (2014). Specific Cx43 phosphorylation events regulate gap junction turnover in vivo. *FEBS Lett.* 588, 1423–1429. doi: 10.1016/j.febslet.2014.01.049

Souness, J. E., Brazdil, R., Diocee, B. K., and Jordan, R. (1989). Role of selective cyclic GMP phosphodiesterase inhibition in the myorelaxant actions of M&B 22,948, MY-5445, vinpocetine and 1-methyl-3-isobutyl-8-(methylamino)xanthine. *Br. J. Pharmacol.* 98, 725–734. doi: 10.1111/j.1476-5381.1989.tb14599.x

Swanson, R. A., Ying, W., and Kauppinen, T. M. (2004). Astrocyte influences on ischemic neuronal death. *Curr. Mol. Med.* 4, 193–205. doi: 10.2174/1566524043479185

Takac, T., Pechan, T., Samajova, O., and Samaj, J. (2013). Vesicular trafficking and stress response coupled to PI3K inhibition by LY294002 as revealed by proteomic and cell biological analysis. *J. Proteome Res.* 12, 4435–4448. doi: 10.1021/pr400466x

Verkhratsky, A., Zorec, R., and Parpura, V. (2017). Stratification of astrocytes in healthy and diseased brain. *Brain Pathol.* 27, 629–644. doi: 10.1111/bpa.12537

Wang, H., Zhang, K., Zhao, L., Tang, J., Gao, L., and Wei, Z. (2014). Anti-inflammatory effects of vinpocetine on the functional expression of nuclear factor-kappa B and tumor necrosis factor-alpha in a rat model of cerebral ischemia-reperfusion injury. *Neurosci. Lett.* 566, 247–251. doi: 10.1016/j.neulet.2014.02.045

Weissman, B. A., and Gross, S. S. (2001). Measurement of NO and NO synthase. *Curr. Protoc. Neurosci. Chapter.* 7:Unit7.13. doi: 10.1002/0471142301.ns0713s05

Wu, L. R., Liu, L., Xiong, X. Y., Zhang, Q., Wang, F. X., Gong, C. X., et al. (2017). Vinpocetine alleviate cerebral ischemia/reperfusion injury by down-regulating TLR4/MyD88/NF-kappaB signaling. *Oncotarget* 8, 80315–80324. doi: 10.18632/oncotarget.20699

Yin, X., Feng, L., Ma, D., Yin, P., Wang, X., Hou, S., et al. (2018). Roles of astrocytic connexin-43, hemichannels, and gap junctions in oxygen-glucose deprivation/reperfusion injury induced neuroinflammation and the possible regulatory mechanisms of salvianolic acid B and carbenoxolone. *J. Neuroinflammation* 15:97. doi: 10.1186/s12974-018-1127-3

Zhang, F., Yan, C., Wei, C., Yao, Y., Ma, X., Gong, Z., et al. (2018). vinpocetine inhibits nf-kappab-dependent inflammation in acute ischemic stroke patients. *Transl. Stroke Res.* 9, 174–184. doi: 10.1007/s12975-017-0549-z

Zhang, Y. S., Li, J. D., and Yan, C. (2018). An update on vinpocetine: new discoveries and clinical implications. *Eur. J. Pharmacol.* 819, 30–34. doi: 10.1016/j.ejphar.2017.11.041

Zhao, H., Sapolsky, R. M., and Steinberg, G. K. (2006). Phosphoinositide-3-kinase/akt survival signal pathways are implicated in neuronal survival after stroke. *Mol. Neurobiol.* 34, 249-270. doi: 10.1385/mn 34, 249.

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership