NEUROPROTECTION IN BRAIN HYPOXIA

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NEUROPROTECTION IN BRAIN HYPOXIA

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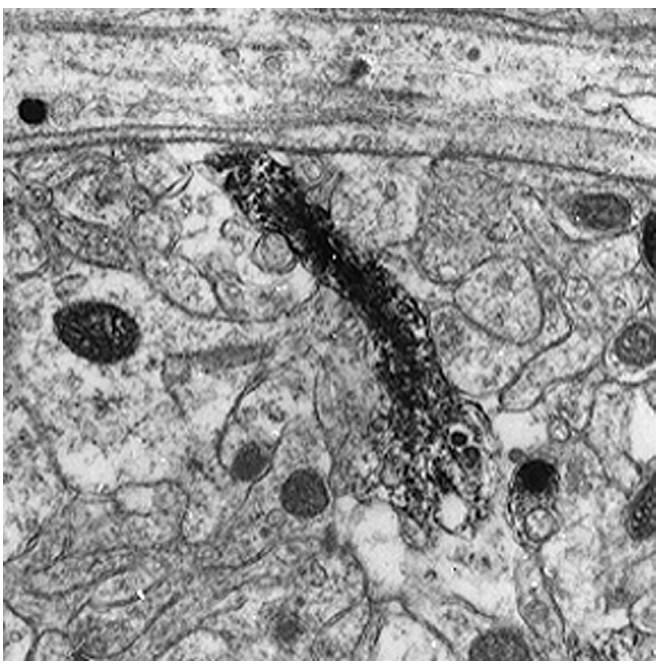

Filopodia formation after perinatal asphyxia. Electron micrograph showing a growing F-Actin-positive filopodium in the hippocampus following perinatal asphyxia. Figure by Francisco Capani

Despite compelling preclinical evidence from laboratory models of brain hypoxia suggesting potential neuroprotective strategies, only scattered data are available from clinical studies. A few candidate neuroprotectants have been studied regarding antioxidant, antiapoptotic, anti-excitotoxic, immunomodulatory, and neurotrophic effects.

In parallel with clinical innovations, preclinical research initiatives are also identifying new animal models more closely resembling the clinical course and pathology of neurodegenerative diseases.

Clarifying the specific mission of the brain cells involved in the damage/repair system in the examined animal models is important to define new therapeutic targets. Following hypoxic damage, detrimental events intermingle with the repairing events in both time- and cellular-dependent fashion. Identifying reliable post-hypoxia brain markers is mandatory to develop potential therapeutic interventions, improving translational research from the experimental observations to the clinical application.

A deeper understanding of the precise participation of neurons, glia, and endothelial cells is expected to offer more clues for designing new therapeutic strategies to reduce the current gap among the experimental and the clinical data. One of the main issues should be to study the epigenetic mechanism of neuroprotective agents and their action on the genetic modification induced by hypoxia. Achieving this goal is presumed critical to obtain more conclusive results in patients that by now do not receive appropriate therapy for mitigating the several diseases generated by brain hypoxia. Although a few ongoing studies are evaluating interesting approaches, future research is necessary to come to a novel mechanism of neuroprotection.

This book encompasses different chapters conveying the goals and aims of the pertaining research.

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EDITORIAL
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Editorial: Neuroprotection in Brain Hypoxia

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Keywords: neuroprotection, brain hypoxia, pathophysioloy, pharmacology, genetics

Editorial on the Research Topic

Neuroprotection in Brain Hypoxia

Despite there is compelling preclinical evidence from laboratory models of brain hypoxia suggesting potential neuroprotective strategies, only scattered data are available from clinical studies. In this regard, a few candidate neuroprotectants have been little studied based on their antioxidant, antiapoptotic, anti-excitotoxic, immunomodulatory, and neurotrophic effects.

In parallel with clinical innovations, preclinical research initiatives are also identifying new animal models that more closely resemble the clinical course and pathology of neurodegenerative diseases.

Clarifying the specific mission of the brain cells involved in the damage/repair system in the examined animal models is important to define new therapeutic targets. Moreover, this is so considering that after hypoxic damage, detrimental events like excitotoxicity, pro-apoptosis, pro-inflammatory reaction, and others intermingle with the repairing events in both time- and cellular-dependent fashion. Identifying more reliable post hypoxia brain markers is mandatory to design and develop potential therapeutic interventions over the follow-up time after the hypoxic episode, improving translational research from the experimental observations to the clinical application.

Thus, a deeper understanding of the precise participation of neurons, glia, and endothelial cells by contrasting and comparing the outcome of studies using animal vs. cellular models might be expected to offer more clues for designing new therapeutic strategies to reduce the current gap among the experimental and the clinical data. One of the main issues should be to study the epigenetic mechanism of neuroprotective agents and their action on the genetic modification induced by hypoxia. Achieving this goal is presumed critical to obtain more conclusive results in patients that by now do not receive an appropriate therapy for mitigating the several diseases generated by brain hypoxia. Although a few ongoing studies are evaluating interesting approaches, future research is necessary to come to a novel mechanism of neuroprotection.

Here we summarize the contributing articles to our Topic conveying the goals and aims of the pertaining research.

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- One of the chapters addresses the issue of metabolic syndrome (MetS) and neuroprotection (Etchegoyen et al.). An advanced outlook of MetS beyond its classical association with cardiovascular disease and Diabetes Mellitus Type 2 is revisited, for MetS also poses a risk factor for the nervous tissue and threatens neuronal function. The characteristically impaired metabolic pathways of MetS lead to hyperglycemia, insulin resistance, inflammation, and hypoxia, all closely associated with an overall pro-oxidative status. Oxidative stress is well-known to cause the wreckage of cellular structures and tissue architecture. Alteration of the redox homeostasis and oxidative stress alter the macromolecular array of DNA, lipids, and proteins, in turn disrupting the biochemical pathways necessary for normal cell function. The authors revise a few essential concepts of MetS pathophysiology, and explore some neuroprotective approaches in MetS concerning brain hypoxia. The prevalence of metabolic syndrome (MetS), a risk factor for neurological disorders, has drastically increased in developing countries over the years as a major byproduct of industrialization. Many factors, like high-calorie diets and a sedentary lifestyle, bolster the spread of this disorder. Undoubtedly, the massive still increasing incidence of MetS places this epidemic as an important public health issue.
- In the same line, another chapter discusses neuroprotection targeting protein misfolding on microvascular dysfunction and chronic cerebral hypoperfusion (CCH) in MetS (Herrera et al.). Persistent reduction in oxygen and energy supply to the brain implies brain hypoxia and consequent protein misfolding. This silent process represents a linking mechanism between CCH and Alzheimer's disease. Several experimental studies using distinct models of CCH revealed neurodegeneration was induced through protein misfolding. The regulation of proteostasis network pathways, such as ubiquitin-proteasome system (UPS), chaperone-mediated autophagy, and macroautophagy, appears as a novel target for neuroprotection. Lipoxin A4 methyl ester, Baclofen, URB597, N-stearoyl-L-tyrosine, and melatonin are potential neuroprotective agents to rebalance proteostasis network in CCH. Therapeutic drug options targeting misfolded proteins offer promising treatments for cognitive impairment following CCH in MetS.
- The neuroprotective role of palmitoylethanolamide (PEA) in perinatal asphyxia (PA) is also examined (Herrera et al.). The incidence of PA is estimated at 1/1,000 live births in developed countries and 5–10/1,000 live births in developing countries, causing not only mortality but also morbidity out of synaptic and cytoskeletal alterations associated with neuronal death. Cerebral palsy, epilepsy, and several neurodevelopmental disorders are common complications of PA. The role of PEA, known to protect in several models of brain injury and neurodegeneration, was studied in a well-established murine model of PA. Treatment with PEA successfully reversed neurodegenerative changes in hippocampus 1 month after experimental PA insult in rats. Data obtained using conventional electron microscopy (EM), immunocytochemistry, and immunohistochemistry for different markers of neuron and glial cells, ethanolic phosphotungstic acid (E-PTA) staining combining with electron tomography, and 3-D reconstruction techniques support the neuroprotective role of PEA in PA.
- Another chapter discusses the activation of the PI3K/Akt/GSK3 pathway by estradiol in long-term neurodegeneration following PA (Saraceno et al.). Based on their findings, the authors suggest that the interaction between ERα and the type 1 insulin-like growth factor receptor (IGF-IR) with the subsequent downstream activation underlies the beneficial effects of estradiol observed in late treatment of PA.
- The lack of impact of the selective inhibition of Janus kinase 3 (JAK3) on anatomical or neurobehavioral outcomes in experimental ischemic stroke is also addressed (DeMars et al.). The Janus kinase 3 (JAK3) is associated with the common gamma chain of several interleukin receptors essential to inflammatory signaling. The potential role of JAK3 in stroke-induced neuroinflammation was studied, and the effects of JAK3 inhibition with decernotinib (VX-509) on infarct size and behavior were investigated. Total and phosphorylated/activated JAK3 dramatically increased after stroke in the ipsilateral hemisphere in mice. However, JAK3 inhibition did not reduce infarct volume 48 h after stroke or altered behavioral outcomes sensitive to neurological deficits.
- Another chapter summarizes the state-of-the-art of the mechanistic target of rapamycin complex 1, mTORC1, in pathological situations, focusing mainly on ischemia/hypoxia (Perez-Alvarez et al.). Downstream the PI3K-Akt pathway, mTORC1 is deregulated after ischemia and neuronal oxygen-glucose deprivation (OGD). Neuroprotective intervention with estradiol or a specific AT2R agonist regulates mTORC1 activity, affecting VEGF levels.
- The antioxidative and antiapoptotic effects of delta-opioid peptide [D-Ala2, D-Leu5] enkephalin on spinal cord ischemia-reperfusion injury in rabbits (Fu et al.), the molecular bases of brain preconditioning (Deryagin et al.), and the effect of danhong (a complementary alternative medicine for chronic stable angina) combined with tissue-plasminogen activator in focal embolic stroke (Li et al.) are other captivating subjects discussed.

AUTHOR CONTRIBUTIONS

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

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MINI REVIEW
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Neuroprotection Targeting Protein Misfolding on Chronic Cerebral Hypoperfusion in the Context of Metabolic Syndrome

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Metabolic syndrome (MetS) is a cluster of risk factors that lead to microvascular dysfunction and chronic cerebral hypoperfusion (CCH). Long-standing reduction in oxygen and energy supply leads to brain hypoxia and protein misfolding, thereby linking CCH to Alzheimer's disease. Protein misfolding results in neurodegeneration as revealed by studying different experimental models of CCH. Regulating proteostasis network through pathways like the unfolded protein response (UPR), the ubiquitin-proteasome system (UPS), chaperone-mediated autophagy (CMA), and macroautophagy emerges as a novel target for neuroprotection. Lipoxin A4 methyl ester, baclofen, URB597, N-stearoyl-L-tyrosine, and melatonin may pose potential neuroprotective agents for rebalancing the proteostasis network under CCH. Autophagy is one of the most studied pathways of proteostatic cell response against the decrease in blood supply to the brain though the role of the UPR-specific chaperones and the UPS system in CCH deserves further research. Pharmacotherapy targeting misfolded proteins at different stages in the proteostatic pathway might be promising in treating cognitive impairment following CCH.

Keywords: metabolic syndrome, chronic cerebral hypoperfusion, neuroprotection, protein misfolding, endoplasmic reticulum stress, chaperones, neurodegenerative diseases

INTRODUCTION

The energy requirements of the brain are high, and neuronal viability critically depends on cerebral blood flow (CBF) delivery of oxygen and nutrients (Daulatzai, 2017). Endothelial cells interact with pericytes, glial cells, and neurons to coordinate functions in a neurovascular unit (NVU) (Hermann and ElAli, 2012). Endothelial dysfunction is typically associated with metabolic syndrome (MetS) (Otero-Losada et al., 2013, 2014), and affects CBF distribution and NVU integrity (McCrimmon et al., 2012) bringing about chronic cerebral hypoperfusion (CCH). CBF impairment increases the risk of cognitive decline shaping a neurovascular pathway to sporadic Alzheimer's disease (AD) (Zlokovic, 2011). Since an adequate protein folding and trafficking in neurons depends on energy supply by CBF (Wang and Kaufman, 2016), protein misfolding might be considered a mechanism linking CCH with AD. On these grounds, targeting proteome homeostasis

portrays a promising neuroprotective approach in AD prevention (Jackrel and Shorter, 2017; Sweeney et al., 2017) in the context of MetS.

CHRONIC CEREBRAL HYPOPERFUSION AS A SILENT CONSEQUENCE OF MetS

MetS is the constellation of vascular risk factors including hypertriglyceridemia, hyperglycemia, and/or insulin resistance, hypertension, and visceral obesity in man (Otero-Losada et al., 2016). These factors embody the prelude to type 2 diabetes (T2D) (Bruce and Hanson, 2010), characterized by hyperglycemia, hyperinsulinemia and low insulin sensitivity (American Diabetes Association, 2002; McCrimmon et al., 2012). Type 2 diabetes is usually concurrent with several features of the MetS, which contribute to its severity (McCrimmon et al., 2012). Defective angiogenesis in T2D leads to immature vascularization (Li et al., 2010), CCH, neuro-glial dysfunction, and degeneration in time (ElAli et al., 2013). Endothelial injury and microvascular dysfunction associated with MetS (McCrimmon et al., 2012) narrows luminal spaces during sustained periods of high metabolic demand (de la Monte, 2014) leading to CCH and cerebral metabolic suffering (Daulatzai, 2017; Obadia et al., 2017).

Insufficient brain perfusion in CCH is chronic, silent, and may last for years, progressively damaging brain tissue and should be distinguished from sudden CBF obstruction due to brain ischemia (de La Torre, 2008; de la Torre, 2012). Besides, moderate hypoperfusion (a 30% CBF rate decrease in De Jong et al., 1999) and mild neuronal damage observed in experimental models of CCH (Farkas et al., 2007) contrast with the full CBF deprivation found in acute ischemia models (Jia et al., 2015; Park and Lee, 2017).

Clinical studies on the circulatory impact of MetS factors' prevalence show that progressive clustering of MetS factors escalate microvascular damage due to further weakening of the cerebral arterial vasodilatation response (Nazzaro et al., 2013). The concurrence of three or more MetS features leads to a substantial CBF decrease mainly in the mediolateral areas of the frontal, parietal, temporal, and occipital gray matter and weakening of the immediate memory (Birdsill et al., 2013), attention, processing speed, executive functions, fluid intelligence, and visuospatial processing (Dik et al., 2007; Muller et al., 2010; Reijmer et al., 2011). Accordingly, on cerebral perfusion matters, MetS should be studied as a whole rather than as the mere concurrency of multiple scattered factors (Mellendijk et al., 2015).

A systematic revision of longitudinal population-based studies on the contribution of different MetS features to the risk of dementia confirmed an association as for hypertension, dyslipidemia, obesity, and T2D, the two latter imposing the greatest risk (Kloppenborg et al., 2008). The progressive brain damage associated with T2D is known as diabetic encephalophathy (Van den Berg et al., 2007). However, identifying the actual factors responsible for diabetic encephalophathy is hampered not only by the presence of multiple vascular comorbidity factors in MetS (McCrimmon et al., 2012) but also by co-variables like glycemic control, disease history, and treatment modality in T2D (Van den Berg et al., 2007).

Murine models of MetS and T2D showed time-dependent cerebrovascular impairment. Mice fed a high-fat diet (HFD) for 12 weeks developed arteriolar damage in the brain and CBF alteration (Lynch et al., 2013), and cerebral endothelial dysfunction appeared around the week 5 in a mouse model of streptozotocin-induced diabetes (Kitayama et al., 2006). In both studies, the endothelial dysfunction in cerebral arterioles preceded that in the carotid arteries (Kitayama et al., 2006; Lynch et al., 2013). We reported carotid damage in another mice model 8 weeks after MetS induction (Otero-Losada et al., 2013), so time-dependency may vary upon the experimental settings. Recent evidence revealed that aging exacerbated cerebrovascular alterations in the hippocampus and the cerebral cortex that ushered cognitive impairment in HFD mice (Tucsek et al., 2014). Furthermore, cells exposed to hypoxia and high glucose underwent autophagy dysregulation, and impaired mitochondrial quality control, reproducing CCH and diabetes *in vitro* (Song et al., 2018).

CHRONIC CEREBRAL HYPOPERFUSION AND SPORADIC AD

Long before cognitive decline becomes apparent, CCH stands for an early sign of sporadic AD (Daulatzai, 2017; de la Torre, 2017). In this regard, the sporadic AD has been described as a vasocognopathy, a vascular-related cognitive disorder (de La Torre, 2004) upon CCH pathogenic requirement (Austin et al., 2011). The long-standing decline in cerebral circulation triggers a neuronal energy crisis and a pathogenic cascade giving way to the characteristic cognitive decline (de La Torre, 2008) in CCH (Tanashyan et al., 2016).

As sporadic AD shows aggravated hypoperfusion from the pre-clinical phases to the advanced stages with the progression of the disease, CCH may represent a promising biomarker in the early diagnosis of AD (Austin et al., 2011). In this regard, the interest on the critical role of vascular risk factors like hypertension, hypercholesterolemia, and diabetes, and the ensuing CCH (Austin et al., 2011) in the early stages of the sporadic AD (Chen et al., 2011) has lately increased. The primary CBF deficiency concept has replaced that of secondary deficiency aiming to better understand the initial memory loss in AD (Mazza et al., 2011).

Most studies have focused on oxidative stress and neuroinflammation to explain the association between CCH and AD (Zhao and Gong, 2015). Beyond them, protein misfolding and aggregation emerges as a novel relevant mechanism (Jackrel and Shorter, 2017). Extraneuronal accumulation of β-amyloid peptide (Aβ) is found in the senile plaques long before cognitive AD deficits. Distinctively, intraneuronal tau protein aggregates in neurofibrillary tangles (NFTs) appear later upon clinical

progression impairing axonal transport and synaptic function (Ashraf et al., 2014).

Not only neuronal cells are particularly vulnerable to protein aggregation, but also their unique cellular structure precludes protein quality control. Post-mitotic neurons are unable to remove cytotoxic proteins after cell division (Ciechanover and Kwon, 2015), and protein aggregates in dendrites and axons need to be packaged into autophagic vacuoles to return to the cell body for lysosomal degradation. While aging slows-down protein quality control systems (Ciechanover and Kwon, 2015), age-related stress and protein misfolding play a major role in cerebral proteopathies, the sporadic forms of the neurodegenerative disease (Saxena and Caroni, 2011). MetS is a high-risk condition for premature aging-related changes (Otero-Losada et al., 2011, 2016).

PROTEOME HOMEOSTASIS AND NEURODEGENERATIVE PROTEIN DISORDERS

ER Stress and Misfolded Proteins Clearance Mechanisms

The endoplasmic reticulum (ER) plays a pivotal role in the high energy-demanding protein folding and trafficking processes. Energy restriction under stressing conditions leads to unfolded or misfolded proteins' accumulation in the ER lumen (Wang and Kaufman, 2016). In this scenario, triggering the unfolded protein response (UPR), an adaptive function of protein quality control that reduces polypeptide synthesis, improves correct protein folding, and promotes misfolded protein degradation, restores cell homeostasis avoiding apoptosis (Sims-Robinson et al., 2016; **Figure 1**). Three main signaling pathways are activated under ER stress conditions: the inositol-requiring enzyme 1a (IRE1α), the protein RNA-like endoplasmic reticulum kinase (PERK), and the activating transcription factor 6 (ATF6) (Lindholm et al., 2017; **Figure 1**). The endoribonuclease IRE1α produces an active form of the transcription factor X-box binding protein-1 (XBP-1) triggering the UPR which upregulates chaperone genes involved in protein folding (Lindholm et al., 2017). Chaperones help new proteins in their timely degradation and adequate folding, without influencing their final structure (Balchin et al., 2016). In this way, proteostasis or proteome functional homeostasis is partially restored (Lindholm et al., 2017; **Figure 1**). Conjoinctly, ER stress activates PERK which phosphorylates the eukaryotic translation initiation factor-2α (eIF2α), down-regulating protein synthesis and decreasing misfolded proteins in the ER (**Figure 1**). Finally, under ER stress conditions, ATF6 migrates to the nucleus and activates genes of ER chaperones (Cybulsky, 2013).

Endoplasmic reticulum stress also increases protein degradation in the ER lumen and membrane by way of the ER-associated degradation (ERAD) mechanism which, together with soluble cytoplasmatic misfolded proteins, produce the activation of clearance mechanisms such as the ubiquitin (Ub)-proteasome system (UPS) (**Figure 1**). In eukaryotic cells, the UPS is the most important degradation pathway for a broad range of short-lived proteins which regulate cellular processes and those of quality control of protein folding and proteotoxic stress. The main protease of the UPS is the proteasome which degrades substrates bearing a poly-ubiquitin chain (Chowdhury and Enenkel, 2015; Ji and Kwon, 2017; **Figure 1**).

Chaperone-mediated autophagy (CMA), another line of defense against misfolded proteins, is a branch of the autophagy-lysosome system (**Figure 1**). The heat-shock cognate 70 (Hsc70) chaperone recognizes misfolded proteins exposing the KFERQ degradation signal, luring them to lysosomes for degradation by hydrolases (Ciechanover and Kwon, 2015).

Substrates escaping the surveillance mechanisms are not vulnerable to the mentioned proteolytic pathways and tend to form aggregates, which are mainly removed by macroautophagy (**Figure 1**). Misfolded protein substrates of macroautophagy are recognized by molecular chaperones, ubiquitinated, and delivered into autophagosomes, which are later incorporated to lysosomes and undergo degradation (Ciechanover and Kwon, 2015).

Misfolded proteins also trigger the UPR (mt) mitochondrial response, characterized by distinctively own protein quality control system (De et al., 2018) whereby nuclear transcription of mitochondrial chaperones takes place for organelle homeostasis (Lindholm et al., 2017; **Figure 1**).

All the above-mentioned quality control systems, a collection of chaperones and protein degradation machinery working to balance the proteome, embody the proteostasis network (**Figure 1**). Nevertheless, there are some aggregated proteins resistant to quality control systems representing a common molecular mechanism reported for a group of so-called protein misfolding disorders (PMD) like the AD, Parkinson's disease (PD), Huntington's disease (HD), and others (Ciechanover and Kwon, 2015). A defective ER regulation of many cellular processes may contribute to the progression of the pathology (Lindholm et al., 2017) while proteostasis alteration leading to chronic activation of the UPR and other surveillance mechanisms may contribute to the pathogenesis of many diseases (Ozcan and Tabas, 2012).

The deposition and accumulation of misfolded proteins is a common sign in many neurodegenerative diseases, characterized by cell signaling impairment and defective neuronal connectivity following cell death (Soto, 2003). Under cellular ER stress, clearance and protein refolding pathways are activated while the UPS and other systems are mitigated by protein aggregates or toxic products including reactive oxygen species (Soto, 2003). The dysfunctional UPS caused by the accumulation of proteins in the cell furthermore aggravates ER stress (Ciechanover and Brundin, 2003). Finally, altered proteostasis in this stressful cell environment affects mitochondria, caspases are activated, and cell death ensues (Rao et al., 2004).

ER Stress in Alzheimer's Disease and Other Proteopathies

The hallmarks of Alzheimer's disease (AD) are the aggregation of extracellular amyloid-β peptides and intracellular phosphorylated tau proteins, and the abnormal intracellular

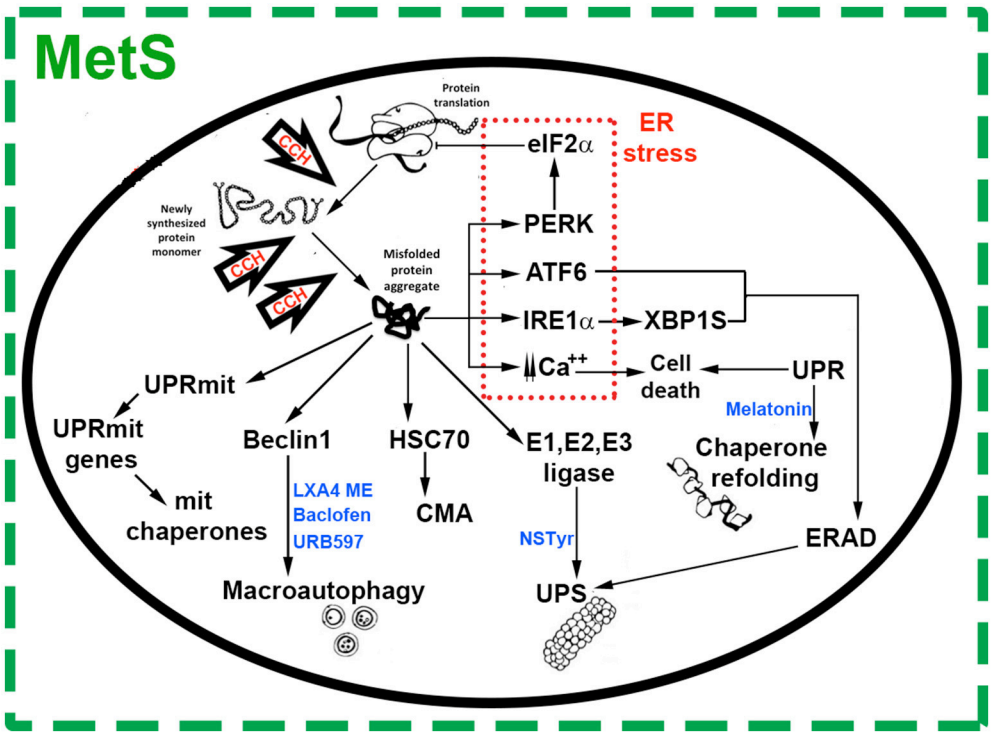

FIGURE 1 | Chronic Cerebral Hypoperfusion (CCH) induces protein misfolding in the context of Metabolic Syndrome (MetS). Under cell stress, proteostasis network surveillance systems refold or degrade proteins through several mechanisms. Accumulation of misfolded proteins in the Endoplasmic Reticulum (ER) triggers the unfolded protein response (UPR) which induces an upregulation in the expression of chaperone genes. This response is characterized by transcription factor 6 (ATF6), inositol-requiring enzyme 1a (IRE1-α) and X box–binding protein 1 (Xbp1). These ER stress components trigger ER-associated degradation (ERAD) genes, which work to restore organelle function and maintain cell survival. Under ER stress, protein RNA-like ER kinase (PERK) mediates the phosphorylation of eukaryotic translation initiator factor 2α (eIF2α), which inhibits translation and attenuates protein synthesis at the ER. Prolonged ER stress increases levels of intracellular calcium, activating signals of cell death. Mitochondria responds to cell stress throughout its own protein quality control system known as UPR(mt), promoting transcription of mitochondrial chaperones and factors for organelle homeostasis. Depending on the nature, size and solubility of substrates, misfolded proteins can also be degraded by the Ubiquitin Proteasome System (UPS), Chaperone Mediated Autophagy (CMA) or macroautophagy. In general, most soluble and monomeric misfolded proteins are degraded by the UPS and CMA. Substrates targeted by CMA are bound by chaperone heat-shock cognate 70 (Hsc70) and degraded into amino acids by lysosomal hydrolases. Misfolded proteins in the cytosol are also conjugated to ubiquitin through an enzymatic cascade involving the ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-protein (E3) enzymes. Then, ubiquitinated substrates are degraded by the proteasome. Other substrates, such as protein aggregates, are recognized by molecular chaperones, ubiquitinated and delivered to the autophagosome via Beclin-1 complex. The autophagosomes are fused with lysosomes to form autolysosomes, where misfolded proteins are degraded. Agents targeting proteostasis network pathways in CCH-induced protein misfolding are shown in blue color text. N-stearoyl- L-tyrosine (NSTyr), Lipoxin A4 methyl ester (LXA4 ME).

calcium levels with neuronal deterioration, that lead to death (Ozcan and Tabas, 2012). The brain of deceased AD patients showed activated UPR expressing chaperone Grp78 (Hoozemans et al., 2005), and immunohistochemical evidence of PERK and eIF2α activation (Unterberger et al., 2006; Scheper and Hoozemans, 2015). Inhibiting PERK decreased p-eIF2α levels and partially reversed memory impairments in an AD mouse model (Ma et al., 2013). The evidence unquestionably confirms the specific ER signaling effect on neurons and neuronal connections not only in the AD but also in other PMD like Parkinson Disease (PD) in which UPR dysfunction has been currently reported. Mutations in the Parkin gene impaired the degradation of unfolded proteins (Ciechanover and Kwon, 2015). Also, the accumulation of a substrate from Parkin gene led to ER stress and apoptosis, and phosphorylated forms of PERK (p-PERK) and eIF2α (p-eIF2α) increased in PD neurons (Ozcan and Tabas, 2012).

EXPERIMENTAL FINDINGS OF CCH-INDUCED NEURODEGENERATION BY PROTEIN MISFOLDING

The impact of CCH on neurodegeneration has been investigated using different animal models (Zhao and Gong, 2015). Increasing evidence shows that protein misfolding is involved in CCH-induced neurodegeneration. Both a bilateral common carotid artery occlusion (BCCAO) murine CCH model (Ozacmak et al., 2009), and another animal model of CCH designed to reproduce human hemodynamic insufficiency (Hai et al., 2002) showed a compensatory defensive neuronal loss in the

hippocampal CA1 region. Years later, ultrastructural analysis using ethanolic phosphotungstic acid (EPTA)-stained electron microscopy confirmed that neurodegeneration was induced by protein aggregation after protein synthesis machinery destruction (Jian et al., 2013).

The stress-sensitive and novel negative modulator of myelination Redd1 (RTP801/Dig2/DDIT4) expresses in response to hypoxia, ER stress, and autophagy. It underwent time-dependent changes in a rat CCH model induced by permanent BCCAO and two-vessel occlusion (2VO) (Park and Lee, 2017). Following surgery, Redd1 increased in pyramidal neurons of the CA1 region by day 7 and gradually decreased by day 28, being associated with CCH-induced neuronal damage (Park and Lee, 2017). Autophagy upregulation by CCH in bilateral common carotid artery ligation (BCCAL) and oxygen-glucose deprivation (OGD) models associated with brain atrophy and neuronal apoptosis in the CA1 area (Liu et al., 2015). Likewise, neuronal damage and high level of the autophagy markers LC3-II and beclin-1 were found in the CA1 area after CCH, along with ultrastructural evidence of an increased number of apoptotic neurons showing the typical pyknotic nuclei, autophagosomes and autolysosomes, and of ER fragmentation among other changes. These observations have been correlated with the spatial working memory impairment (Wang et al., 2017).

Cognitive and pathological features and the associated autophagic modifications were evaluated from week 2 to 20 after BCCAO to elucidate the long-term neurological effects of CCH-induced autophagy after CBF recovery, and CBF changes were monitored in a two-step BCCAO rat model. Unlike the sustained increase in the autophagy markers Beclin-1, light chain 3B, and P62, CBF returned to baseline. Regardless CBF recovery, a striking cognitive decline, and neuronal damage were observed revealing the early contribution of the early autophagy impairment to the later neurodegeneration and cognitive decline. Autophagic dysfunction may hamper the successful clearance of the Aβ peptide, leading to cognitive alterations. Other interesting findings suggested that cortical neurodegeneration and autophagic changes precede those in the hippocampus, the same as white matter damage precedes gray matter degeneration. However, only hippocampal deposition of Aβ plaques was related to cognitive failure (Zou et al., 2017). The hippocampus is the archetypical brain area of learning and memory that becomes dysfunctional in AD (Ashraf et al., 2014), and the CA1 area is specifically vulnerable to hypoperfusion (Liu et al., 2015). Hippocampal neurodegeneration also developed in a rat model of BCCAL and 2VO (Jia et al., 2015). Sustained ER stress due to CCH was inferred from the expression of the CCAAT/enhancer binding protein, C/EBP, homologous protein. Besides, CCH stimulated macroautophagy based on the ratio of microtubule-associated protein light chain 3 II (LC3-II) to LC3-I and beclin1 marker level (Jia et al., 2015).

Over and above, dysfunction of the UPS might be related to hippocampal CA1 degeneration after CCH as concluded after finding long-term locally decreased proteasome peptidase activity, and accumulation of ubiquitinated protein aggregates in a rat model (Hai et al., 2013). Earlier studies had reported similar results suggesting that the reduced activity of the UPS might impair the removal of misfolded proteins leading to protein aggregation and eventual neurodegeneration (Hai et al., 2011). The following cognitive impairment might compromise both learning and spatial memory skills concurrently with long-term potentiation (LTP) inhibition (Hai et al., 2009). Right common carotid artery permanent ligation also induced protein aggregation and mild CCH resulted in NVU dysfunction and rapid Aβ deposition in ipsilateral brain capillaries (ElAli et al., 2013). The same as observed in amyloid protein precursor (APP)- transgenic (APP-Tg) mice, bilateral carotid artery stenosis (BCAS) induced CCH-accelerated Aβ deposition. Accordingly, CCH might precipitate the neurodegenerative process in AD (Kitaguchi et al., 2009). Previous findings in a rat model of BCCAL and 2VO demonstrated that CCH fostered the aberrant processing of APP (Bennett et al., 2000). Unilateral common carotid artery occlusion (UCCAO) resulted in CCH and induced tau hyperphosphorylation, memory deficits, dysregulation of synaptic proteins, and decreased post-translational tau O-GlcNAcylation by β-N-acetylglucosamine (Zhao et al., 2014). Earlier studies had suggested that brain glucose metabolic dysfunction down-regulated tau O-GlcNAcylation mediated by tau hyperphosphorylation (Liu et al., 2004; Liu F et al., 2009; Liu Y et al., 2009).

CCH in AD leads to increased tau protein hyperphosphorylation and intracellular aggregation upon conformational changes (Šimić et al., 2016; Lathuilière et al., 2017), same as found after UCCAO in mice or 2VO in rats (Li et al., 2015; Qiu et al., 2016). Accordingly, stepwise BCCAO-induced CCH increased the cortical expression of proteins involved in protein synthesis and folding like glycine-tRNA ligase (GARS), heterogeneous nuclear ribonucleoprotein K (HNRNPK), nitrilase homolog 1 (NIT1), histidine triad nucleotide-binding protein 1 (HINT1), ATP-dependent RNA helicase DDX1, and the protein disulphide-isomerase A6 (PDIA6). Proteins involved in ubiquitin-mediated degradation also increased, including the COP9 signalosome complex subunit 2 (COPS2), the proteasome subunit alpha type-1 (PSMA1), the 26S protease regulatory subunit 6A (PSMC3), and the 26S protease regulatory subunit 6B (PSMC4) (Völgyi et al., 2017).

Genetic risk factors like the apolipoprotein E (APOE) gene cause vascular impairment (Farrer et al., 1997). In the elderly, mounting evidence suggests that AD links to atherosclerosis under brain hypoperfusion (de la Torre, 2002). In the general population, the e4 allele of the APOE gene poses the highest risk for sporadic AD (Farrer et al., 1997). It is a modest genetic risk factor for atherosclerosis (Wilson et al., 1996), associated with decreased efflux of cholesterol from cultured neurons (Michikawa et al., 2000). It might also boost APP-to-Aβ production (Casserly and Topol, 2004), contribute to proteostasis dysregulation impairing Aβ plaques' clearance, foster Aβ oligomer formation, and increase tau hyperphosphorylation (Casserly and Topol, 2004; Inbar et al., 2010; Argon and Gidalevitz, 2015).

NEUROPROTECTIVE AGENTS TARGETING PROTEIN MISFOLDING IN CCH

Lipoxin A_4 methyl ester (LXA_4 ME) ameliorated hippocampal degeneration in rat under CCH induced by BCCAL and 2VO, attributable to regulation of ER stress and macroautophagy decreasing the level of C/EBP homologous protein, beclin-1, and the LC3-II-to-LC3-I ratio (Jia et al., 2015). Alternatively, the activation of the extracellular signal-regulated kinase/nuclear factor erythroid 2-related factor 2 (ERK/Nrf2) pathway might account for LXA_4 ME neuroprotection (Jin et al., 2014). Also, baclofen has protective properties against hippocampal atrophy and neuronal apoptosis after CCH. Chronic treatment with baclofen induced suppression of the cytodestructive autophagic activity through protein kinase B (Akt)/ERK- B-cell lymphoma 2 (Bcl2)-beclin-1 signaling pathway and up-regulation of the protective autophagy activating the ionotropic metabotropic γ-aminobutyric acid $(GABA)_A$ receptor-connexin $(CX)_{43}/CX_{36}$ signaling pathway. Since autophagy is a double-edged sword mechanism, bi-directional regulative effects on autophagy render neuroprotection (Liu et al., 2015). The modulation of autophagy also provides neuroprotection in a murine model of CCH through BCCAO. Treatment with the fatty acid amide hydrolase (FAAH) inhibitor URB597, might regulate autophagy, suppress apoptosis, and ameliorate ultrastructural neurodegeneration and cognitive decline in the CA1 area via the m-TOR pathway. This FAAH inhibitor also reversed the CCH-induced decrease in cannabinoid receptor (CB)1 level (Wang et al., 2017).

Melatonin administration modulated CCH-induced stress protein expression restoring chaperone HSP70 level in the hippocampus in a rat model of BCCAO (Ozacmak et al., 2009). N-stearoyl-L-tyrosine (NSTyr), an analog of the endogenous endocannabinoid anandamide (AEA), regulated the UPS and induced neuroprotection in rat hippocampus increasing proteasome peptidase activity and consequently inhibiting ubiquitinated proteins' intracellular aggregation (Hai et al., 2013). Previously, NSTyr had mitigated the cognitive deficits and restored hippocampal levels of the microtubule-associated protein 2 (MAP-2) and the synaptophysin protein in rats subjected to CCH (Lin et al., 2010). Besides, NSTyr induced neuroprotective effects on rat brain slices under OGD as well (Yao et al., 2009) (**Table 1**). In our laboratory, we have also found an AEA analog, Palmitoylethanolamide, could reverse behavioral dysfunctions and attenuate alterations in hippocampal MAP-2 levels in a murine model of acute hypoxia (Herrera et al., 2018). This experimental model of hypoxia is also known for inducing protein ubiquitination (Herrera et al., 2017).

FUTURE DIRECTIONS

Neuroprotective targets in protein misfolding are represented by the different steps in the production and processing of proteins: synthesis, folding, repair, and degradation. The down-regulation of translation stands for an initial approach, aimed at reducing the load on molecular chaperones system. Enhancing and potentiating this system might represent an alternative approach since chaperones are responsible for the adequate protein folding and conformational repair when necessary. The up-regulation of the degrading pathways is another possibility along with non-toxic inclusions formation (Sweeney et al., 2017). Additionally, ER stress attenuation may protect from protein misfolding and aggregation. To date, increasing evidence pinpoints this strategy as a promising intervention in different animal models of neurodegeneration via genetic or pharmacological therapy (Hetz and Mollereau, 2014).

CCH is a chronic and silent disease characterized by years-standing of insufficient brain perfusion, concurrent with the worldwide highly prevalent MetS. Several studies were published using different models of CCH focusing mainly on inflammatory processes and cell death. Little research has paid attention to early signs of neurodegeneration like protein misfolding. The adequate function of the protein folding machinery critically depends on CBF and appears as a potential mechanism linking CCH with AD. With regard to CCH, active research is ongoing to uncover the mechanisms responsible for proteostatic network disbalance like the chaperones, UPR, and autophagy. Cellular autophagy is one of the most studied pathways of proteostatic cell response to insufficient brain blood supply. Future studies are encouraged to evaluate how mTOR affects autophagy and ER stress. Also, regulating the degree of eIF2α phosphorylation, which can be modified using specific compounds, may offer a promising approach to control ER stress in various diseases (Lindholm et al., 2017). Further investigation is required to explore the role of specific chaperones and the UPR system in CCH models. Conjointly, different animal models should be brought

TABLE 1 | Neuroprotective agents targeting protein misfolding in CCH.

	Agent	Pathway	Proteostatic complex	References
NSTyr	N-stearoyl- L-tyrosine		UPS	Hai et al., 2013
LXA4 ME	Lipoxin A4 methyl ester	m-TOR	ER stress—macroautophagy	Jia et al., 2015
Melatonin		HSP70	HSP70	Ozacmak et al., 2009
Baclofen	$GABA_B$ receptors agonist		Autophagy	Liu et al., 2015
URB597		m-TOR	Autophagy	Wang et al., 2017

This table summarizes the most relevant findings on targeting protein misfolding in CCH over the last few years. Chronic Cerebral Hypoperfusion (CCH), Ubiquitine Proteosome System (UPS), Heat Shock Protein (HSP).

about to broaden our knowledge in this matters. Therapeutic drug options targeting misfolded proteins at different points in the proteostatic pathway are likely to emerge as promising neuroprotective treatments for cognitive impairment following CCH.

AUTHOR CONTRIBUTIONS

MH: writing—original draft; LU: supervision; NT-U: supervision; CK: Supervision; JL: writing—review and editing; MO-L: original idea—review and editing—grammar, style and language; FC: funding acquisition, writing—review and editing.

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REVIEW
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Metabolic Syndrome and Neuroprotection

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Introduction: Over the years the prevalence of metabolic syndrome (MetS) has drastically increased in developing countries as a major byproduct of industrialization. Many factors, such as the consumption of high-calorie diets and a sedentary lifestyle, bolster the spread of this disorder. Undoubtedly, the massive and still increasing incidence of MetS places this epidemic as an important public health issue. Hereon we revisit another outlook of MetS beyond its classical association with cardiovascular disease (CVD) and Diabetes Mellitus Type 2 (DM2), for MetS also poses a risk factor for the nervous tissue and threatens neuronal function. First, we revise a few essential concepts of MetS pathophysiology. Second, we explore some neuroprotective approaches in MetS pertaining brain hypoxia. The articles chosen for this review range from the years 1989 until 2017; the selection criteria was based on those providing data and exploratory information on MetS as well as those that studied innovative therapeutic approaches.

Pathophysiology: The characteristically impaired metabolic pathways of MetS lead to hyperglycemia, insulin resistance (IR), inflammation, and hypoxia, all closely associated with an overall pro-oxidative status. Oxidative stress is well-known to cause the wreckage of cellular structures and tissue architecture. Alteration of the redox homeostasis and oxidative stress alter the macromolecular array of DNA, lipids, and proteins, in turn disrupting the biochemical pathways necessary for normal cell function.

Neuroprotection: Different neuroprotective strategies are discussed involving lifestyle changes, medication aimed to mitigate MetS cardinal symptoms, and treatments targeted toward reducing oxidative stress. It is well-known that the routine practice of physical exercise, aerobic activity in particular, and a complete and well-balanced nutrition are key factors to prevent MetS. Nevertheless, pharmacological control of MetS as a whole and pertaining hypertension, dyslipidemia, and endothelial injury contribute to neuronal health improvement.

Conclusion: The development of MetS has risen as a risk factor for neurological disorders. The therapeutic strategies include multidisciplinary approaches directed to address different pathological pathways all in concert.

Keywords: metabolic syndrome X, neuroprotection, hypoxia, brain, oxidative stress, antioxidants

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INTRODUCTION

Definition

Metabolic syndrome (MetS) is a disorder characterized by a cluster of conditions that increases the risk of developing cardiovascular disease (CVD) and Type 2 Diabetes Mellitus (DM2) (International Diabetes Federation, 2006). Currently, there are still controversies among the different Health Organizations on the selection criteria for this syndrome. The most accepted diagnostic tool is the global consensus described by the International Diabetes Federation (IDF) (International Diabetes Federation, 2006) that entails the presence of:

- Central obesity (based on waist circumference): ≥80 cm for women and ≥90 cm for men from the Hispanic background (values vary with ethnicity).
- Plus two or more of the following parameters:

1. **Hypertriglyceridemia**: ≥ 150 mg/dL, or under treatment for this lipid abnormality.
2. **HDL-cholesterolemia below recommendations**: <40 mg/dL in men and <50 mg/dL in women, or history of specific treatment for this lipid abnormality.
3. **Hypertension**: SBP ≥ 130 mmHg and/or DBP ≥ 85 mmHg over 24 h.
4. **Fasting Hyperglycemia**: ≥100 mg/dL, hyperinsulinemia, or DM2.

Metabolic syndrome (MetS) has been associated with hepatic steatosis, respiratory illness, osteoarticular disease, and cancer (Siegel and Zhu, 2009). The main mechanism implicated in the pathogenesis of MetS is the resistance to insulin (IR), namely the insufficient response to physiological insulin levels (Eckel, 2005). Other aspects that can shape MetS pathology are environmental stressors, mostly chemical, infections, lifestyle (sedentary habits, smoking, and nutritional factors), genetic predisposition, and other chronic diseases. These characteristics have one common denominator which is the generalized pro-oxidative status, in turn favoring free radical generation and resulting in oxidative stress (Roberts and Sindhu, 2009). The specific metabolic pathways typically affected by the development of MetS are discussed in more detail later in this work.

Epidemiology

Metabolic syndrome (MetS) affects nearly 30% of the world population, associated with a 2–3-fold increase in morbidity and mortality compared with healthy people (Engin, 2017). In regard to the global statistics of MetS, an important item to highlight is the lack of unanimity on the diagnosis of this syndrome, specifically derived from the regional variation of the cut-off values for waist circumference linked to ethnicity (Borch-Johnsen, 2013). Therefore, the percentage calculated on a worldwide scale is only approximated and should be adjusted based on the prevalent ethnicity in a national scope.

Despite any criteria discrepancy, the need to reduce the prevalence of this disorder on a global scale becomes relevant. Not only does the presence of MetS reduce life expectancy and quality of life, but it also causes a financial burden derived from high health costs (Rask-Madsen and Kahn, 2012). For this reason, the World Health Organization (WHO) has as a priority to lower the worldwide prevalence of non-communicable diseases like CVD, DM2, and cancer, globally accounting for 63% of overall deaths. One example of this intended approach is the taxing of sweetened beverages.[1]

As aforementioned, MetS increases mortality and morbidity and is associated with accelerated aging (Rask-Madsen and Kahn, 2012). The aging process *per se* increases an individual's susceptibility to developing CVD or DM2. Interestingly, some reports substantiate how MetS even in the absence of CVD or DM2 also renders higher morbimortality (Borch-Johnsen, 2013).

The relevance of MetS in the modern industrialized society is undeniable. Its staggering global prevalence and concomitantly diminished quality of life rank this disorder as a major public health concern.

Effects of Metabolic Syndrome on the Nervous System

Over the years, the importance of MetS pertaining cardiovascular risk and progression to DM2 has been carefully studied and extensively divulged, for CVD is the leading cause of death worldwide.[2] However, research has been scarce with regard to the effects of MetS on nervous tissue. In the recent years, the ever-growing evidence suggests a correlation between Alzheimer's disease (AD) and other cognitive impairments, and MetS. These results suggest that this syndrome does not only act as a risk factor for CVD and DM2 but also contributes to the progression toward AD (Kim and Feldman, 2015).

The nervous tissue has two vastly different cell populations: neuronal and glial cells. Neurons are highly specialized cells that propagate electrical stimulus in order to accomplish synaptic transmission, while the glia (composed mainly by astrocytes, oligodendrocytes, and microglia) is responsible for maintaining the homeostasis in nervous tissue. The brain depends upon glucose as its main source of energy, and a tight regulation of glucose metabolism and ATP reserves are critical for brain physiology (Mergenthaler et al., 2013; Brusco et al., 2014).

The aim of this review is:

1. First, to revise the pathophysiology of MetS and the consequences of the intrinsically altered metabolism in the nervous tissue.
2. To propose and explore different therapeutic approaches aimed at reducing the compromised neuronal function and neurodegenerative damage in MetS.

PATHOPHYSIOLOGY

Overview

It is imperative to acknowledge that MetS develops in susceptible individuals bearing genetic factors and engaging in certain epigenetic unhealthy habits like a sedentary lifestyle, excessive

[1]WHO Mid-term Programmatic and Financial Report for 2016–2017 including audited financial statements for 2016 (WHA 70/40) (http://www.who.int/about/finances-accountability/reports/en/).

[2]World Health Organization. The top 10 causes of death (Fact sheet) Updated January 2017 http://www.who.int/mediacentre/factsheets/fs310/en/.

consumption of high energy foods and drinks, smoking, and many others. This complex disorder is characterized by a sustained positive energy balance, which progressively breeds a mild inflammatory environment due to the activation of abnormal metabolic pathways (Kaur, 2014). Pivotal mechanisms implied in MetS were described in this review: hyperglycemia, insulin resistance (IR), inflammation and oxidative stress.

Patients with long-term MetS may be prone to develop diabetic encephalopathy due to the diabetogenic milieu, entailing moderate cognitive deficits, and both neurophysiological and structural changes in the brain (Biessels et al., 2002). Passos et al. demonstrated that the senescent cells had higher reactive oxygen species (ROS) concentration, dysfunctional mitochondria, more DNA double-strand breaks and shorter telomeres. It was also shown that mitochondrial ROS enhanced telomere-dependent senescence (Passos et al., 2007). Likewise, some authors showed the link between telomere length and metabolic disease suggesting increased cellular turnover and therefore accelerated cell aging (Bonomini et al., 2011; Kong et al., 2013). The increasing abdominal adiposity typical of the MetS is accompanied by accelerated telomere attrition (Révész et al., 2015).

Hyperglycemia

It is well-known that a high concentration of blood glucose triggers diverse metabolic pathways. The most evident is the tricarboxylic acid (TCA) cycle, due to an abundant amount of substrate that in turn feeds the succeeding electron transport chain (ETC). This energy surplus creates an imbalance of partially reduced oxygen species favoring oxidant over antioxidant compounds and resulting in oxidative stress (Kawahito et al., 2009).

Another thoroughly described mechanism is the network of glycation reactions bringing forth oxidative stress alongside glucose toxicity (Kaneto et al., 1996). Advanced glycation end-products (AGEs) are formed as a result of these non-enzymatic reactions and promote inflammation by interacting with the receptor of AGEs (RAGE) in cells of the immune system (Gkogkolou and Böhm, 2012). The activation of the RAGE triggers abundant intracellular signaling pathways including kinases (e.g., MAP kinases, PI3 kinase), transcription factors as the nuclear factor-κB (NFκB), and the activator protein-1. This signaling cascade activates the further expression of cytokines, chemokines, enzymes, and growth factors resulting in an overall proinflammatory environment which leads to oxidative stress (Medzhito and Horng, 2009).

Moreover, hyperglycemia reduces antioxidant levels and increases the production of free radicals. The enzymes superoxide dismutase (SOD) and catalase or glutathione peroxidase involved in the antioxidant defense are down-regulated in the diabetic brain (Suresh Kumar and Menon, 1993; Makar et al., 1995; Miranda et al., 2007; Alvarez-Nölting et al., 2012). The possible source of oxidative stress in brain injury, however, also includes auto-oxidation of glucose, lipid peroxidation, and decreased tissue concentrations of low molecular weight antioxidants like reduced glutathione (GSH) (Reagan et al., 2000; Grillo et al., 2003; Ulusu et al., 2003; Muriach et al., 2006). This alteration of reduced glutathione levels may be related to an increased activity of the polyol pathway as this leads to a depletion of the NADPH required for enzymatic reduction of the oxidized glutathione (Preet et al., 2005).

Overall, hyperglycemia creates a pro-oxidative environment in live tissue involving various mechanisms. This condition causes a detrimental effect on various cells and tissues, especially those more vulnerable due to an insufficient antioxidant defense like the pancreatic β-cells (Kaneto et al., 1999).

Insulin Resistance

Insulin is well-known for its role in the regulation of glucose metabolism in the body. However, insulin has other roles in the central nervous system (CNS) pertaining cognitive processes, memory and synaptic plasticity (Zhao and Alkon, 2001). Accordingly, either the deficiency of insulin or hyperinsulinemia characteristic of type 1 or type 2 diabetes mellitus respectively could be associated with degenerative events in the brain (Xu et al., 2004).

Recent studies have suggested that the nervous system is also capable of developing resistance to insulin. This is possible even though neurons are not dependent on insulin, they bear insulin receptors and are insulin-responsive (Belfiore et al., 2009). Insulin receptors are expressed in brain areas as the olfactory bulb, cerebral cortex, hippocampus, hypothalamus and amygdala. Resistance to insulin in sensory neurons affects the cellular response to growth factors, leading to neurodegeneration, and neuropathy over time. In regard to mitochondrial metabolism, insulin regulates the PI3K/Akt (phosphatidylinositol 3-kinase/serine/threonine-specific Protein Kinase B) intracellular transduction signaling pathway (Stiles, 2009; Cheng et al., 2010) which promotes survival and growth in response to extracellular signals. This, in turn, affects neuronal mitochondria, resulting in increased oxidative stress (Fisher-Wellman and Neufer, 2012).

In addition, evidence has convincingly reported the involvement of the mechanistic mammalian target of rapamycin (mTOR) in cellular senescence. This pathway is activated by an insulin dependent signal. The mTOR modulates cell growth and cellular metabolism in response to growth factors, nutrients, and cellular energy conditions. The loss of mTOR signaling disrupts multiple responses in glucose metabolism, energy production, mitochondrial function, and cell growth (Blagosklonny, 2013).

Inflammation

Inflammation is a biologically essential process which stands as a common denominator in various pathological circumstances. The inflammatory reaction is triggered in response to tissue damage in an attempt to restore tissue homeostasis via repairing mechanisms. In physiological conditions, the inflammatory reaction is controlled and self-limited. However, when the fine orchestrated regulation of these mechanisms is disrupted, the uncontrolled inflammatory response usually ends in collateral damage (Goldszmid and Trinchieri, 2012).

The amazingly complex immune system mediates the response to unknown stimuli. When the inflammatory response is unable to restore homeostasis, systemic, and cellular stress

persists and physiological abnormalities develop (Okin and Medzhitov, 2012). Diabetes is an example of a chronic inflammatory disease (Pacher et al., 2007).

The production of ROS is a typical response to the stimulation of immune system cells (Meier et al., 1989, 1990). Both, acute and chronic inflammatory states have redox equilibrium alterations due to the increased generation of oxidizing agents (Pacher et al., 2007; Roberts et al., 2010; Li et al., 2013; Rochette et al., 2013). Toll-like receptors (TLRs) activate NFκB, a regulator of inflammation that is controlled by hundreds of genes and is also a redox-sensitive nuclear factor, are at a key juncture between the regulation of oxidative stress and inflammation.

Different experimental models from studies have described that the activation of NFκB and proinflammatory cytokines is associated to neuronal dysfunction, neuronal loss and impaired cognitive function (Mattson and Camandola, 2001; Vincent et al., 2002; Cai and Liu, 2012; Li et al., 2013). Activated NFκB can lead to oxidative stress-induced cell dysfunction or death due to the induction of cytotoxic products, which exacerbate inflammation and promote apoptosis (Pahl, 1999; Morgan and Liu, 2011).

Overnutrition is considered as an independent environmental factor which activates the innate immune system and triggers an atypical form of inflammation leading to metabolic dysfunction in the CNS (Cai and Liu, 2012). This inflammatory cascade can also affect the hypothalamus and impair appetite control, energy expenditure, carbohydrate and lipid metabolism, and blood pressure homeostasis (Kahn and Flier, 2000; Lam et al., 2005; Meister, 2007; Schenk et al., 2008; Zhang et al., 2008; Shoelson and Goldfine, 2009). The molecular pathway involved in this dysfunction is the activation of IKKβ/NFκB (Sonoda et al., 2008; Cai, 2009; Lumeng and Saltiel, 2011).

The endoplasmic reticulum (ER) also plays a key role in the metabolic imbalance caused by oxidative stress since it depends on IKKβ/NFκB pathway activity (Zhang et al., 2008; Purkayastha et al., 2011) and causes ROS accumulation (Cullinan and Diehl, 2006). Sustained exposure to high levels of blood glucose promotes oxidative stress generating oxidative free radicals and aberrant protein folding (Cullinan and Diehl, 2006).

Zhang et al. have reported an increase in the expression of C/EBP (CCAAT/enhancer-binding protein) homologous protein (CHOP) in the hippocampus of diabetic rats. This increase suggests that CHOP-ER stress-mediated apoptosis could be caused by hyperglycemia, impairing hippocampal synapses, and promoting diabetic cognitive dysfunction (Zhang et al., 2013).

Oxidative Stress

As previously described, IR, inflammation, and hyperglycemia all induce oxidative stress. The current concept of oxidative stress conceived by Helmut and Jones is an "imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control, and/or molecular damage" (Sies and Jones, 2007). Free radicals are highly unstable and reactive molecules which disrupt protein structure and modify the physicochemical properties of membranes resulting in organelles and cell damage. Some cardinal examples of free radicals are the well-known ROS, which are byproducts of the partial reduction of O_2 (**Figure 1**). This fundamental reaction occurs in Complex IV of the ETC during physiological respiration (Mitchell, 2012).

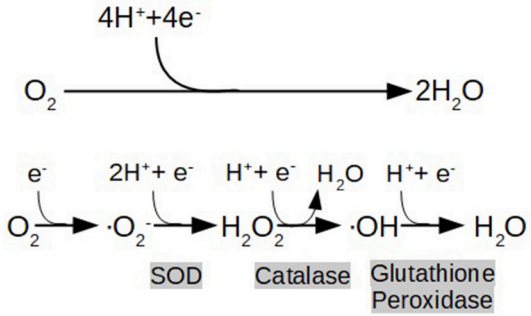

FIGURE 1 | End metabolic pathway of electron transport chain (ETC), depicting the reduction of oxygen (O_2) to water (H_2O), and the formation of reactive oxygen species (ROS) as a consequence of its partial reduction: O_2 (Superoxide) H_2O_2 (hydrogen peroxide), OH (hydroxyl radical). Highlighted in gray are shown the enzymes that catalyze the reactions (SOD:Superoxide Dimutase).

It is known that the appearance of oxygen in our world's atmosphere is directly correlated with the origin of the powerhouse organelles in cells: the mitochondria (Poderoso, 2016). The mitochondria not only supply the cell with a greater amount of ATP compared with other metabolic pathways but also play important roles in signaling apoptosis, thermoregulation, and other vital processes. The adaptation to an aerobic lifestyle benefited eukaryotic cells in many ways but also brought complications. Unfortunately, the aerobic environment generates ROS (Mitchell, 2012), and mitochondria are the main cellular source of ROS. Metabolically active tissues as the liver, heart, and brain are the major contributors of ROS to the body (Boveris and Repetto, 2016).

The amount of ROS, normally produced in low concentrations as byproducts of physiological processes like respiration, is regulated by a variety of enzymes and other molecules with antioxidant properties (Glutathione Peroxidase, Glutathione Reductase, SOD, Vitamin C, Vitamin E, and the β-carotenes) (Birben et al., 2012). When the redox homeostasis is affected, ROS are overproduced exceeding the physiological antioxidant capacity resulting in oxidative stress and causing structural and conformational changes in mitochondrial proteins, lipids, and nuclear material, thereby impairing their function. Mitochondrial dysfunction in particular, eventually leads to nerve cell damage. Since the nervous tissue is highly metabolically active and critically dependent on energy supply, it is unable to work properly in ATP shortcoming conditions (Bhat et al., 2015).

In diabetic patients, aging may be associated with brain dysfunction supported by the link between aging with cell death and oxidative stress mediated by free radicals (Beckman and Ames, 1998). In these pathological conditions, cellular stress triggers oxidative mitochondrial damage, which in turn may lead to apoptosis and/or necrosis (Merad-Boudia et al., 1998). Apoptosis-induced oxidative stress has been

linked to neurogenesis inhibition (Cui et al., 2006). Alteration in mitochondrial the ETC, ROS formation, dysfunction of mitochondrial metabolism, and oxidative stress are recognized as the main protagonists in complications related to diabetes (Moreira et al., 2009).

Likewise, many studies have revealed that homocysteine, associated with endothelial dysfunction, is responsible for the release of hydrogen peroxide which causes cellular oxidative stress and inflammation mediated by cell injury *in vitro* (Rozycka et al., 2013). There is consensus that the activated microglia is an important key mediator of proinflammatory and neurotoxic factors involved in the progression of PD and AD. These proinflammatory and neurotoxic factors include cytokines like interleukin-6 (IL-6) and interleukin-1β (IL-1β), the tumor necrosis factor-α (TNF-α) and ROS, consequently contributing to oxidative stress (Bayarsaikhan et al., 2015).

Inexorably, altered metabolic homeostasis affecting multiple metabolic pathways in MetS becomes the driving force toward oxidative stress. The MetS should be regarded not only as a predictor of CVD and DM2 but also as a silent threat to cognitive performance and a risk for neurodegeneration (Kim and Feldman, 2015). Timely assessment of risk for MetS and its related conditions may be recommended.

Hypoxia

Metabolic syndrome (MetS) is associated with an increased risk of cerebrovascular diseases, including cerebral ischemia (Aoqui et al., 2014). The abnormal metabolic pathways involved in MetS affect a broad variety of tissues, organs and systems, including the cardiovascular system. Microvascular dysfunction is particularly associated with MetS (Czernichow et al., 2010). This is not surprising since DM2 progresses toward micro and macrovascular dysfunction (Mitchell, 2012). Furthermore, high blood pressure, increased pulse wave velocity suggesting increased arterial stiffness, and low capillary density were found in individuals with MetS (Greenstein et al., 2009). Vascular degeneration brings forth a flawed circulation, which in turn leads to hypoxia in target organs like the brain.

There is ever-growing evidence for obesity associated with changes in perivascular adipose tissue, which gives forth an altered vasoactive tone of the microvasculature (Obadia et al., 2017). Some key factors that may play a crucial role regarding these vascular modifications are cardinal molecules that are increased substantially in MetS: free fatty acids and adipokines (TNF-α) (Greenstein et al., 2009). Additionally, not only do individuals with MetS have a higher likelihood of microvascular dysfunction, but they are also more susceptible to damage during ischemia-reperfusion events (Aoqui et al., 2014).

The cluster of these elements poses important risk factors toward producing hypoxia in tissues that have a strict oxygen supply requirement, such as nervous tissue. The neurovascular unit (composed of neurons, astrocytes and endothelial cells) is the structure in charge of maintaining the brain's homeostasis (Gorelick et al., 2011). Studies show that the dysfunction of this unit plays a crucial role regarding the onset of neurodegenerative conditions, such as AD (Zlokovic, 2008; Grammas, 2011; Marchesi, 2014). In fact, reports have associated stroke patients with progression toward AD, where hypoxic/ischemic injury promotes the formation of β-amyloid plaque (Guglielmotto et al., 2009).

NEUROPROTECTION

Neuroprotection encompasses the therapeutic actions to prevent or limit the progression of neuronal degeneration (Orsini et al., 2016). A vast array of noxious stimuli can trigger damage in nervous tissue. Among these, altered metabolic pathways and hypoxia are the main focus of this review (Marcano Torres, 2004).

The therapeutic approaches regarding neuroprotection discussed in this review comprise three instances: lifestyle habits, MetS symptoms medication (hypertension, hyperglycemia, and dyslipidemia), and antioxidant treatment.

Lifestyle: Nutritional Habits and Exercise

A fundamental cornerstone of neuroprotection in the context of MetS concerns striving toward physical wellness obtained by a balanced diet and exercise through a multidisciplinary approach. This item alone can revert hypertension, hyperglycemia, and dyslipidemia, without the need to implement medication (Pitsavos et al., 2006).

Nutritional Habits

Reducing daily calorie intake and adopting a dietary style like the Mediterranean diet or the Dietary Approaches to Stop Hypertension (DASH) is advisable. While both dietary strategies improve the patient biochemical profile, the DASH was more beneficial in normalizing blood pressure (Kaur, 2014). The Adult Treatment Panel III (ATP III) recommends a diet for cholesterol management containing 25–35% of total fat to reduce the low density-cholesterol (LDL-C) fraction level (National Cholesterol Education Program, 2001). We have reported that chronic consumption of cola beverages impairs metabolic homeostasis increasing glycemia, cholesterolemia, triglyceridemia, and systolic blood pressure. Systolic blood pressure and most of the biochemical parameters normalize after switching cola beverages to tap water over a sustained washout period. However, hypertriglyceridemia is resistant and persists long after discontinuing cola consumption (Milei et al., 2011; Otero-Losada et al., 2014, 2015, 2016a).

Exercise

Health professionals should indicate exercise programs, such as 30 min or more of moderate-intensity physical activity and preferably all days of the week (Thompson et al., 2003). Continuing evidence from our research reports a beneficial effect of exercise on pancreatic morphology in long-term cola-drinking rats. These results support the by now accepted positive correlation between exercise and physical wellness (Otero-Losada et al., 2016b).

However, when modifying lifestyle habits does not revert pathological values, it is time to take the next level of action including a pharmacological therapeutic approach. Nevertheless, physical wellness should always compliment the medication.

Medication Targeted Toward Mets Cardinal Symptoms

The cardinal symptoms of MetS with indicated pharmacological treatment are hypertension, dyslipidemia, and hyperglycemia.

Hypertension

At present, a vast array of antihypertensive drugs is available (Gupta and Guptha, 2010). However, the election of a specific pharmacological association should consider a holistic view of each patient, always bearing in mind the individual idiosyncrasy and any other relevant factor (Gutiérrez, 2001). There is a certain structure regarding the course of drug administration that is validated by clinical trials in hypertensive patients. Treatment should initiate with a mono-drug treatment and only escalate indicating additional drugs with different mechanism of action in patients that do not normalize their blood pressure (Rivas-Chávez et al., 2007).

The first line drugs in pharmacological therapy[3]:

- Angiotensin Converting Enzyme (ACE) inhibitors/Angiotensin II Receptor Blockers (ARBs) which inhibit receptor binding to angiotensin II.
- Dihydropyridine Calcium Channel Blockers (CCBs) which inhibit Ca^{2+} influx into smooth muscle cells in vessels.
- Thiazide Diuretics which inhibit the reabsorption of Na^{+} and Cl^{-} in the distal tubules of the nephron.

The second line drugs in pharmacological therapy are administered to patients with risk factors or developing side effects from the first-line drugs (Gutiérrez, 2001; Sweitzer, 2003; De Luis Román et al., 2008).

Dyslipidemia

The pharmacological approach to normalize blood lipids comprises a variety of drugs indicated according to the patient's specific abnormality (Tonkin and Byrnes, 2014). In patients with MetS, the pharmacological goal is to decrease the LDL-C fraction and triglycerides, and increase the HDL-C fraction in blood. The classical first-line drugs involve statins, fibrates and inhibitors of cholesterol absorption while thiazolidinediones, GLP-1 agonists, and DPP-4 inhibitors seem to be promising therapeutic alternatives (Binesh Marvasti and Adeli, 2010).

Statins decrease LDL-C level acting as inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is the key enzyme in cholesterol endogenous synthesis (Baigent et al., 2010). Most frequent adverse side effects of long-term treatment with statins are hepatotoxicity and myopathy (Pescio, 2001).

Ezetimibe, the standard cholesterol absorption inhibitor, impairs cholesterol luminal transport in the small intestine by the enterocyte (Tonkin and Byrnes, 2014). This drug lowers blood level of LDL-C and its effect increases when associated with a statin (Binesh Marvasti and Adeli, 2010).

Another drugs used in MetS are the fibrates, which activate peroxisome proliferator-activated receptors (PPARs) eventually improving lipid and carbohydrate metabolism. The fibrates are very effective at lowering blood triglycerides, reducing LDL-C, and increasing HDL-C as well (Staels et al., 1998). The thiazolidinediones are insulin-sensitizing drugs (i.e., rosiglitazone) that also act as PPARs stimulators and are used to normalize glycemia (Krentz et al., 2008).

The incretins, typically, the glucagon-like peptide-1 (GLP-1) and the glucose-dependent insulinotropic peptide (GIP), are gut hormones secreted by the enteroendocrine cells within minutes after ingestion and they stimulate insulin secretion after eating (Ahrén, 2003). Their use is promising in patients with DM2 (Meier and Nauck, 2006). The dipeptidyl peptidase-4 (DPP-4) is the enzyme responsible for lowering the level of incretin in blood to the basal level. Hence, the administration of DPP-4 inhibitors increases insulinemia (Kendall et al., 2006).

Hyperglycemia

The leading non-insulin antidiabetic pharmacological groups are the insulin-sensitizing drugs (biguanides and thiazolidinediones), insulin secretagogues (sulfonylureas, meglitinides, and incretins), and starch blockers (α-glucosidase inhibitors) (Rockville, 2007; Zárate et al., 2010; Powers and D'Alessio, 2012). The preferred drugs in MetS are those that do not have a direct effect on pancreatic β-cells, such as biguanides (metformin), thiazolidinediones (rosiglitazone), and α-glucosidase inhibitors (acarbose) (Mamedov and Shishkova, 2007). The main advantage of these euglycemic agents is avoiding hypoglycemia as a side effect (Vlckova et al., 2009).

Data describing clinical evidence concerning anti-MetS treatment and its effect on cognitive impairment is summarized in **Table 1**.

Medication Targeting Oxidative Stress

As described in this review, the common denominator of various pathological pathways is an overall pro-oxidative status. The use of diverse antioxidative strategies has been evaluated to mitigate oxidative stress, mainly in diabetic neuropathy (Spychalowicz et al., 2012).

Antioxidant Drugs

Among the wide variety of antioxidant compounds, the following have been thoroughly studied:

- Vitamins A, C, and E
- Flavonoids
- Alpha-lipoic acid (ALA)
- Lutein and docosahexaenoic acid (DHA)

Other antioxidants have shown neuroprotection as well. Such is the case of the amino acids Taurine, acetyl L-carnitine, N-acetylcysteine (Negre-Salvayre et al., 2008; Shakher and Stevens, 2011; Wang et al., 2011; Hosseini and Abdollahi, 2013), synthetic ROS scavengers like tempol and SOD mimetics (De Silva and Miller, 2016), and resveratrol which decreases the activity of NF-κβ. Also, TNF-α, IL-6, and Cox-2, angiopoietin-1 which protects against brain stroke (Nguyen et al., 2012), the anti-inflammatory cytokines IL-10 and IL-8 (Shukla et al., 2017), mitochondrial antioxidants as the coenzyme Q10 (Chew and

[3]National Institute for Health and Clinical Excellence Hypertension (CG127). http://www.nice.org.uk/guidance/cg127. Accessed October 10, 2017.

TABLE 1 | Effect of anti-MetS treatment on cognitive processes, and brain circulation and metabolism in clinical trials.

Symptoms	Drug	*n*	Sample Characteristics	Study Time	Results	References
HYPERTENSION	ACE inhibitor	616	Mild/Moderate AD	4-year prospective	Mini-Mental State Examination (MMSE) decline between control and placebo	Soto et al., 2013
	CCB	18,423	Elderly hypertensive patients older than 60 years	12-year prospective	CCB use significantly reduced the risk of total dementia, Alzheimer's dementia, and vascular dementia	Hwang et al., 2016
	Thiazide Diuretics	3,417	Elderly hypertensive population with AD	7-year prospective	Use of any anti-hypertensive medication was associated with lower incidence of AD. Thiazides were associated with the greatest reduction of AD risk	Chuang et al., 2014
HYPERGLYCEMIA	Metformin (Biguanides)	20	Mild cognitive impairment or mild dementia due to AD	16-week prospective	Metformin was associated with improved executive functioning, and trends suggested improvement in learning/memory and attention	Koenig et al., 2017
	Pioglitazone (TZD)	42	Mild AD with type II Diabetes Mellitus	6-month prospective	The pioglitazone group improved cognition and regional cerebral blood flow in the parietal lobe	Sato et al., 2011
	Rosiglitazone (TZD)	30	Mild AD or amnestic mild cognitive impairment	6-month prospective	Relative to the placebo, better delayed recall, and selective attention	Watson et al., 2005
	GLP-1 agonists	9	Healthy, Caucasian males with a mean age of 22 years old	4-week prospective	Ensures less fluctuation of brain glucose levels	Gejl et al., 2012
DYSLIPIDEMIA	Simvastatin	46	45–64 year old participants with normal cognition, grouped by high or normal cholesterol	12-month prospective	Reduction of p-tau in CSF seen in patients who had high initial baseline LDL cholesterol	Li et al., 2017

ACE, angiotensin converting enzyme; CCB, calcium channel blockers; TZD, thiazolidinedione; CSF, cerebral-spinal fluid; LDL, low-density lipoprotein.

Watts, 2004), and uncoupler proteins (UCPs) (Harper et al., 2001; Green et al., 2004) have neuroprotective effects. Some of these drugs as tempol and the anti-inflammatory cytokines have improved the response to hypoxic events reducing the spread of microhemorrhages (Han et al., 2015; Shukla et al., 2017).

Vitamins A, C, and E are dietary antioxidants capable of directly reducing free radicals and participating in the recycling of antioxidant cofactors (Maritim et al., 2003). The antioxidant vitamins also boost the immune system, preserve DNA structure avoiding oxidative damage, and alleviate diabetic neuropathy symptomatology, all linked with oxidative stress reduction (Niedowicz and Daleke, 2005; Rahman et al., 2006; Valko et al., 2007; Salah et al., 2010).

Several subclasses of flavonoids are free radical scavengers found in plants (Nijveldt et al., 2001; Arts and Hollman, 2005; Nettleton et al., 2006; Lukačínová et al., 2008). Some examples are: proanthocyanidin, luteolin, hesperidin, fisetin, epigallocatechin-gallate, rutin, and quercetin have been shown to possess antioxidant activities which protect against diabetic neuropathy (Al-Enazi, 2003; Cui et al., 2008; Ibrahim, 2008; Maher et al., 2011; Wang et al., 2011; Baluchnejadmojarad and Roghani, 2012; Ferreira et al., 2013).

Alpha-lipoic acid (ALA), an amphiphile antioxidant molecule, has proved to be likely the most successful antioxidant in clinical trials (Vallianou et al., 2009). Both ALA and its active metabolite (DHLA), act at different levels as antioxidants; they are free radical scavengers, inhibit the hexosamine and AGEs pathways, and are also involved in the intracellular regeneration of ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), and oxidized glutathione (GSSG) (Packer et al., 2001; Du et al., 2008; Vallianou et al., 2009). Many experimental models support the beneficial effects of ALA in diabetic neuropathy (Nagamatsu et al., 1995; Evans et al., 2002; Ametov et al., 2003; Baydas et al., 2004; Ziegler et al., 2004, 2006, 2011; Tankova et al., 2005; Du et al., 2008; Huang and Gitelman, 2008; Gianturco et al., 2009; Vallianou et al., 2009).

The advantageous treatment with lutein and DHA in the brain of diabetic animals, and the way that these substances were able to mitigate the intrinsically oxidative environment in diabetes has also been studied (Muriach et al., 2006; Arnal et al., 2010). Moreover, treatment with DHA improved memory and learning skills in patients with Alzheimer's disease, related to a decrease in the concentration of lipid peroxide and ROS (Hashimoto et al., 2005).

Mitigating Oxidative Stress Pathways

Diverse research projects have studied the downstream sequence of reactions and biochemical avenues undergoing oxidative stress as possible targets for the treatment of oxidative neuropathy. For the purpose of this review, we will focus on Aldose Reductase Inhibitors (ARIs), PKC Inhibitors, and Anti-AGE Agents.

Aldose Reductase Inhibitors (ARIs)

The aldose reductase enzyme participates in the synthesis of sorbitol and fructose. The ARIs decrease the amount of glucose entered into the polyol pathway avoiding neuronal accumulation of sorbitol and fructose. Based on the positive effect of ARIs administration in neuropathies caused by oxidative stress (Hotta et al., 1996; Yagihashi et al., 2001), clinical trials were conducted to test the effects of Fidarestat (SNK-860) (Hotta et al., 2001), Epalrestat (Hotta et al., 2006; Ramirez and Borja, 2008; Sharma and Sharma, 2008), and Ranirestat (AS-3201) (Bril and Buchanan, 2004; Bril et al., 2009). To date, only Epalrestat has a license in Japan while the other two were removed from the market (Casellini and Vinik, 2006; Kawai et al., 2010; Schemmel et al., 2010).

Protein Kinase C (PKC) Inhibitors

The PKC enzyme participates in the activation of key regulatory proteins responsible for the synthesis of neurotransmitters, and it is essential for nerve impulse conduction. Different studies have shown that PKC participates in alleviating neuropathic pain (Chattopadhyay et al., 2008; Norcini et al., 2009). The specific PKC-1b inhibitor Ruboxistaurin improved axonal velocity and endoneurial blood flow in diabetic rats (Nakamura et al., 1999). In clinical trials, Ruboxistaurin slowed down the progression of diabetic neuropathy but was not effective in suppressing the neuropathic symptomatology (Vinik et al., 2005).

Anti-advanced glycation end products (AGEs) Agents

Some drugs may prevent or inhibit AGEs formation and accumulation. The anti-AGEs drug's family is also responsible for disrupting the interaction between AGEs and AGEs receptors (RAGEs), which would otherwise magnify oxidative damage. Some examples of these agents are Benfotiamine, Aminoguanidine, Aspirin, and Rapamycin (Haupt et al., 2005; Edwards et al., 2008).

- Benfotiamine increases the activity of the Transketolase enzyme which is responsible for directing AGE substrates to the phosphate pentose pathway, consequently reducing hyperglycemic damage. Benfotiamine also inhibits the increase in UDP-N-acetylglucosamine (UDP-GlcNAc), which in turn limits the substrate's capacity to enter the hexosamine pathway, reducing AGEs production (Stirban et al., 2007; Balakumar et al., 2010).
- Aminoguanidine reacts with 3-deoxyglucosone, an AGE precursor, inactivating the reactive carbonyl residues essential to enzymatic activity, and preventing AGEs formation. Studies on this compound, however, have been discontinued due to its toxicity (Yan et al., 2008).
- *In Vitro* studies have shown that aspirin inhibits the production of pentosidine (an AGE) by accepting free radicals and ionic chelants in the presence of collagen (Urios et al., 2007).
- Ding et al. studied the activity of Rapamycin, an inhibitor of the proapoptotic mTOR-p53-Bax pathway, showing that Rapamycin injection 4 h after causing an injury, improved functional recovery. Recovery consisted of a reduction in microglial and macrophage activation, a decreased rate of apoptosis, and an improved neurobehavioral function (Ding et al., 2015).

Our Research

As already mentioned, one of our research lines investigates the effects of chronic cola beverages consumption in murine models. Clear evidence of beneficial effects of a wash-out period (diet) and exercise have been reported (Milei et al., 2011; Otero-Losada et al., 2014, 2015, 2016a,b).

Another study conducted in our institution included elderly patients of both sexes that attended periodical routine check-ups, with the purpose of evaluating the effectiveness of short-term antioxidant supplementation. The outcome of our study confirmed that antioxidant supplementation improved plasma biochemistry as a result of changes in oxidative metabolism, typically in those patients having low basal endogenous antioxidants concentration in plasma. Based on these results, we recommended measuring the basal level of plasma antioxidants before starting any supplementation with antioxidants in elderly cardiovascular patients, a particularly vulnerable population with special precautions to be endorsed. Not only adverse effects were not reported during the course of our study, but subjective observations as "feeling more vital" or experiencing a state of "general well-being" were declared (Otero-Losada et al., 2013).

Using an experimental model, we assessed the therapeutic benefits of medical grade ozone, a mixture of 0.05–5% of pure O_3 and 95–99.5% of pure O_2 according to the pharmaceutical legislation. We reported promising effects of medical ozone auto-hemotransfusion on the cardiovascular system injury. Pretreatment with ozone auto-hemotransfusion decreased neointimal proliferation and induced reendothelialization following a metal stent insertion (Barone et al., 2016). The protective mechanism whereby ozone reduces restenosis appears to involve the ozone well-described oxidative preconditioning upregulating the antioxidant enzymes, and improving the antioxidant response to an eventual injury (Barone et al., 2016).

Cognitive impairment or skills were not assessed in these experimental models. However, the promising outcome encourages pursuing further study of cognitive abilities as well.

Clinical Relevance Regarding Neurodegenerative Diseases

Among neurodegenerative disorders, AD has been extensively studied regarding cognitive impairment in the setting of MetS (Hashimoto et al., 2005; Moreira et al., 2009; Mayeux and Stern, 2012; Zhang et al., 2013; Jayaraman and Pike, 2014; Kleinridders et al., 2014; Kim and Feldman, 2015). This tendency is reasonable since there is genetic evidence showing the apolipoprotein E (ApoE) as a common gene which links dementia, MetS, and diabetes (Zhang et al., 2017). Moreover, it is also crucial to bear

in mind the extent of hardships that come hand in hand with AD (i.e., long-term functional dependence of the affected patients, the cost of living, quality of life, etc.), making this disorder an important public health issue (Agüero-Torres et al., 1998; Geldmacher et al., 2006). One of the challenges for public health is to identify risk factors, and the MetS is definitely a prevailing one. The compelling association between altered metabolic states and dementia may provide insight on a therapeutic approach that can delay cognitive impairment, targeting the underlying pathophysiological pathways of MetS (de la Monte, 2017).

Staggering evidence demonstrates that AD pathogenesis is strongly associated with oxidative stress, inflammation, and insulin, glucose, and lipid dysregulation; all of these pathological pathways are present in MetS and other altered metabolic states (Rojas-Gutierrez et al., 2017). Consequently, a vast number of studies have shown anti-MetS treatments to improve diverse aspects of cognition in patients with AD (Chen et al., 2016; de la Monte, 2017; Rojas-Gutierrez et al., 2017). An example of said treatments include drugs like gut incretins, thiazolidinediones, and metformin, which show promising results regarding cognitive impairment in animal and clinical trials, improving the effects of insulin via different mechanisms (Chen et al., 2016). **Table 1** summarizes some data observed in clinical trials pertinent to anti-MetS treatment. Furthermore, studies on anti-oxidative treatment in patients with AD also present supportive results. For instance, flavonoids scavenge free radicals and have shown to promote neuronal survival in the hippocampus (Venkatesan et al., 2015). Another promising antioxidant treatment involves the administration of the synthetic S-acyl derivative of thiamine (vitamin B1) Benfotiamine in clinical trials reporting a long-term cognitive improvement, suggesting a possible disease-modifying therapy (Pan et al., 2016). Also, both AD and IR led to deregulation of the mTOR pathway, and treatment with the mTOR inhibitor Rapamycin resulted in learning and memory improvement in AD (Vieira et al., 2017).

Ultimately, the emerging global epidemic of neurodegenerative disorders as the world population ages is of great concern (Brookmeyer et al., 2007). Any insight regarding neuroprotection and delaying the onset of cognitive impairments is of utmost value. The ever-growing evidence of the association between AD and MetS not only allows a deeper comprehension of the pathogenic circuits but also lays the foundations for developing new therapeutic approaches aimed at normalizing both shared and intertwined pathological pathways underlying the two conditions (Agüero-Torres et al., 1998; Geldmacher et al., 2006; Venkatesan et al., 2015; Chen et al., 2016; Pan et al., 2016; de la Monte, 2017; Rojas-Gutierrez et al., 2017; Vieira et al., 2017; Zhang et al., 2017).

CONCLUSION

Metabolic Syndrome (MetS) is an ever-growing disorder affecting up to 25% of the population in industrialized countries increasing morbimortality. Given these proportions, it has become an epidemic of public health concern. Increasing evidence shows that MetS is a risk factor for neurological disorders, beyond its classical association with CVD and DM2.

Hyperglycemia, IR, inflammation, oxidative stress, and hypoxia are key pathological pathways associated with MetS. It is a question of time until this cluster of conditions results in tissue damage in target organs such as the brain and the microvasculature that irrigates the nervous system. Oxidative stress and hypoxia are actually linked to neurological diseases like Alzheimer's and Parkinson's (Kim and Feldman, 2015).

The therapeutic strategies suggested in this review entail multidisciplinary interventions involving different pathological pathways in concert. These include improving lifestyle and daily habits (diet and exercise), treating the cardinal symptoms of MetS, and reducing the pro-oxidative load in affected patients. Antioxidant therapy is not routinely used in MetS, although extensive research relative to its benefits in diabetic neuropathy is available. Hence, we consider that diminishing the pro-oxidative status in patients with MetS may play a critical role in reducing brain hypoxic damage and behavioral deficits.

AUTHOR CONTRIBUTIONS

ME: Lead author; MN and BP (B.Sc.): Data collection; FB: Proofreading; JG: Laboratory research; JM: Editing; MO-L: Editing and proofreading. NL: Has interpreted the data, revised for intellectual content, approved the final version of the work to be published, and may account for a research work properly accomplished.

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Estradiol Activates PI3K/Akt/GSK3 Pathway Under Chronic Neurodegenerative Conditions Triggered by Perinatal Asphyxia

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Perinatal asphyxia (PA) remains as one of the most important causes of short-term mortality, psychiatric and neurological disorders in children, without an effective treatment. In previous studies we have observed that the expression of different neurodegenerative markers increases in CA1 hippocampal area of 4-months-old male rats born by cesarean section and exposed for 19 min to PA. We have also shown that a late treatment with 17β estradiol (daily dose of 250 μg/kg for 3 days) was able to revert the brain alterations observed in those animals. Based on these previous results, the main aim of the present study was to explore the mechanism by which the estrogenic treatment is involved in the reversion of the chronic neurodegenerative conditions induced by PA. We demonstrated that estradiol treatment of adult PA exposed animals induced an increase in estrogen receptor (ER) α and insulin-like growth factor receptor (IGF-1R) protein levels, an activation of the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3 beta/β-catenin signaling pathway and an increase in Bcl-2/Bax ratio in the hippocampus in comparison to PA exposed animals treated with vehicle. Taking together, our data suggest that the interaction between ERα and IGF-IR, with the subsequent downstream activation, underlies the beneficial effects of estradiol observed in late treatment of PA.

Keywords: hippocampus, neuroprotection, western blot, signaling pathway, neuronal survival

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INTRODUCTION

During perinatal lifetime, different factors selectively impair certain aspects of neurodevelopment, having long-lasting deleterious consequences in adult brain functioning (Basovich, 2010). Developing brains are susceptible to hypoxia-ischemia insults (obstetrical problem known as PA), which are associated with neurodevelopmental disorders (NDDs) (van Handel et al., 2007).

Abbreviations: Akt, serine/threonine protein kinase; ER, estrogen receptor; GSK-3, glycogen synthase kinase 3; IGF-IR, insulin-like growth factor-I receptor; PA, perinatal asphyxia; PI3K, phosphoinositide 3-kinase.

The combination of an elevated vulnerability of the immature brain and the long-consequences in life quality, together with the limited availability of therapeutic tools to attenuate brain damage, supports the search for new preventive and therapeutic strategies (Manthey and Behl, 2006). During the last decade, the neuroprotective effects of the ovarian hormone 17β estradiol has been studied by several groups in different experimental pathological conditions (Bourque et al., 2009; Garcia-Segura and Balthazart, 2009; Kipp and Beyer, 2009; Kruse et al., 2009; Lebesgue et al., 2009; Pike et al., 2009; Suzuki et al., 2009). Previous studies from our laboratory showed that the ovarian hormone is able to reduce, in adult animals, chronic reactive astrogliosis and neuronal alterations in the hippocampus caused by PA (Saraceno et al., 2010). However, it is still unknown how estradiol triggers these reparative effects.

Most of the estradiol neuroprotective effects in the brain are mediated by activating several complementary signaling pathways by ERs (Arevalo et al., 2015). Classical ERs are transcription factors and two main isoforms, ERα and ERβ, have been characterized in mammals (Walter et al., 1985; Kuiper et al., 1996). Besides the ligand-binding domain, those ERs could be regulated by kinases activated by signaling pathways of several growth factors, such as IGF-1, due to their second activation domain (Ma et al., 1994; Mendez and Garcia-Segura, 2006). Finally, membrane-associated ERα and ERβ and non-classical ERs, such as G protein-coupled ER (GPER), also allow communication with the signaling cascade of other neuroprotective molecules (Ruiz-Palmero et al., 2013; Arevalo et al., 2015). Thus, considering the neuroprotective mechanisms of estradiol and ERs, it is important to recognize ERs as a key convergent point for estradiol signaling and the signaling of others neuroprotective factors.

Parallel neuroprotective mechanisms could be triggered in the brain by the activation of ERα, ERβ, and GPER. Moreover, ER activation also involves interactions with the protective pathways induced by other neuroprotective factors. On this regard, estradiol signaling and IGF-1 receptor (IGF-1R) signaling interact through ERα, allowing the formation of a multimolecular complex composed by ERα, IGF-1R and components of the IGF-1R signaling pathway such as, PI3K, Akt, and GSK3β (Mendez et al., 2003; Cardona-Gomez et al., 2004). This ERα/IGF-1R signaling interaction allows the regulation of PI3K-Akt-GSK3β-β-catenin signaling pathway by estradiol in the brain (Cardona-Gomez et al., 2004; D'Astous et al., 2006). It has been shown that inhibition of PI3K signaling abolishes the neuroprotective actions of estradiol in global cerebral ischemia and experimental stroke models (Jover-Mengual et al., 2010; Yang et al., 2010). Moreover, previous studies showed that estradiol and IGF-1 have a synergistic effect on Akt phosphorylation (Cardona-Gomez et al., 2002a), which regulates several transcription factors involved in the control of neuronal survival (Kane et al., 1999; Pugazhenthi et al., 2000). In addition, Akt activation by estradiol inhibits GSK3 activity promoting neuronal survival (Cardona-Gomez et al., 2004), inducing the activation of neuronal survival pathways (Cross et al., 1995) and decreasing tau protein phosphorylation (Cardona-Gomez et al., 2004).

Growing body of evidence postulates that the neuroprotective relevance of ERs depends on the implemented pathological model (Simpkins et al., 2012). Although we partially know how estradiol exerts neuroprotective actions in focal or global ischemia models when it was administrated before or shortly after the induction of the brain damage (Dubal et al., 2001; Carswell et al., 2004; Miller et al., 2005; Suzuki et al., 2007), the mechanism by which the hormone exerts reparative actions in the brain of adult animals exposed to PA (Saraceno et al., 2010) remain to be explored. Our main goals in the present study were to determine: (i) whether PA could affect the expression of ERs and their downstream signaling pathway activation, and (ii) whether estradiol treatment in adult animals exposed to PA exerts reparative actions by activating the neuroprotective mechanisms of ERs in parallel with the protective pathways induced by IGF-1.

MATERIALS AND METHODS

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Buenos Aires (CICUAL, #4091/04) and conducted according to the principles of the Guide for the Care and Use of Laboratory Animals (Animal Welfare Assurance, A-3033-01 protocol #S01084). Pregnant Sprague Dawley female rats and their male offspring were used in this study. Female rats in the 15th day of pregnancy were placed in individual cages and maintained on a 12:12 h light/dark cycle in a controlled temperature (21 ± 2°C) and humidity (65 ± 5%) environment. The animals had access to food (Purina chow) and tap water *ad libitum*. One group of animals (n = 10) was used as surrogate mothers, another group (n = 12) was assigned to PA procedures, and the remaining animals (n = 8) were the mothers of the control pups.

Induction of Asphyxia

Twelve full-term pregnant rats on gestational day 22, were anesthetized (Saraceno et al., 2010), rapidly decapitated and the uterus horns were isolated through an abdominal incision and placed in a water bath at 37°C for 19 min (sub-severe PA) (Bjelke et al., 1991; Capani et al., 2001; Saraceno et al., 2010). Following PA, the uterine horns were rapidly opened, the pups were removed, the amniotic fluid was cleaned and the pups were stimulated to breathe by performing tactile intermittent stimulation with pieces of medical wipes for a few minutes until regular breathing was established. The umbilical cord was ligated and the animals were left to recover for 1 h under a heating lamp. When their physiological conditions improved, they were given to surrogate mothers that had delivered normally within the last 24 h. The different groups of pups were marked and mixed with the surrogate mothers' normal litters (control animals that were left undisturbed). We maintained litters of 10 pups with each surrogate mother. Rats were weaned at 21 days of age and housed in groups of four animals per cage through the experiment. Only male animals were retained for the present study, while female animals were used for other research projects.

Estradiol Treatment

Thirty-two adult male rats were i.p. injected, 117 days after PA, with 17β estradiol (water soluble, E4389, Sigma, St. Louis, MO, United States; 250 μg/kg) or vehicle (0.9% saline solution) as described previously (Saraceno et al., 2010). The injections were repeated daily for 3 days consecutively (up to 119 days after PA) (**Figure 1**). Animals were distributed in four experimental groups: (i) control animals injected with vehicle, (ii) control animals injected with 17β estradiol, (iii) animals submitted to PA and injected with vehicle, and (iv) animals submitted to PA and treated with 17β estradiol.

Immunoblotting

Sixteen male animals (120 days old; n = 4 per group) were killed by decapitation and the hippocampus was dissected. Subcellular fractionation was performed as previously described (Cardona-Gomez et al., 2002a,b). Briefly, dissected hippocampus was homogenized in a Dounce homogenizer with 1 ml of homogenization buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1.25 μg/mL pepstatin A, 10 μg/mL leupeptin, 2.5 μg/mL aproptionin, 0.5 mM PMSF) containing 320 mM sucrose. Then, the homogenate was centrifuged at 1000 × g for 10 min and the supernatant was centrifuged at 16000 × g for 15 min to obtain the cytoplasmic cellular fraction. Protein concentration was estimated by Bradford technique (Bradford, 1976). The subcellular fractions were stored at −80°C.

Western blot analysis was carried out using cytosolic fractions separated on 8, 10, or 12.5% SDS-PAGE, in accordance with the molecular weight of the interested protein. Samples containing 20 μg of protein from each groups were applied to each lane. After electrophoresis (120 V for 90 min), proteins were transferred to polyvinylidene difluoride (PVDF) membrane for 2 h at 260 mA as described previously (Dunah et al., 1996). Cytoplasmic cellular fractions (n = 4 per group) were incubated overnight at 4°C with the following primary antibodies: anti-GPER (1:1000, mouse-IgG, Sigma-Aldrich), anti-ERα (mouse-IgG, 1:500, Sigma-Aldrich), anti-ERβ (mouse-IgG, 1:800, Sigma-Aldrich), anti-IGF-1R (rabbit-IgG, 1:1000, Santa Cruz), anti-Akt (1:1000, mouse-IgG, Cell Signaling), anti-GSK3-beta (1:500, mouse-IgG, Abcam), anti-GSK3 beta-phospho S9 (GSK3β-p-Ser, 1:500, rabbit-IgG, Abcam), anti-phospho-Akt-Thr308 (p-Akt, 1:1000, rabbit-IgG, Cell Signaling), anti PI3 (1:2000, mouse-IgG, Santa Cruz), anti β-catenin (1:1000, rabbit-IgG, Abcam). We used anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, rabbit-IgG, Sigma-Aldrich) as loading control. Blots were rinsed three times in PBS with 0.5% Tween-20 buffer (PBST), and then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000, Bio-Rad) for 2 h at RT. Immunoreactive bands were detected using an ECL™ Western Blotting Analysis System (Amersham™, GE/Healthcare). Films were scanned and the optical density of protein bands was quantified using Gel Pro Analyzer software 3.1.00.00 (Media Cybernetics).

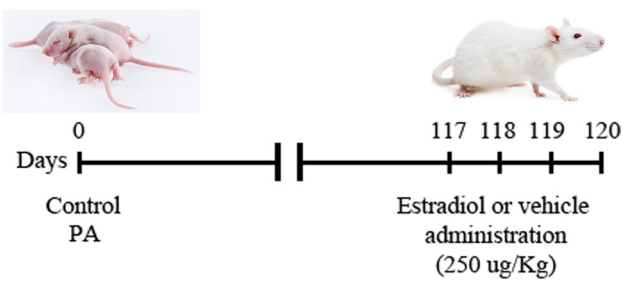

FIGURE 1 | Schematic illustration of the experimental design. Dam rats that delivered no more than two pups (vaginally delivered controls, CTL) were hysterectomized, the uterus horns were immersed in a water bath at 37°C during 19 min [pups born by cesarean section plus asphyxia (PA)]. Intraperitoneal (i.p.) administration of 17β estradiol (250 μg/kg) or vehicle (0.9% saline solution) started 117 days after PA and it was repeated daily for 3 days consecutively (up to 119 days after PA). Animals were distributed in four experimental groups: (i) control animals injected with vehicle, (ii) control animals injected with 17β estradiol, (iii) animals submitted to PA and injected with vehicle and, (iv) animals submitted to PA and treated with 17β estradiol.

Immunostaining, Confocal Microscopy, and Morphometric Analysis

Sixteen male animals (120 days old; n = 4 per group) were anesthetized with chloral hydrate (28% w/v; 0.1 ml/100 g body weight), and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and post-fixed in the same fixative solution for 2 h at room temperature and then immersed overnight at 4°C in 0.1 M phosphate buffer, pH 7.4. Coronal hippocampal sections (40 μm thick) were obtained using a Vibratome (VT 1000 S, Leica Microsystems, Wetzlar, Germany).

Immunofluorescence was performed on free floating sections under moderate shaking. Tissue sections were blocked for 30 min in phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (BSA; Sigma, St. Louis, MO, United States) and 0.3% Triton X-100. Sections were incubated overnight at 4°C with a rabbit polyclonal anti-GFAP antibody (1:2000, Sigma, St. Louis, MO, United States) and a mouse monoclonal anti-ERα antibody (mouse-IgG, 1:500, Sigma-Aldrich). After washing in buffer, tissue sections were incubated for 2 h at room temperature with Alexa 594 goat anti-rabbit IgG (1:200, Molecular Probes) and Alexa 488 goat anti-mouse IgG (1:200, Molecular Probes). Sections were counterstained with DAPI (Vector Laboratories, Inc., Burlingame, CA, United States) to label cell nuclei and mounted with Vectashield mounting medium. As specificity controls, some sections were incubated with the secondary antibody only. Under these conditions no immunostaining was observed. In order to minimize inter-assay variations, samples from all experimental groups were processed in parallel. Fluorescence was acquired on a commercial inverted confocal SPT-5 microscope (Leica Microsystems), with magnification of 63X oil DIC with excitation at 405, 488, 561 nm. Percentage of reactive area and colocalization analysis were estimated using Image J Program (Image J1.41o, NIH, United States). Briefly, images were obtained separately for each channel and analyzed. Single-channel images were thresholded

and a binary image was created from each thresholded single-channel image. We measure the percentage of pixels in the selection that have been highlighted in red as an indicator of the percentage of reactive area. For colocalization analysis, binary images were then added together to display overlap, and a third binarized image of strict pixel overlap (colocalization) was used for analysis. Percentage of colocalization was calculated as colocalized ERα/GFAP divided by the total number of GFAP positive cells.

Statistical Analysis

Material from eight rats was analyzed for each experimental group and for each parameter studied (total $n = 32$). All statistical analyses were performed by two-way analysis of variance (ANOVA) with birth condition (CTL and PA) and treatment (Vhi and 17β) as the main factors. When interaction effects were significant, analyses of the simple effects were carried out by *post hoc* comparisons using Student's *t*-test (two-tailed) adjusted by Bonferroni correction. In any case neither the assumption of normal distribution (Shapiro–Wilk test) nor equality of variances (Levene's test) was rejected. Results were expressed as the mean ± SEM. Differences with a $p < 0.05$ were considered to be significant. Statistical analyses were performed using the GraphPad Prism 5.03 statistical package for Windows (GraphPad software).

RESULTS

Estradiol Increased the Expression of ERα in the Hippocampus of Adult Male Rats Subjected to PA

As shown in **Figure 2**, the protein levels of ERα, ERβ, and GPER were not significantly different ($p > 0.05$) between the animals subjected to PA and injected with vehicle (PA-Vhi) and the control animals injected with vehicle (CTL-Vhi). Estradiol treatment induced a significant increase in the protein levels of ERα in PA exposed animals (PA-17β) (**Figure 2**), but did not affect the expression of ERβ and GPER (**Figure 2**).

Immunolocalization studies by confocal microscopy revealed ERα immunoreactivity in astrocytes in both vehicle and estradiol injected animals. The percentage of colocalization of ERα and GFAP immunoreactivities was increased in PA exposed animals

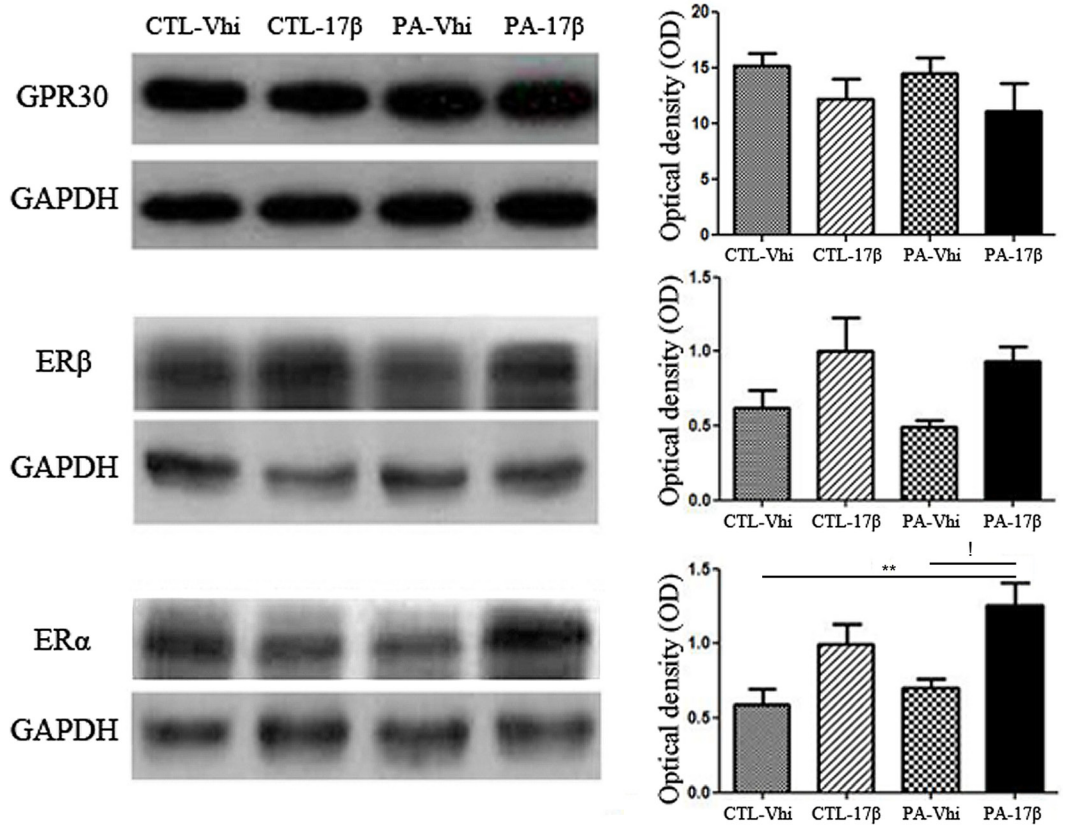

FIGURE 2 | Hippocampal ERs expression analysis in adult male rats subjected to PA or treatment. Immunoblots were analyzed using different antibodies against GPER, ERβ, ERα, and GAPDH (loading control) in cytoplasmic fractions ($n = 4$ per group). The figure shows that PA does not induce any significant change regarding ERs and GPER level expression compared to control animals treated with vehicle or estradiol. In contrast, ERα protein level increased after the treatment with estradiol respect to asphyctic animals injected with vehicle and control animals treated with vehicle (!$p < 0.05$ PA-17β vs. PA-Vhi; **$p < 0.01$ PA-17β vs. CTL-Vhi after two-way ANOVA and Bonferroni *post hoc* test). Error bars represent the mean ± SEM.

injected with vehicle compared to control animals injected with vehicle. This suggests an induction in the expression of ERα in astrocytes by PA. The treatment with estradiol induced an increased ERα cytoplasmic immunoreactivity in CA1 pyramidal neurons and increases the percentage of ERα immunoreactive area in control and PA exposed animals (**Figure 3**).

Estradiol Reverts the Effects of PA on IGF-1R/PI3-K/Akt/GSK3/β-Catenin Signaling Pathway

Some of the estradiol effects on the brain are mediated by the interaction of ERs with the signaling of growth factors, such as IGF-1. We observed that PA decreased the expression of IGF-1 receptor, an effect that was reverted by estradiol treatment (**Figure 4**). Since estradiol treatment increased the levels of IGF-1R and ERα in PA exposed animals, we decided to study the effect of the hormone on the phosphatidyl inositol 3 kinase (PI3K) signaling pathway, which is downstream of IGF-1R. PA exposed animals showed a significant decrease in the protein levels of PI3K compared to the control groups (CTL-Vhi and CTL-17β). Estradiol treatment increased the expression of PI3K in PA exposed animals (PA-17β) to control levels (**Figure 4**).

The PI3K enzyme promotes the phosphorylation of the serine-threonine kinase enzyme Akt and its activation. In turn, Akt phosphorylates and inhibits GSK3β, which regulates the levels of β-catenin. No significant differences in total Akt and GSK3β protein levels were found between the experimental groups (**Figure 4**). However, PA induced a marked decrease in Akt phosphorylation (pAkt), determined as the ratio between its phosphorylation level at Ser473 and total Akt level (**Figure 4**). Estradiol treatment significantly reversed the downregulation of pAkt levels induced by PA (**Figure 4**). Moreover, PA decreased the levels of phosphorylated GSK3β (pGSK3β) and the levels of β-catenin and these effects of PA were reverted by estradiol (**Figure 4**). Together, all these findings suggest that PA induced a permanent downregulation of the PI3-K/Akt/GSK3/β-catenin survival pathway in the hippocampus and that estradiol treatment recovers this pathway.

Estradiol Reverts the Effect of PA on Bcl-2/Bax Ratio

Given the changes in Akt caused by PA and estradiol treatments, we evaluated the levels of Bcl-2 and Bax. PA induced a decrease in Bcl-2 protein levels. In contrast, estradiol induced a significant increase in Bcl-2 protein expression. Bax protein levels were unaffected by the treatments (**Figure 5**). Together, all these findings indicate that PA decreased the Bcl-2/Bax ratio, while

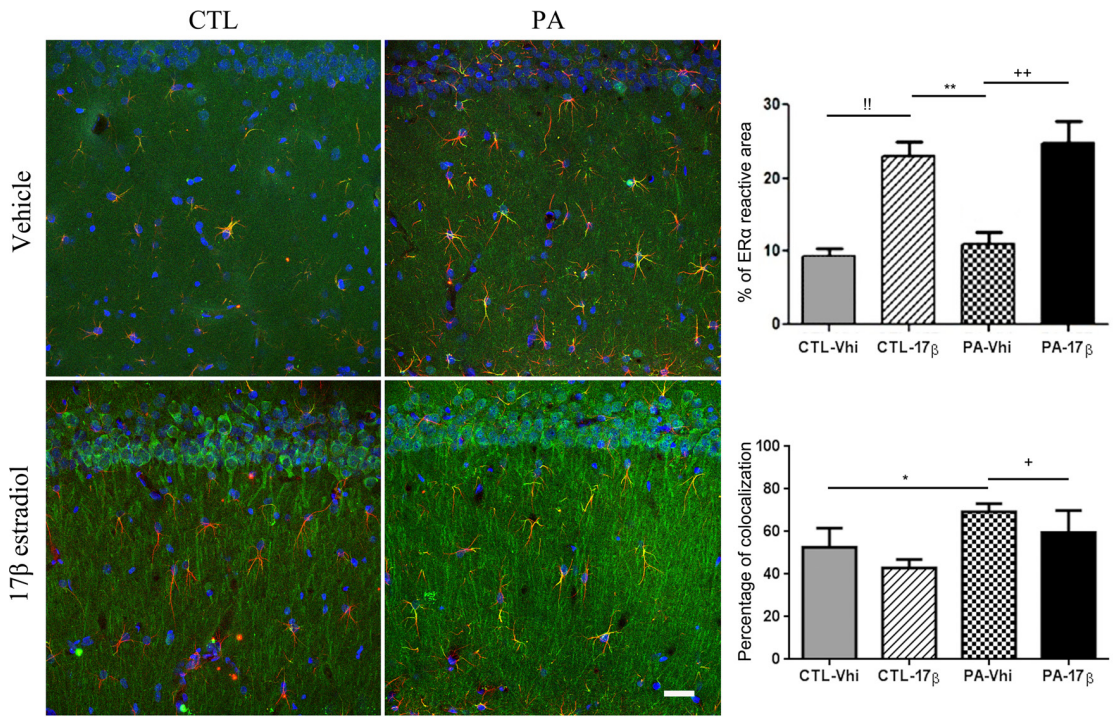

FIGURE 3 | ERα immunoreactivity is modified by PA and estradiol treatment. Confocal microscope images of GFAP (red) and ERα (green) immunostaining from the *striatum radiatum* of CA1 hippocampal area ($n = 4$ per group). Percentage of ERα immunoreactive area was increased by estradiol in both control and estradiol treated animals. !! CTL-Vhi vs. CTL-17β; $^{**}p < 0.01$, PA-Vhi vs. CTL-17β; $^{++}p < 0.01$, PA-Vhi vs. PA-17β after two-way ANOVA and Bonferroni *post hoc* test. Moreover, colocalization analysis revealed an increase in ERα immunoreactivity in astrocytes from PA exposed animals injected with vehicle respect to control group injected with vehicle and PA exposed animals treated with estradiol. Error bars represent the mean ± SEM. $^{*}p < 0.05$, PA-Vhi vs. CTL-Vhi; $^{+}p < 0.05$, PA-Vhi vs. PA-17β after two-way ANOVA and Bonferroni *post hoc* test. Scale bar: 10 μm.

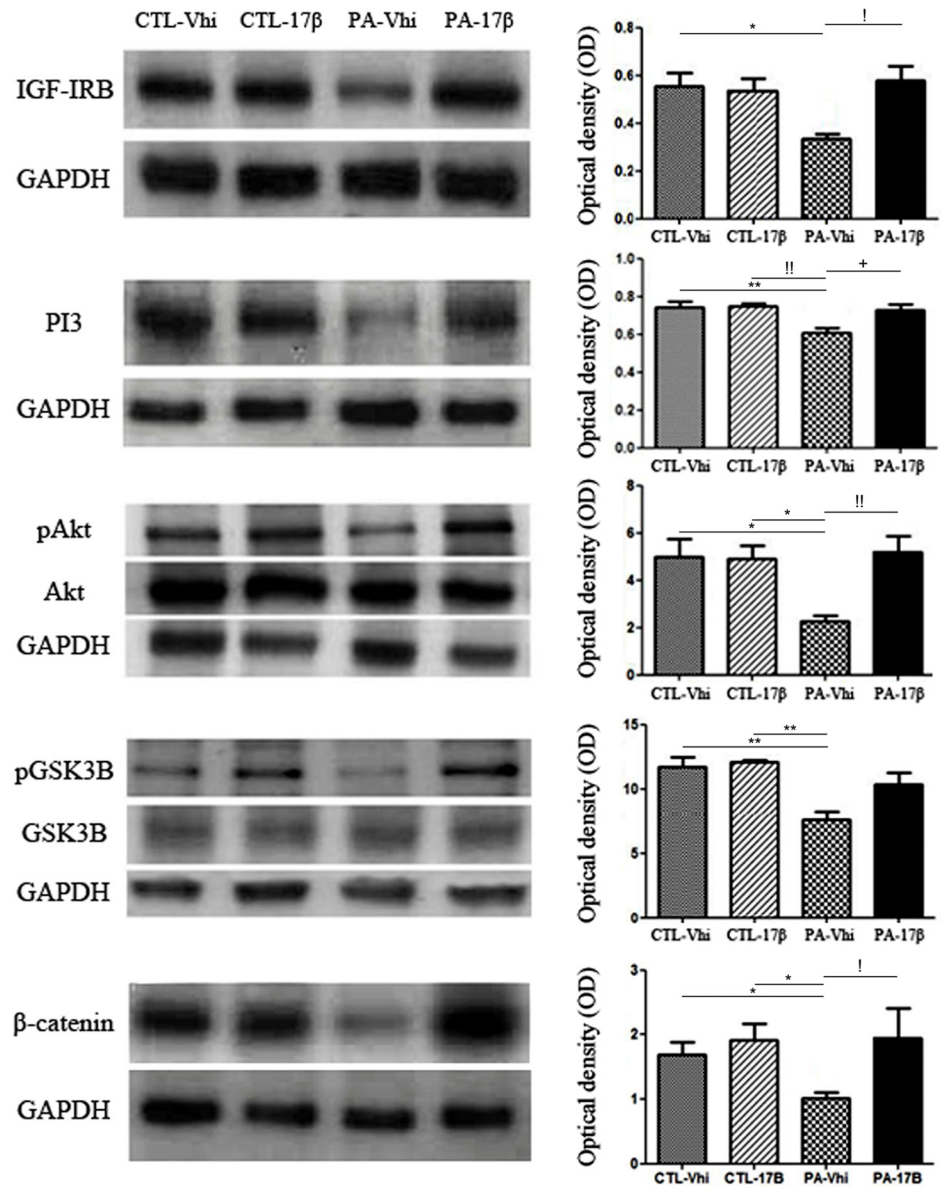

FIGURE 4 | Perinatal asphyxia affects the IGF-1R/PI3-K/Akt/GSK3/β-catenin signaling pathway. Immunoblots were analyzed using different antibodies against IGF-IRB, PI3K, Akt, pAkt, GSK3B, pGSK3B, β-catenin, and GAPDH (loading control) in cytoplasmic fractions (n = 4 per group). The figure shows that PA affected the entire signaling pathway inducing a decrease in the protein expression/phosphorylation respect to control group injected with vehicle or estradiol (IGF-IRB: *$p < 0.05$ PA-Vhi vs. CTL-Vhi; PI3K: **$p < 0.01$ PA-Vhi vs. CTL-Vhi, !!$p < 0.01$ PA-Vhi vs. CTL-17β; pAkt/Akt: *$p < 0.05$; PA-Vhi vs. CTL-Vhi, PA-Vhi vs. CTL-17β; pGSK3B/GSK3B: **$p < 0.01$ PA-Vhi vs. CTL-Vhi, PA-Vhi vs. CTL-17β; β-catenin: *$p < 0.05$ PA-Vhi vs. CTL-Vhi, PA-Vhi vs. CTL-17β after two-way ANOVA and Bonferroni *post hoc* test). Estradiol reverted most of the changes induced by PA (IGF-IRB: !$p < 0.05$ PA-Vhi vs. PA-17β; PI3K: +$p < 0.05$ PA-Vhi vs. PA-17β; pAkt/Akt: !!$p < 0.01$ PA-Vhi vs. PA-17β; β-catenin: !$p < 0.05$ PA-Vhi vs. PA-17β after two-way ANOVA and Bonferroni *post hoc* test). Error bars represent the mean ± SEM.

estradiol treatment significantly increased this ratio, suggesting a rescue response against the asphyctic insult.

DISCUSSION

Our findings indicate that adult rats submitted to PA present a decrease in the expression of IGF-1R, PI3K, β catenin, and Bcl2 and a decreased phosphorylation of Akt and GSK3β in the hippocampus. These changes observed in adult rats suggest that PA induces a permanent alteration in hippocampal IGF-1R signaling (Lee et al., 1996; Kartal et al., 2016). Interestingly, estradiol administration to adult rats submitted to PA was able to revert the decrease in the levels of IGF-1R, PI3K, pAkt, β-catenin, and Bcl2 in the hippocampus, suggesting that the IGF-1R/PI3K/Akt/GSK3β/β catenin signaling pathway is

involved in the hormonal reversion of hippocampal neural deficits caused by early brain damage (Saraceno et al., 2010). These findings suggest potential new therapeutic targets for neuroprotection in infants who suffer PA.

Previous studies have shown that ERα is critical to determine the ability of physiological levels of estradiol to exert neuroprotection (Dubal et al., 2001). ERα mRNA levels are increased shortly after the induction of ischemic injury both in the presence and absence of estradiol (Dubal et al., 1999). In addition, estradiol induces the expression of IGF-1 (Kapur et al., 1992; Michels et al., 1993) and IGF-1R (Cardona-Gómez et al., 2000; El-Bakri et al., 2004) in the brain and activates the signaling cascade of IGF-1R via ERα (Kahlert et al., 2000; Marin et al., 2009). On the other hand, IGF-1R activation is essential for several actions of estradiol in the brain, such as neuroprotection in the hippocampus (Garcia-Segura et al., 2000). It has been shown that estradiol induces the formation of a multimolecular complex composed by ERα, IGF-1R and components of the IGF-1R signaling pathway in the brain (Mendez et al., 2003; Cardona-Gomez et al., 2004). This complex may be involved in the synergistic activation of Akt by IGF-1 and estradiol in the brain through ERα (Cardona-Gomez et al., 2002b).

Taken in consideration the previously discussed interaction of ERα with IGF-1R signaling in the brain, our present results may offer a mechanistic explanation for the reparative effects of estradiol in the hippocampus of adult animals exposed

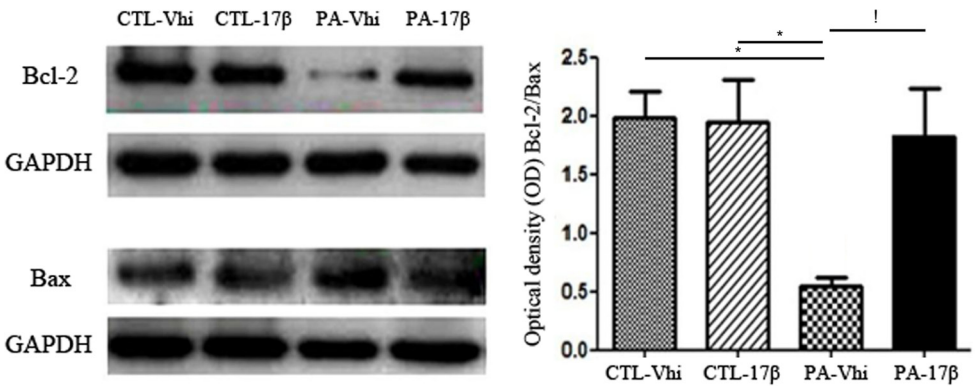

FIGURE 5 | Bcl-2/Bax ratio is affected by PA and estradiol treatment. Immunoblots were analyzed using different antibodies against Bcl-2, Bax, and GAPDH (loading control) in cytoplasmic fractions ($n = 4$ per group). The figure shows that Bcl-2 expression was decreased under PA compared to control groups treated with vehicle or estradiol, affecting the Bcl-2/Bax ratio. Estradiol treatment increased the expression of Bcl-2, reverting the effects of PA (*$p < 0.05$; PA-Vhi vs. CTL-Vhi, PA-Vhi vs. CTL-17β; !$p < 0.05$ PA-Vhi vs. PA-17β after two-way ANOVA and Bonferroni *post hoc* test). Error bars represent the mean ± SEM.

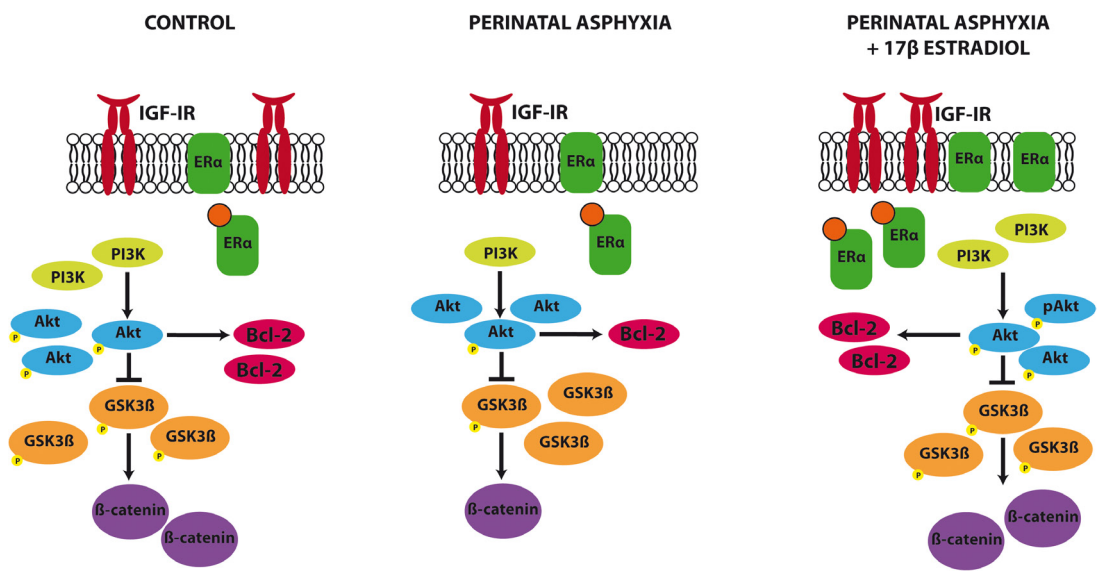

FIGURE 6 | Summary of the changes induced by PA and estradiol treatment in the IGF-1R signaling pathway. PA induced a decrease in IGF-1R, PI3K, β catenin, and Bcl2 expression and a decrease in the phosphorylation of Akt and GSK3β in the hippocampus. Estradiol administration to adult rats submitted to PA was able to revert these changes, restoring the protein levels to similar values to those seen in control animals.

to PA. Thus, estradiol increased the expression of ERα in the hippocampus of adult animals that were submitted to PA. This may facilitate the activation of neuroprotective signaling pathways mediated by ERα (Arevalo et al., 2015). Indeed, estradiol restored the expression or phosphorylation of components of the PI3K/Akt/GSK3β/β-catenin signaling pathway that were decreased by PA. The restoration of this signaling pathway by estradiol may have direct consequences in the reparative process, including the reversion of dendritic and cytoskeletal alterations (Saraceno et al., 2010). Indeed, Akt activation regulates estradiol-induced synaptic plasticity (Znamensky et al., 2003) and is involved in the neuroprotective effects of the hormone (Zhang et al., 2001; Yu et al., 2004). Moreover, Akt activation modulates GSK3β activity (Cardona-Gomez et al., 2004), which regulates microtubule dynamics, neuritic growth, synaptogenesis, and synaptic plasticity (Hall et al., 2000). Therefore, estradiol regulation of GSK3β phosphorylation in the hippocampus of PA exposed animals may be involved in the reparative process. Furthermore, sustained activation of GSK3β may result in degenerative damage (Bhat et al., 2000), while its inhibition, regulated by estradiol (Cardona-Gomez et al., 2004), is associated with the activation of neuronal survival pathways in the hippocampus (Cross et al., 1995).

In this regard, inhibition of GSK3β activity by the *Wnt* signaling pathway has been shown to allow nuclear translocation and transcriptional activity of β-catenin, whereas GSK3β activation redirects β-catenin into the proteasome pathway (Barth et al., 1997). On the other hand, the activation of Akt by estradiol in the hippocampus of animals exposed to PA may also affect the expression of molecules that regulate apoptosis. Thus, we observed an increase in the relative concentration of Bcl-2 and a decrease in Bax, affecting the Bcl-2/Bax ratio in the hippocampus of PA exposed animals treated with estradiol. It has been shown that Akt activation also promotes phosphorylation of BAD, a member of the Bcl-2 family, and can suppress BAD-induced cell death (del Peso et al., 1997). Furthermore, the activation of Akt increases the activity of the Bcl-2 promoter, and estradiol induces the expression of Bcl-2 in neurons (Garcia-Segura et al., 1998; Pugazhenthi et al., 2000) by IGF-1R activation in the adult brain (Cross et al., 1995). Thus, the increase in Bcl2 expression in the hippocampus of PA exposed rats treated with estradiol may be a consequence of the restoration of Akt signaling and may also contribute to the reparative process.

Based on these encouraging findings, further experiments are needed to determine whether the effect of estradiol is permanent or transient since we just analyzed one single time point after the estradiol administration. In this line, since only male rats were included in our analysis, it will be important to examine the effects of PA and estradiol in females, because sex differences have been reported in the outcomes of different forms of perinatal brain injuries (Nuñez, 2012). Although these findings could help to determine a potential new therapeutic target for neuroprotection in infants, we know that the use of estradiol as therapeutic treatment is limited by its peripheral hormonal effects, such as increasing the risk of breast, endometrial, and ovarian cancer. Therefore, further studies should determine whether other estrogenic compounds that are free of these limitations, exert brain reparative actions after PA.

CONCLUSION

Our findings indicate that PA affects one key signaling pathway that promotes neuroprotection and modulates neuronal plasticity, the IGF-1R/PI3K/Akt/GSK3β/β catenin signaling pathway (**Figure 6**). The rescue of this signaling pathway by estradiol may contribute to the reparative actions of the hormone in the hippocampus of adult animals exposed to PA.

AUTHOR CONTRIBUTIONS

GS contributed to the conception and design of the research, performed the experiments, analyzed the data, and wrote the manuscript. MB performed data analysis and provided insightful ideas. LG-S contributed to the design, provided insightful ideas, wrote the manuscript, and provided financial support. FC provided financial support for animal housing.

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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.

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Danhong Injection Combined With t-PA Improves Thrombolytic Therapy in Focal Embolic Stroke

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Background: Hemorrhagic transformation, neurotoxicity, short treatment time windows, and other defects are considered as the major limitations for the thrombolytic therapy. This study is devoted to figure out whether Danhong injection (DHI) combined with tissue-plasminogen activator (t-PA) could extend the treatment time windows and ameliorate brain injury, hemorrhagic complication and BBB disruption after focal embolic stroke.

Methods: *In vitro*, the combined concentrations of DHI and t-PA were added to wells reacted with plasminogen and D-Val-Leu-Lys-AMC. The optimum ratio of the combination of DHI plus t-PA was explored by detecting relative fluorescent. *In vivo* experiments, we firstly investigated the optimal dose of t-PA and Danhong injection for focal embolic stroke. The neurological deficit score, infarct volume and brain edema were assessed. Secondly, we proved that the combination group extended the thrombolytic window for treatment of focal embolic stroke. The neurological deficit score, infarct volume, brain edema and hemorrhagic complication were assessed, while levels of BAX, Bcl-2 and caspase-3 in brain tissue were analyzed by real-time polymerase chain reaction. Finally, to ask whether combination therapy with DHI plus t-PA protected the blood–brain barrier in a rat model of focal embolic stroke, neurological deficit score, ELISA, RT-PCR, western blot and fluorescence were used to detect the indicators of blood–brain barrier, such as tight junction protein, blood–brain barrier permeability and related gene expression.

Results: *In vitro*, plasmin activity assays showed that the combination of t-PA with DHI at about 1:1.6 w/v ratio increased by almost 1.4-fold the plasmin-generating capability of t-PA. *In vivo* experiments, the results showed that the combination of Danhong injection (4 mL/kg) and t-PA (2.5 mg/kg) could extend the t-PA treatment time windows to 4.5 h. And the combination t-PA (2.5 mg/kg) with DHI (4 mL/kg) ameliorated neurological score, cerebral infarction, brain edema, brain hemorrhage, and BBB disruption.

Conclusion: Combination therapy with Danhong injection (4 mL/kg) plus t-PA (2.5 mg/kg) could extend the t-PA treatment time windows to 4.5 h, ameliorate BBB disruption, reduce infarction, brain swelling and hemorrhage after ischemic stroke.

Keywords: Danhong injection, t-PA, combination therapy, embolic, cerebral ischemia

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INTRODUCTION

Ischemic stroke is a leading cause of death, disability, and massive socioeconomic loss worldwide (Wang et al., 2014). By stimulating thrombolysis and rescuing the ischemic brain via reopening occluded vessels, intravenous administration of recombinant tissue plasminogen activator (t-PA) remains the most effective intervention with FDA approval for emergency treatment of stroke (Whiteley et al., 2014). However, its efficacy and safety only ensured when administered in 3 h after the onset of symptoms. Only a small percentage of patients with ischemic stroke are eligible for t-PA treatment. In addition, t-PA thrombolytic therapy is inevitable with hemorrhage, reperfusion injury and other complications. The risk of symptomatic intracranial hemorrhage, poor thrombolytic perfusion rate, neurotoxicity, and a short treatment time window comprise the major challenges of its application (Bambauer et al., 2006; Weintraub, 2006). Improving t-PA thrombolytic therapy is a high priority in stroke research (Zhu et al., 2010), and it may both lengthen the time-to-treatment window and make reperfusion therapy safer and more efficacious (Marder and Stewart, 2002; Dávalos, 2005). The pathological mechanism of ischemia stroke is complex, involving excitotoxicity, oxidative damage, inflammation and Ca^{2+} overload. The combination therapy in the treatment of acute cerebral infarction has achieved good therapeutic effect. Minocycline combined with t-PA could extend the treatment time window and decrease hemorrhagic transformation after focal embolic stroke in type 1 diabetic rats. Therefore, it is imperative to seek combination therapies that will truly extend the treatment time window, while reducing the risk of t-PA-associated hemorrhagic transformation, and enhancing thrombolytic efficacy (Whiteley et al., 2014). For this perspective, Danhong injection may be a compelling candidate.

Danhong injection (DHI), a Chinese Materia Medica standardized product extracted from *Radix Salviae miltiorrhizae* and *Flos Carthami tinctorii*, is used extensively for the treatment of cerebrovascular diseases such as acutely cerebral infarction in clinic (Du et al., 2011; He et al., 2012). *Radix Salviae miltiorrhizae* and *Flos Carthami tinctorii* are both well-known Chinese herbal medicinal formula for treated cardiovascular and cerebrovascular diseases. The main bioactive constituents of DHI are danshensu, salvianic acid A and B, protocatechuic aldehyde, rosmarinic acid and some saccharides. These compositions are the material basis of neuroprotective effect of DHI. More experimental evidences have shown that DHI exhibited diverse pharmacological effects, including protection against vascular endothelium injury (Gao et al., 2011; Zhi et al., 2012), improvement in microcirculation (Jung et al., 2011), inflammation, and neuroprotective (He et al., 2012).

Combination therapy to extend the treatment time window may have the following aspects. The combination approach of t-PA and DHI may reduce the expression of PAI-1 and the dosage of t-PA, thereby reducing the side effects of t-PA, and prolonging the treatment time window. DHI of cerebrovascular expansion in combined treatment may reduce the t-PA-induced cerebral vasoconstriction and improve the efficiency of thrombolysis. DHI could inhibit the activation of MMP-9 and protect the blood–brain barrier, which may reduce the MMP-9 activation caused by t-PA and the destruction of the blood–brain barrier. It might lengthen the treatment time windows, enhance t-PA thrombolytic efficacy and reduce the side effects of t-PA-induced. In this study, we aimed at evaluating a novel combination approach of low-dose t-PA and Danhong injecting in a rat model of focal embolic stroke, which might lengthen the treatment time windows, enhance t-PA thrombolytic efficacy, while reducing its associated complications related to intracerebral hemorrhagic transformation.

MATERIALS AND METHODS

Plasmin Activity Assay

A 96-well plate was pre-loaded with fibrinolytic solution at 37°C for 1 h. After washing plate, 50 μL of the indicated individual or combined concentrations of Danhong injection, t-PA and BSA were added. Thereafter, a fluorogenic plasmin substrate, D-Val-Leu-Lys-AMC, was added to wells of 96-well plate in a final volume of 100 μL PBS. It was incubated at 37°C for 30 min. Plasmin generation was read on a fluorescent plate reader with excitation set at 360 nm and emission at 460 nm (Zhu et al., 2010). The fluorescence intensity in the very dilute solution was proportional to the concentration of the fluorescent substance, so the final result was represented as fold of plasmin activity generated by t-PA.

Animal Models of Focal Embolic Cerebral Ischemia

All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (body weight, 250–280 g) were purchased from the Animal Center of Zhejiang Chinese Medical University, Hangzhou, China (Laboratory Animal Certificate: SCXK:2014-0001). All rats were subjected to a focal embolic stroke by occluding the MCA with a fibrin-rich allogeneic clot (Fan et al., 2012). Its thrombolytic reperfusion time window and hemorrhagic transformation closely mimic the clinical situation, which has been most commonly used for thrombolytic stroke studies. Expose the common carotid artery (CCA), internal carotid artery (ICA), external carotid artery (ECA) with careful blunt dissection. Necessary prevention and care should be provided to avoid injury to the vagal nerve during dissection. Clamp the CCA and the ICA with an aneurysm clip, and apply a 4-0 silk suture loosely around the trunk of the ECA near the bifurcation. Create a partial arteriotomy on the ECA, and then insert the tip of the clot into the arteriotomy. Meanwhile, the regional cerebral blood flow was used to monitor for up to 1 h after treatment (Henninger et al., 2009). The inclusion criteria were stable 50% or less rCBF of pre-ischemic baseline for up to 1 h after embolization. It's worth noting that rectal temperature was monitored and maintained at 37°C using a heating blanket.

Experiments and Treatment Groups

All drug treatments and outcome assessments were performed by an investigator blinded to the surgical groups (Lapchak et al., 2013). Three sets of experiments were performed. In the first set of experiments, rats were used to test the optimal dose of t-PA and Danhong injection (DHI) in the embolic stroke animal model. Four hours after initiation of ischemia, 70 animals (n = 5 per group) were treated intravenously with saline, high-dose t-PA (10 mg/kg), DHI (4 mL/kg), combination of t-PA (1.25 mg/kg) plus DHI (2 mL/kg) (DHI-L + tPA-L), t-PA (2.5 mg/kg) plus DHI (4 mL/kg) (DHI-M + tPA-M), and t-PA (5 mg/kg) plus DHI (8 mL/kg) (DHI-H + tPA-H). Our initial choice of combination doses for t-PA plus DHI *in vivo* was based on plasmin generation data *in vitro*, where the ratio of combining t-PA with DHI was about 1:1.6. Brain infarction volume, brain edema and neurologic outcomes, was quantified at 24 h after stroke.

In the second set of experiments, a total of 90 rats were used to investigate whether Danhong injection could extend the t-PA treatment time windows. Rats were divided randomly into nine groups: (1) sham group; (2) vehicle group; (3) t-PA (10 mg/kg, treated at 4 h after stroke); (4) DHI (4 mL/kg, treated at 4 h after stroke); (5) DHI + tPA + 2 h (dosage: experiment 1 results, treated at 2 h after stroke); (6) DHI + tPA + 4 h (dosage: experiment 1 results, treated at 4 h after stroke); (7) DHI + tPA + 4.5 h (dosage: experiment 1 results, treated at 4.5 h after stroke); (8) DHI + tPA + 5 h (dosage: experiment 1 results, treated at 5 h after stroke); (9) DHI + tPA + 6 h (dosage: experiment 1 results, treated at 6 h after stroke). In this segment, we tested hemorrhagic transformation, neurotoxicity, infarct volumes, edema volume and expression of apoptosis-related gene.

In the third set of experiments, rats were tested to protect the blood–brain barrier in a rat model of focal embolic stroke. Four hours after initiation of ischemia, 75 animals (n = 5 per group) were treated intravenously with saline, t-PA (5 mg/kg), DHI (4 mL/kg), combination of t-PA (2.5 mg/kg) plus DHI (4 mL/kg). High-dose t-PA treated at 4 h after stroke caused higher 24 h mortality. To avoid unacceptable high mortality, the dose of t-PA to 5 mg/kg was adjusted. ELISA, RT-PCR, western blot and fluorescence were used to detect the indicators of blood–brain barrier, such as tight junction protein, blood–brain barrier permeability and related gene expression.

Analysis of Neurological Score, Infarct Volumes and Edema Volume

At 24 h after ischemia, rats were assessed with a 4-point neurologic deficit scale that has been extensively used for rat models of stroke. The neurological deficit scoring system, based on the Bederson (Bederson et al., 1986) and Garcia score (Garcia et al., 1995), used a scale from 0 to 4: (0) no observable deficit; (1) decreased forelimb resistance to a lateral push; (2) forelimb flexion; (3) circling behavior in addition to the former symptoms; and (4) deficiency in spontaneous walking.

Rats were euthanized at 24 h after ischemia. The rat brain was immediately removed and placed at −20°C for 10 min. Coronal brain sections were cut at 2 mm from the frontal tips, and stained with 2% 2.3.5-triphenyltetrazolium chloride (TTC, Sigma, United States) at 37°C for 15 min in the dark (Wang et al., 2014). The infarct area in each slice was photographed via a digital camera and quantified via computer-assisted image analysis. Infarct volume was expressed by a percentage of the total volume of slices.

In paralled with infarct volume, brain edema with determined used the wet/dry method as previously described (Guo et al., 2014), in which percentage brain water = [(wet weight-dry weight)/wet weight]*100%.

Quantification of Intracerebral Hemorrhage

T-PA thrombolytics alone exhibited a trend for infarct reduction, but significantly elevated intracerebral hemorrhage. In order to study whether the treatment of combination could decrease intracerebral hemorrhage treated by t-PA alone, we measured the intracerebral hemorrhage as we described previously (Ploen et al., 2014). Unstained coronal sections were taken every 400 μm, and scored by investigators blinded to the experiment. Hemorrhage scores range from 0 to 4: 0 = no hemorrhage; 1 = single petechial hemorrhage; 2 = confluent petechial; 3 = a single space occupying parenchymal hemorrhage encompassing < 30% infarction area; and 4 = multiple space-occupying parenchymal hemorrhage > 30% of infarction area.

Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay

Total RNA was separated from the ischemic brain by using TRLzol reagent, and detected concentration by NanoVue Plus (A260/A280). Then, total RNA subsequently was reverse-transcribed to cDNA by using TaqMan Reverse Transcription Reagents. The reaction condition was installed for 37°C for 15 min, 85°C for 5 s, 4°C at 10 min. Real time PCR was performed using SYBR Green PCR Master Mix reagent kits and the specific primers. After the reaction, the fusion curve was analyzed to identify the specificity of PCR products. Using GAPDH as an internal control, and the data were analyzed by using the comparative threshold cycle (*C*t) method.

Measurement of MMP-9, PAI-1 and P-Selectin Levels

To determine changes of blood–brain barrier permeability and t-PA inhibition, blood plasma samples were collected at 24 h after ischemia. 10 μl of plasma sample was applied in each well and all the samples were run in duplicates. The MMP-9, PAI-1, and P-selectin levels were measured by ELISA kits according to the manufacturer's instructions, respectively. The content of MMP-9, PAI-1, and P-selectin in plasma was calculated by establishing standard curve.

Blood–Brain Barrier Permeability for Evans Blue

Evans blue had high affinity with plasma albumin. They could form an Evans Blue -albumin complex, which could limit Evans

Blue to penetrate BBB. Therefore, Evans Blue permeability was an objective index to measure the variation of BBB permeability. To quantify postischemic blood brain barrier (BBB) permeability, a previously established fluorometric assay were used to assess extravasation of albumin-bound Evans Blue (Ploen et al., 2014). Animals were briefly reanesthetized 22 h after stroke, and 2% Evans Blue in phosphate buffered saline (4 mL/kg) was injected into the femoral vein. Two hours later, rats were transcardially perfused with saline until the outflow of liquid was limpid. Centrifuge tubes containing brain tissues were added 2 mL/g formamide, and took water bath in a light resistant container. After centrifugation centrifugal, supernatant was collected in centrifuge tubes. Evans Blue content of each hemisphere was quantified by fluorometry. The results were expressed by μg/g of brain tissue.

Western Blotting Analysis

Rats were euthanized 24 h after ischemia. Brain tissues were homogenized in lysis buffer including protease inhibitors on ice. After centrifugation, supernatant was collected to centrifuge tubes, and total protein concentrations were determined using the NanoVue Plus. Protein samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membrane was subsequently blocked with 5% BSA in Tris-buffered saline with Tween 20 solution and incubated with polyclonal occludin (Abcam, ab167161) and MMP-2 (Abcam, ab92536) for 12 h at 4°C. Peroxidase-linked anti-rabbit IgG (Boster, BST12B08A54) and ECL reagents (Boster, 11F16B11) were used as a detection system. The expression of MMP-2 and occludin protein was analyzed by using gel imaging analysis system.

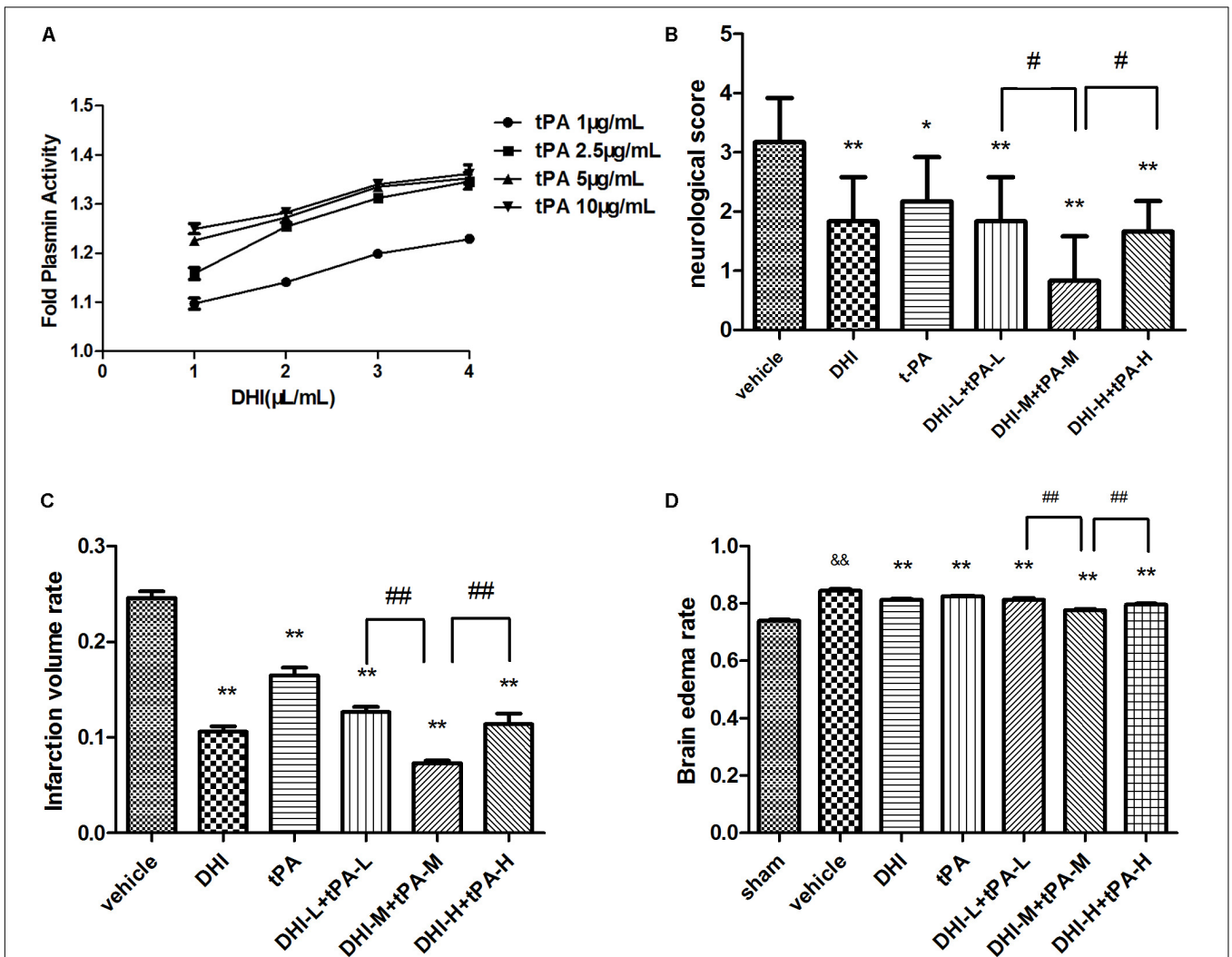

FIGURE 1 | Effect of DHI on tPA-dependent plasmin generation *in vitro* and acute brain tissue outcomes of different combination therapy in stroke rats. **(A)** A range of concentrations of tPA (1, 2.5, 5, and 10 μg/mL) with or without the indicated concentrations of DHI (1, 2, 3, and 4 μL/mL) were added to wells of 96-well plate. Plasmin activity was represented as fold of plasmin activity related to 1 μg/mL of tPA alone. **(B)** At 24 h after stroke, neurological score were quantified. **(C)** Ischemic infarct volume rates were quantified at 24 h after stroke. **(D)** Brain edema was assessed at 24 h after stroke. Data were expressed as mean ± SD, $n = 6$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle group. $^{\&\&}P < 0.01$ vs. sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. DHI-M+tPA-M.

Statistical Analysis

Data were expressed as mean ± SD. Statistical analysis was carried out by using a one-way analysis of variance as well as the least significant difference test. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Danhong Injection Amplifies t-PA-Mediated Plasmin Generation *in Vitro*

In vitro, plasmin activity assays showed Danhong injection (4 μL/mL) combined with t-PA (2.5 μg/mL) significantly amplified t-PA-converted plasmin generation (**Figure 1A**). Equal activity of plasmin could be reached by different combinations with lower dose t-PA with DHI, such as t-PA 10 μg/mL plus DHI 4 μL/mL, t-PA 5 μg/mL plus DHI 4 μL/mL, t-PA 2.5 μg/mL plus DHI 4 μL/mL. The data of assay indicated that the plasmin-generating capability of combining t-PA with DHI at 1:1.6 w/v ratio was increased by almost 1.4-fold of t-PA *in vitro*. To explore the optimal dose of t-PA and Danhong injection, we selected appropriate dosage for animal experiment in the ratio of t-PA and DHI to 1:1.6.

Explore the Optimal Dose of t-PA and Danhong Injection for Focal Embolic Stroke

Brain infarction volume, brain edema and neurologic outcomes, were quantified at 24 h after stroke. The infarct size was observed by using vital staining with 2, 3, 5-triphenyltetrazolium chloride (TTC). Representative images for TTC stained ischemic brain infarctions were shown in **Figure 2**. Normal brain tissue was

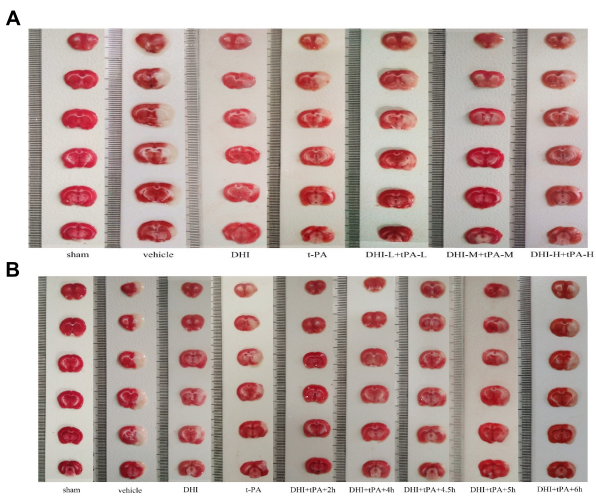

FIGURE 2 | Representative images for TTC stained ischemic brain infarctions. **(A)** Effect of different combination groups on cerebral infarction volume. **(B)** Effect of treatment time window on cerebral infarction volume.

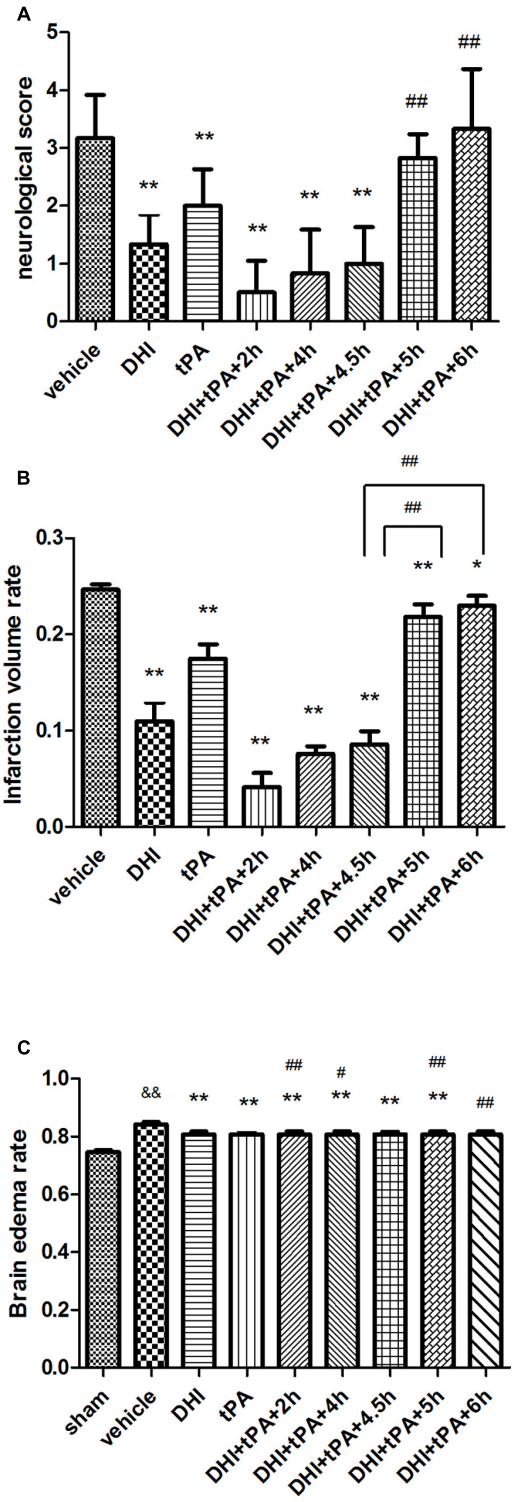

FIGURE 3 | Effect of different treatment time on neurological score, cerebral infarction volume and cerebral edema. **(A)** At 24 h after stroke, neurological score were quantified. **(B)** Ischemic infarct volume rates were quantified at 24 h after stroke. **(C)** Brain edema was assessed at 24 h after stroke. Data were expressed as mean ± SD, $n = 6$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle group. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. DHI+tPA+4.5 h.

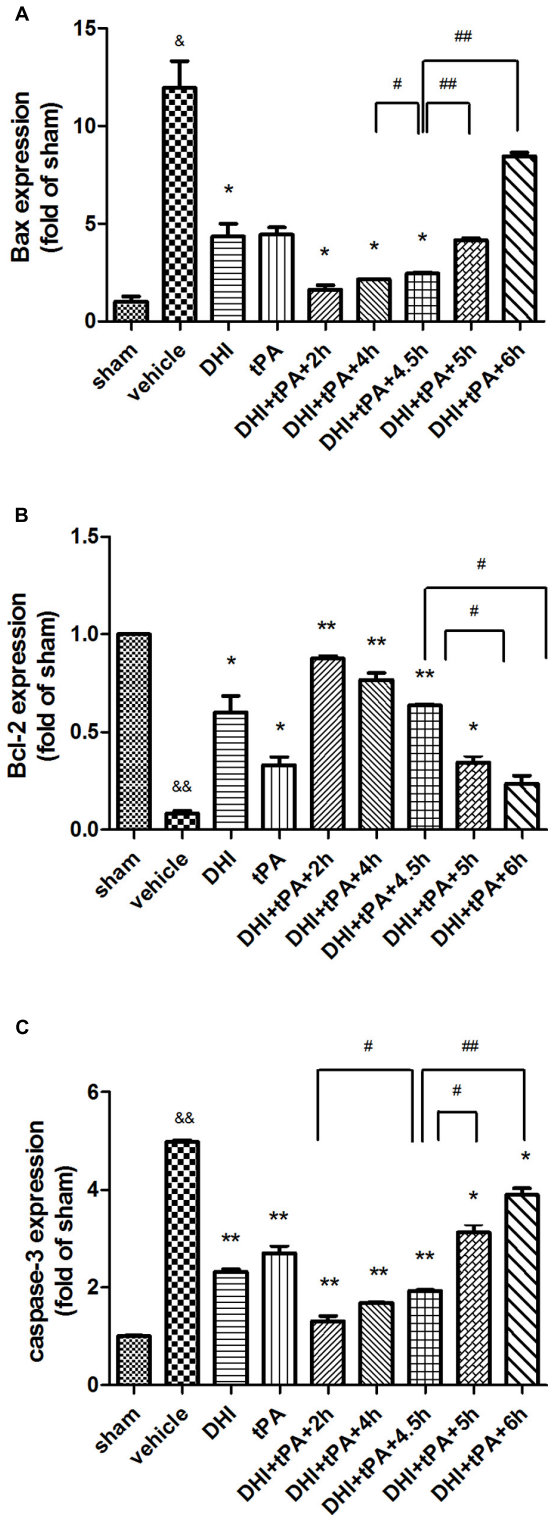

FIGURE 4 | Effect of different treatment time on the mRNA transcriptions of Bax, Bcl-2, caspase-3. **(A–C)** The real time PCR was performed to determine the relative levels of Bax, Bcl-2, caspase-3 mRNA transcriptions at 24 h after stroke. Data were expressed as mean ± SD, $n = 6$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle group. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. DHI+tPA+4.5 h.

stained deep red, whereas the infarct tissue was not stained. Compared to model group, the infarct volume was significantly reduced in each treatment group. Infarction volume in the t-PA (2.5 mg/kg) plus DHI (4 mL/kg) combination group was significantly smaller than the other combination groups (**Figure 1C**). Meanwhile, the Brain edema in the model group was significantly increased in comparison to the sham group. The combination of DHI (4 mL/kg) plus t-PA (2.5 mg/kg) showed the smallest Brain edema, followed the combination of t-PA (1.25 mg/kg) plus DHI (2 mL/kg) and the combination of t-PA (5 mg/kg) plus DHI (8 mL/kg), when compared with the model group (**Figure 1D**).

Similarly, neurological score was also significantly reduced in the treatment groups. Neurological deficit score in the t-PA (2.5 mg/kg) plus DHI (4 mL/kg) combination group was significantly lower than that of the other treatment groups (**Figure 1B**). These data indicated that this combination thrombolytic therapy was more effective and specific for fibrinolysis. So, the combination of t-PA (2.5 mg/kg) plus DHI (4 mL/kg) was selected as a combined group for subsequent research.

Low-Dose of t-PA, in Combination With Danhong Injection, Extends the Thrombolytic Window for Treatment of Focal Embolic Stroke in Rats

Focal stroke dramatically increased the infarct volume in the ischemic brain hemisphere. Cerebral infarct volume was decreased after the administration of medicine. The combination of t-PA plus DHI administered at 2, 4, and 4.5 h after stroke significantly lowered infarction volume compared with t-PA alone treatment. At the same time, the combined group administered at 2, 4, and 4.5 h after stroke, had the similar ability to reduce the volume of cerebral infarction, and achieved the same effect of protecting brain tissue. However, infarction volume in the combination groups treated at 5 and 6 h after stroke was significantly bigger than the other treatment groups (**Figures 2, 3B**). As the results of cerebral infarction volume, the neurological scores of the combination groups administered at 2, 4, and 4.5 h were better than the combination groups administered at 5 and 6 h after stroke ($P < 0.01$). There was no statistically significant difference between the combination therapy treated at 4.5 h after ischemia and combination therapy administered at 2 and 4 h after stroke (**Figure 3A**). It indicated that DHI combinated t-PA indeed extended the thrombolytic window to 4.5 h for treatment of focal embolic stroke in rats.

Then, the brain edema was examined in the different treatment groups (**Figure 3C**). In addition to the combination group treated at 6 h after stroke, brain edema of the other combination groups was significantly reduced as compared with the model group. Meanwhile, the rate of brain edema in the combination group treated at 5 and 6 h after stroke was significantly higher than that of the combined group administrated 4.5 h after stroke.

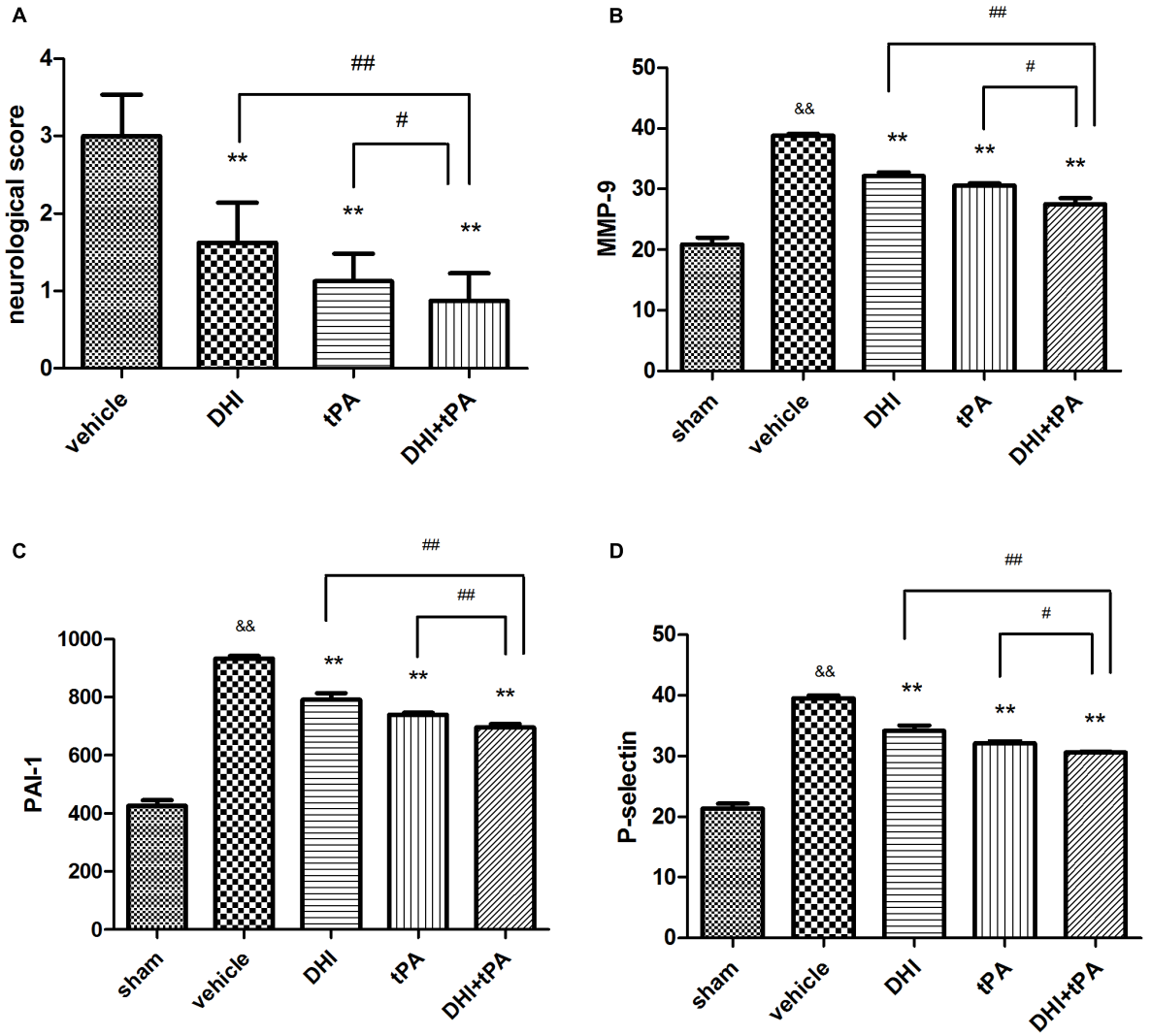

FIGURE 5 | Effect of DHI combinated with tPA in neurological core and the levels of MMP-9, PAI-1, P-selectin at 4.5 h after initiation of ischemia. **(A)** At 24 h after stroke, neurological score were quantified. **(B–D)** The levels of MMP-9, PAI-1, P-selectin were tested at 24 h after stroke. Data were expressed as mean ± SD, $n = 6$ per group. $^{**}P < 0.01$ vs. vehicle group. $^{\&\&}P < 0.01$ vs. sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. DHI+tPA.

t-PA increased macroscopic hemorrhage on unstained coronal cryosections compared with the vehicle group (mean hemorrhage score 2.3 ± 0.7 vs. 1.1 ± 0.5). The combination of t-PA plus DHI treated at 4.5 h after stroke decreased hemorrhage compared with the other combination groups.

In order to determine the Danhong injection plus t-PA prolonged the thrombolytic window to 4.5 h for treatment of focal embolic stroke in rats, real time RT-PCR was performed to determine the relative levels of BAX, Bcl-2, and caspase-3 mRNA transcriptions in rat tissue. After stroke, the expression of BAX and caspase-3 mRNA was significantly upregulated. The combination group treated at 4.5 h after ischemia was significantly reduced the expression of BAX and caspase-3 mRNA, as same as the combination group treated at 2 and 4 h after stroke (**Figures 4A,C**). However, the combination group treated at 5 and 6 h after ischemia could not reduce the BAX mRNA transcription. Furthermore, compared with combination group treated at 5 and 6 h after ischemia, the Bcl-2 signals were strengthened in combination group treated at 4.5 h after stroke (**Figure 4B**). Thus, Danhong injection extended the t-PA treatment time windows to 4.5 h.

Combination of DHI Plus t-PA Protects the Blood–Brain Barrier in Focal Embolic Stroke

To increase the validity of our findings, we examined the effect that combination of DHI plus t-PA protected the blood–brain barrier. At 24 h after stroke, neurological score was

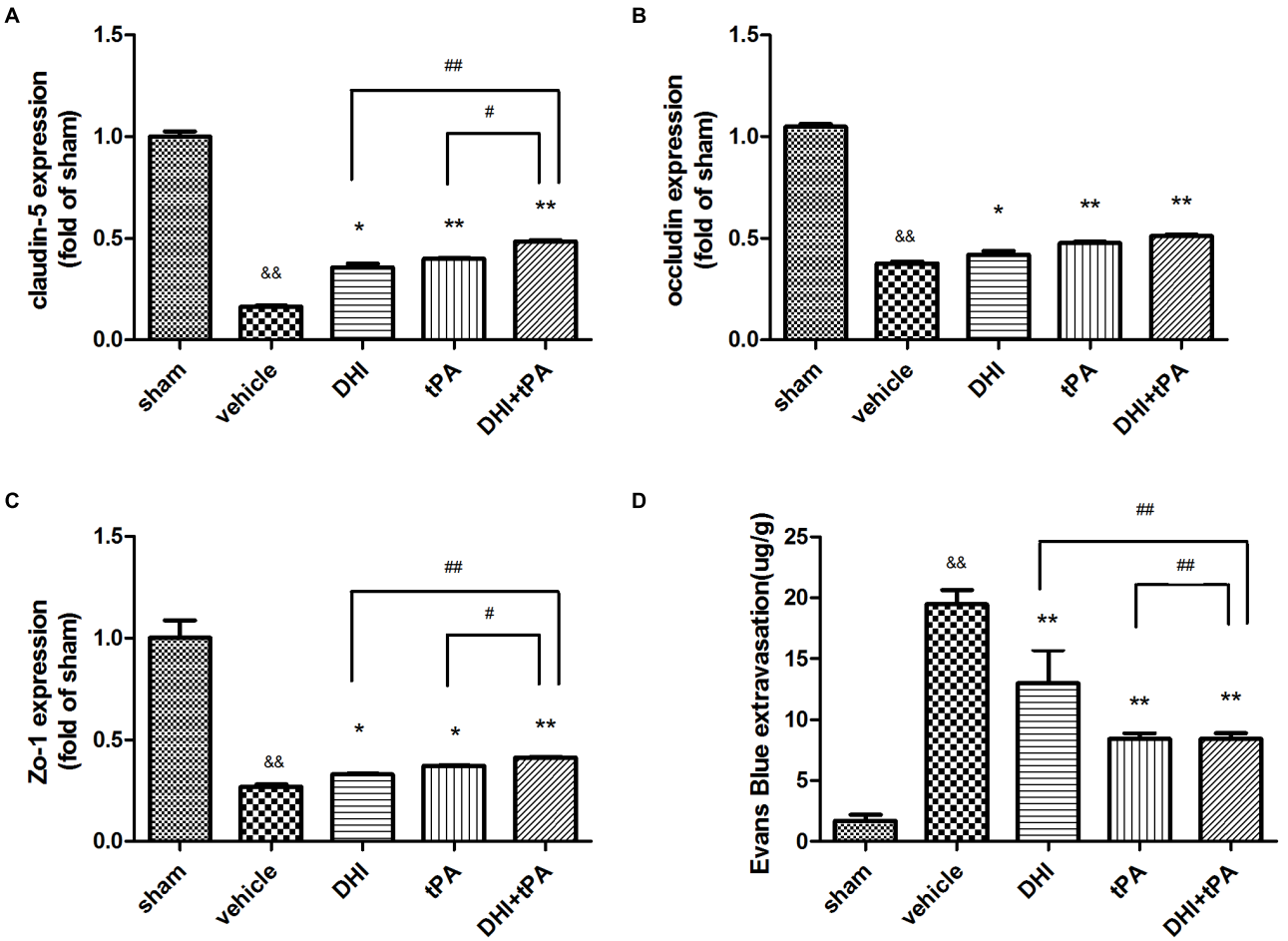

FIGURE 6 | (A–C) The real time RT-PCR was performed to determine the relative levels of claudin-5, occludin, ZO-1 mRNA transcriptions at 24 h after stroke. **(D)** Evans Blue extravasation in the ischemic hemi sphere was quantified by fluorometry. Data were expressed as mean ± SD, n = 6–8 per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle group. $^{\&\&}P < 0.01$ vs. sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. DHI+tPA.

significantly reduced in DHI group, t-PA group, and combined group. Neurological score in the combination of t-PA plus DHI was significantly smaller than the other treatment groups (**Figure 5A**). After stroke onset, plasma MMP-9, PAI-1, and P-selectin were significantly elevated, and the combination of t-PA plus DHI significantly suppressed the plasma MMP-9, PAI-1, P-selectin levels compared with t-PA alone treatment (**Figures 5B–D**). These data indicated that this combination thrombolytic therapy was more effective and specific for cerebral ischemic stroke. Then, RT-PCR was performed to determine the relative levels of claudin-5, occludin, ZO-1 mRNA transcriptions in rat brain tissue. The results showed weak claudin-5, occludin, ZO-1 positive signals in the brain tissues in model group, indicating a degradation of tight junction integrity in microvessels. In contrast, significant increase of claudin-5, occludin, and ZO-1 mRNA transcriptions was found in DHI group, t-PA alone treatment and combination group (**Figures 6A–C**). Compared with t-PA group, the claudin-5, ZO-1 signals were strengthened in the combination of t-PA plus DHI. Western blot analysis showed weak MMP-2 signals and strong occlusion signals (**Figure 7**). After stroke, significant increasement of the MMP-2 protein and reduction of the occluding protein were found in vehicle group. Compared with the vehicle group, the signal of MMP-2 was reduced in the combination group of t-PA plus DHI, while the signal of occludin was strengthened in the combination group.

To further evaluate the blood–brain barrier permeability of the combination group at 24 h after stroke, Evans Blue extravasation in the ischemic hemi sphere was quantified by fluorometry. After cerebral ischemia, rats had substantially more Evans Blue extravasate. Each treated group led to a pronounced decrease of postischemic BBB permeability after thrombolysis. The combination of t-PA plus DHI significantly lowered Evans Blue extravasate compared with t-PA alone treatment (**Figure 6D** and **Supplementary Figure S1**). These data showed that the combination group improved blood – brain barrier permeability.

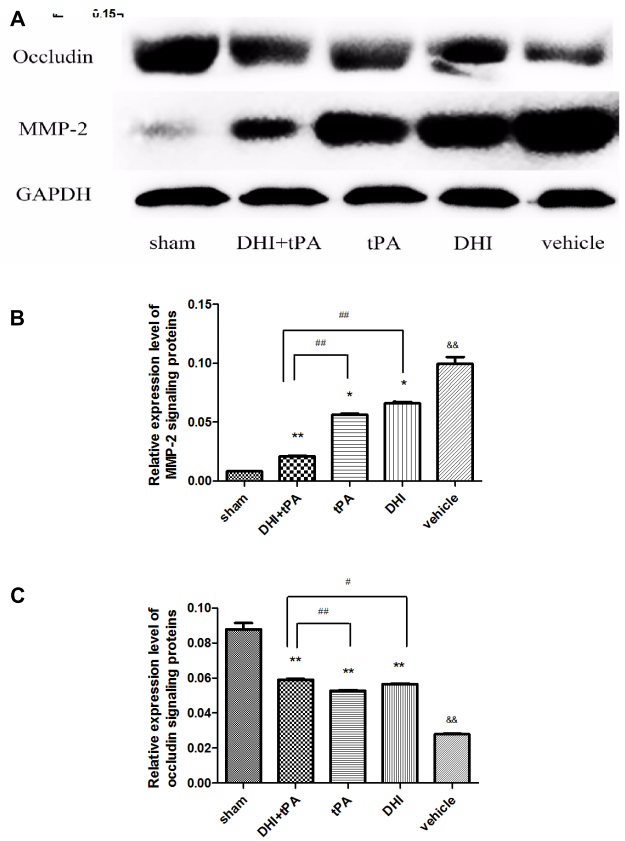

FIGURE 7 | At 24 h after stroke, the expression of MMP-2 and occludin protein treated with saline, DHI, tPA, and DHI combinated with tPA. **(A)** Representative blots of the MMP-2, occludin in the five groups. **(B,C)** The relative expression level of MMP-2 and occluding signaling proteins. Data were expressed as mean ± SD, n = 6–8 per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle group. $^{\&\&}P < 0.01$ vs. sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. DHI+tPA.

DISCUSSION

Clinical studies have demonstrated that t-PA therapy is the fundamental method of treating ischemic stroke (Zhang et al., 2015). It can quickly and directly reopen occluded vessels and restore blood flow by lysis of the thrombus (Fan et al., 2010). However, besides thrombolysis *per se*, emerging data showed that exogenous t-PA may have some other pleiotropic actions in brain (Kaur et al., 2004). These actions of t-PA may increase ischemic neurotoxicity, disrupt the blood brain barrier, aggravate brain edema and hemorrhage (Kaur et al., 2004), which compromise its usefulness as a thrombolytic drug (Yepes et al., 2009; Armstead et al., 2011). So how to extend the t-PA treatment time window and reduce the risk of bleeding deserves further investigation? Reducing the dose of t-PA may overcome the dose-dependent side effects such as cerebral hemorrhage, cerebral edema and neurotoxicity, but at the same time, it would be likely to lower perfusion effect (Fan et al., 2010). Therefore, optimization of t-PA thrombolytic efficiency may require one balance into account that is thrombolytic efficiency and t-PA complication. This balance can reduce t-PA complications such as neurotoxicity, cerebral edema, and cerebral hemorrhage while ensures thrombolytic efficiency. Clearly, it prolongs the time-to-treatment window, making t-PA safer and more effective (Marder and Stewart, 2002; Dávalos, 2005).

Drug combination is a research hotspot to resolve this contradiction. The combination therapy of minocycline plus t-PA significantly reduced brain infarction, intracerebral hemorrhage and hemispheric swelling at 24 h after stroke (Fan et al., 2013). The combined application of Annexin A2 and t-PA decreased the effective thrombolytic dose of tPA, reduced hemorrhage and brain infarction, and prolonged the reperfusion time window for stroke (Zhu et al., 2010; Jiang et al., 2015). Traditional Chinese medicine (TCM) is focused on this multitargets therapy. TCM has been used in ancient medical systems for treating various neurological diseases, especially stroke, and has exerted its distinctive neuroprotective effects on cerebral ischemia (Guo et al., 2012; Liu et al., 2013). Danhong injection is widely used in traditional Chinese preparation to treat cardiovascular and cerebrovascular diseases (Du et al., 2011; He et al., 2012). We previously have demonstrated the protective effect of DHI on cerebral ischemia reperfusion injury via anticoagulant, antithrombotic, antifibrinolytic, antiinflammatory and antioxidant activities (He et al., 2012). Based on the above discussion, we speculated DHI combined with t-PA may lessen ischemic brain damage, reduced risk of intracerebral hemorrhagic transformation and lengthened the treatment window of t-PA stroke therapy.

Our study provided three major researches. (1) The optimal dose of t-PA and Danhong injection was explored for Focal Embolic Stroke. The therapeutic effects of DHI plus t-PA were systematically evaluated by neurological function evaluation, infarct volume assessment, and cerebral edema assessment. The combination of t-PA plus DHI could significantly improve neurological function, cerebral infarction, and cerebral edema in ischemia rats. The result indicated that combination thrombolytic therapy of t-PA (2.5 mg/kg) plus DHI (4 mL/kg) was more effective and specific for fibrinolysis, which was selected as a combined group for subsequent research. (2) We explored whether combination of t-PA plus DHI could extended the thrombolytic treatment time window after stroke. Rats were treated at different time after stroke. By assessment of neurological function deficits, infarct volume, cerebral edema, cerebral hemorrhage and expression of apoptotic gene, we found that combination of t-PA (2.5 mg/kg) plus DHI (4 mL/kg) which treated at 4.5 h after stroke significantly improved neurological function recovery, reduced infarct volumes, cerebral edema, inhibited the expression of proapoptotic genes, and enhanced that of inhibiting-apoptotic genes as compared the other combination groups of delayed treatment. Thus, we speculated DHI extended the t-PA treatment time windows to 4.5 h. (3) The study of protective blood–brain barrier was explored after stroke. Experimental animal studies have suggested that the disruption of blood–brain barrier may be the major contributor to increase hemorrhagic transformation

and worse the neurological outcomes after stroke (Fan et al., 2017). In this study, we examined commonly used biomarkers for cerebrovascular damage, i.e., degradation of claudin-5, occludin and ZO-1, keys integral membrane proteins and components of tight junction strands. The tPA-DHI combination significantly reduced degradation of claudin-5, occludin and ZO-1 at 24 h after stroke. After cerebral ischemia, inflammatory cytokines or oxygen free radicals increased, the expression of MMPs activated, which leaded to the degradation of blood–brain barrier basement membrane and extracellular matrix. It will lead to the destruction of the blood brain barrier, aggravate cerebral edema and cerebral infarction, thus forming a vicious circle (Wang et al., 2008; Gu et al., 2012). MMP-9, and MMP-2 played the most prominent role in cerebral ischemia (Elgebaly et al., 2010; Alluri et al., 2015).

Then, MMP-9 and MMP-2 activities were tested after stroke. Results from the present study showed that the increment of plasma MMP-9 and brain MMP-2 activity after stroke were significantly inhibited by the combination of DHI with t-PA. Experimental results from this study showed that DHI (4 mL/kg) plus t-PA (2.5 mg/kg) combination therapy significantly reduced brain infarct volume, hemorrhage and brain edema at 24 h after stroke. Furthermore, our initial findings also suggest that these beneficial effects of DHI plus t-PA may be mediated by the amelioration of MMP, Evans blue higher permeability, and tight junction degradation.

The present study also has its limitations. On the one hand, we only examined the treatment of t-PA combined with Danhong injection by detecting the cerebral infarction and nervous system at 24 h after stroke. Acute neuroprotection may not always correspond to improvements of long-term outcomes. For animal experiments and clinical applications, further studies are needed to determine whether the protective effect of t-PA-DHI on nerve and the blood–brain barrier in the acute phase would be sustained. Further studies are required to confirm that t-PA combined with DHI truly correlate with improved long-term neurological outcomes, and how it interfaces with t-PA effects in extending treatment time windows after stroke. On the other hand, the underlying molecular mechanism of the combination therapy in this study is unclear, and further studies are necessary to elucidate this issue. It is hypothesized that the combination group may reduce t-PA-associated brain edema, cerebral hemorrhage and blood–brain barrier damage by reducing the dose of t-PA. Moreover, DHI has the function of improving blood flow and promoting thrombolysis. Therefore, Danhong injection combined with low-dose t-PA could improve cerebral ischemia and nerve injury, prolong t-PA treatment time window, and thereby, reduce the cerebral hemorrhage risk and neurotoxicity of t-PA. However, further investigations are needed to dissect and potentially optimize on the molecular mechanism of the combination therapy.

Our experimental results suggest that DHI (4 mL/kg) combined with t-PA (2.5 mg/kg) may reduce ischemic brain damage, ICH and blood brain barrier damage. We believe this combination of t-PA (2.5 mg/kg) plus DHI (4 mL/kg) may be ultimately facilitate new therapeutic approaches to enhance t-PA thrombolysis in stroke patients.

AUTHOR CONTRIBUTIONS

YZ conceived the idea and designed the study. ML and JZ participated in the experimental study and wrote the manuscript. WJ participated in the data collecting and statistical analysis. XL revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00308/full#supplementary-material

FIGURE S1 | The images of the evans blue infiltration after 1 day of different groups.

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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.

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Palmitoylethanolamide Ameliorates Hippocampal Damage and Behavioral Dysfunction After Perinatal Asphyxia in the Immature Rat Brain

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Perinatal asphyxia (PA) is an obstetric complication associated with an impaired gas exchange. This health problem continues to be a determinant of neonatal mortality and neurodevelopmental disorders. Palmitoylethanolamide (PEA) has exerted neuroprotection in several models of brain injury and neurodegeneration. We aimed at evaluating the potential neuroprotective role of PEA in an experimental model, which induces PA in the immature rat brain. PA was induced by placing *Sprague Dawley* newborn rats in a water bath at 37°C for 19 min. Once their physiological conditions improved, they were given to surrogate mothers that had delivered normally within the last 24 h. The control group was represented by non-fostered vaginally delivered pups, mimicking the clinical situation. Treatment with PEA (10 mg/kg) was administered within the first hour of life. Modifications in the hippocampus were analyzed with conventional electron microscopy, immunohistochemistry (for NeuN, pNF-H/M, MAP-2, and GFAP) and western blot (for pNF H/M, MAP-2, and GFAP). Behavior was also studied throughout Open Field (OF) Test, Passive Avoidance (PA) Task and Elevated Plus Maze (EPM) Test. After 1 month of the PA insult, we observed neuronal nucleus degeneration in CA1 using electron microscopy. Immunohistochemistry revealed a significant increase in pNF-H/M and decrease in MAP-2 in CA1 reactive area. These changes were also observed when analyzing the level of expression of these markers by western blot. Vertical exploration impairments and anxiety-related behaviors were encountered in the OF and EPM tests. PEA treatment attenuated PA-induced hippocampal damage and its corresponding behavioral alterations. These results contribute to the elucidation of PEA neuroprotective role after PA and the future establishment of therapeutic strategies for the developing brain.

Keywords: perinatal asphyxia, palmitoylethanolamide, neuroprotection, hippocampus, CA1 region

INTRODUCTION

Perinatal asphyxia (PA) is an obstetric complication occurring when oxygen supply to the newborn is temporally interrupted (Adcock and Papile, 2008). This health problem remains as a condition of high prevalence (1 to 10/1000 live births) worldwide (Douglas-Escobar and Weiss, 2015). PA continues to be a determinant of neurological morbidity (Morales et al., 2011). If the region affected is immature, the insult interferes with the initial plastic changes necessary for the establishment of brain circuitries (Herrera-Marschitz et al., 2014). Several neurodevelopmental disorders (NDDs) have been associated with PA, including Attention Deficit Disorder (Perna and Cooper, 2012), Autism Spectrum Disorder (Schieve et al., 2014; Fezer et al., 2017) and distinct learning disorders (van Handel et al., 2007). Symptomatology becomes apparent during childhood, as environmental demands increase (Herrera-Marschitz et al., 2017).

An experimental model of PA was established several years ago (Bjelke et al., 1991). This rat model of global asphyxia involves the submersion of pup containing uterine horns in a water bath immediately after caesarean section, mimicking delayed labor. This method presents other translational advantages (Barkhuizen et al., 2017; Herrera et al., 2017; Herrera-Marschitz et al., 2017), such as the induction of PA at a time point when rodent brain maturity resembles the brain of a human fetus at 22–32 gestational weeks (Semple et al., 2013). This aspect is of clinical relevance since prematurity is associated with PA and increases the risk of neurological morbidity (Kuzniewicz et al., 2014). In the last 26 years, this murine model contributed to scientific advances on the neuropathological, functional (Barkhuizen et al., 2017) and synaptic effects of PA on the CNS (Herrera et al., 2017), and on mechanisms of neuroprotection (Herrera-Marschitz et al., 2011; Muñiz et al., 2014). Research on neuroprotection is based on the concept of secondary lesion, which offers a therapeutic window for early intervention (Blanco et al., 2011).

Palmitoylethanolamide (PEA) is an endogenous lipid compound derived from the reaction between ethanolamine and palmitic acid (Guida et al., 2017a). PEA is member of the N-acylethanolamides (NAEs) family, characterized for inhibiting mechanisms of secondary injury (Ahmad et al., 2012a). This pro-homeostatic mediator (Petrosino et al., 2010; Petrosino and Di Marzo, 2017) exerted neuroprotective effects when administered exogenously in models of brain injury and neurodegeneration (Herrera et al., 2016), including spinal cord injury (Genovese et al., 2008; Esposito et al., 2011), traumatic brain injury (Ahmad et al., 2012a; Guida et al., 2017a), stroke (Ahmad et al., 2012b), Alzheimer's Disease (D'Agostino et al., 2012; Scuderi et al., 2014), and Parkinson's Disease (Esposito et al., 2012). These effects are apparently mediated by the nuclear Peroxisome Proliferator-Activated Receptor (PPAR)-α (Lo Verme et al., 2005).

Recent studies from our laboratory reported a dysregulation of NAEs signaling system in rat hippocampus at post-natal day 30 (P30) after PA, and suggested PEA might exert neuroprotective effects when administered exogenously (Blanco et al., 2015). P30 constitutes an interesting time point for testing the effect of neuroprotective agents since it is equivalent to 4–11 human years (Semple et al., 2013), a period of NDDs onset (Herrera-Marschitz et al., 2014, 2017). In addition, the hippocampus represents a brain region of major vulnerability to PA (Petito and Pulsinelli, 1984; Pulsinelli, 1985; Van de Berg et al., 2000) and of considerable implication in the pathophysiology of NDDs (Rojas et al., 2004; Saraceno et al., 2016). Therefore, the aim of the present work was to study the neuroprotective role of PEA in rat hippocampus at P30 after PA, contributing to the establishment of treatments for PA and the consequent prevention of NDDs.

MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Buenos Aires (CICUAL#4091/04) and conducted according to the principles of the Guide for the Care and Use of Laboratory Animals (Animal Welfare Assurance, A-3033-01/protocol#S01084).

Animals

Subjects consisted of 20 pregnant *Sprague Dawley* rats obtained from the central vivarium at the School of Veterinary Sciences, Universidad de Buenos Aires. Animals arrived 1 week prior to delivery to our local vivarium in order to acclimate to the new environment. They were housed in individual cages with constant temperature (21 ± 2°C) and humidity (65 ± 5%) conditions. A light/dark cycle of 12:12 h was employed. The light period began at 7 a.m. Food and tap water were provided.

Induction of PA

Rat pups were subjected to PA using the experimental model originally developed by Bjelke et al. (1991) and modified in our laboratory (Capani et al., 2009). At the expected day of delivery, gestational day 22, pregnant rats were observed and when the first pup was delivered, the dam was immediately euthanized by decapitation. Uterine horns were rapidly isolated through an abdominal incision, and submerged in a water bath at 37°C for 19 min, inducing severe asphyxia. When time elapsed, horns were rapidly opened and pups were subjected to tactile intermittent stimulation until regular breathing was established. Umbilical cord was tied and the animals were left to recover 1 h under a heat lamp.

Protocol of Neuroprotection

Sixty-three male rat pups were treated with PEA or vehicle (VHI) in a dose of 10 mg/kg by subcutaneous injection. This dose has shown effective results in several models of brain injury and neurodegeneration (Genovese et al., 2008; Esposito et al., 2011, 2012; Ahmad et al., 2012a,b; Sayd et al., 2014; Scuderi et al., 2014). The respective treatment was performed within the first hour of life. VHI used was 1:1:8 DMSO, Tween 80 and NaCl. The formulation of PEA tested was C18H37NO2 from Tocris Bioscience (Bristol, UK). This formulation crosses the blood-brain barrier because it is proven that this compound possess neuromodulatory effects *in vivo* through inhibition of nicotine-induced excitation on dopamine neurons in the ventral tegmental area of rats (Melis et al., 2008). Besides, this compound exhibits

antinociceptive, antiepileptic and anticonvulsant properties *in vivo* (Lambert et al., 2001; Sheerin et al., 2004). On the other hand, it is widely accepted that this PEA formulation is involved in the modulation of neuroinflammation produced in the central nervous system (Sayd et al., 2014). It has shown to be useful in the control of neuropathic pain (Re et al., 2007) and it could exert neuroprotective properties in neurological disorders such as stroke (Ahmad et al., 2012b), traumatic brain injury (Ahmad et al., 2012a), Alzheimer and Parkinson's disease (D'Agostino et al., 2012; Esposito et al., 2012) or addiction (Coppola and Mondola, 2013).

Once pups improved their physiological conditions, they were given to surrogate mothers that had delivered normally within the last 24 h. Four experimental groups were established: rats subjected to PA and injected with VHI (PA group, $n = 15$), rats born vaginally and injected with VHI (control – CTL- group, $n = 13$), rats subjected to PA and injected with PEA (PA+PEA group, $n = 18$) and rats born vaginally and injected with PEA (CTL+PEA group, $n = 17$). The different groups were marked and mixed in litters with a surrogate mother. After weaning, rats were housed in cages of 3–4 animals from the same group. Cesarean controls were not used since previous studies revealed this group did not present significant differences with vaginal controls (Galeano et al., 2011; Blanco et al., 2015).

Behavioral Testing

General Procedure

At P30, rats ($N = 63$) were subjected to behavioral tests in an isolated behavioral room from 8 a.m. to 5 p.m. In order to avoid the confounding effect of time of day, testing order of the groups was counterbalanced. The apparatus of each test was cleaned with 70 % ethanol between sessions to minimize the olfactory stimulus. White noise was provided during tests (Galeano et al., 2015). Before any of the tests, the assessed rat was placed in the behavioral room for acclimatization during 5 min (Molina et al., 2016). In this way, potential confounding variables were controlled. The following tests were performed.

Elevated Plus Maze (EPM) Test

The EPM test evaluates anxiety-related behaviors, which are hippocampal dependent (Montgomery, 1955; Brenes et al., 2009; Violle et al., 2009; Molina et al., 2016). The apparatus was made of black melamine and consisted of a central square platform (11 × 11 cm). Four illuminated arms radiated from this platform: two closed arms (50 × 11 × 40 cm) and two open arms (50 × 11 × 0.25 cm), conforming a maze in the shape of a plus. This maze was elevated to a height of 100 cm above floor-level (Galeano et al., 2015). Procedure: The test began by placing the rat on the central platform facing an open arm. The rat was allowed to freely explore the apparatus for 5 min. When time elapsed, the rat was removed from the apparatus and returned to the home cage. A rat was considered to enter an arm when its 4 paws were inside (Galeano et al., 2015). Total distance traveled and time spent in closed arms were quantified as indicators of locomotion. Time spent in open arms was measured in order to assess anxiety levels. The stretching of rats over the edge of an open arm, known as head-dipping (HD), was also recorded as a risk-assessment-behavior (RAB) (Rodgers and Cole, 1993; Molina et al., 2016). Prototypical behaviors, rearing and grooming, were assessed. Time spent rearing was recorded as a hippocampal-dependent indicator of vertical exploration (Lever et al., 2006). Rats that fell down from the maze during testing were excluded from the study. Testing sessions were filmed using a digital camera (Sony HDR-AS100V) and later analyzed with ANY-Maze video-tracking software (version 5.29). Rearing, grooming and HD were registered by blind evaluation and time was quantified using the key resource of ANY-Maze.

Open Field (OF) Test

The OF test was used to assess locomotion, exploratory activity and changes in emotionality induced by exposure to a novel environment (Molina et al., 2016). These behavioral parameters also depend on hippocampal integrity (Vianna et al., 2000; Barros et al., 2006; Molina et al., 2016). The apparatus consisted of a black melamine square (60 × 60 × 40 cm) surrounded by walls of 40 cm high (Galeano et al., 2015). Procedure: Rats were placed on the center of the apparatus and allowed to explore it for 5 min. (Molina et al., 2016). Total distance traveled and number of line crossings were quantified in order to measure locomotion. Prototypical behaviors, rearing and grooming, were assessed. Time spent rearing was recorded as a hippocampal-dependent indicator of vertical exploration (Lever et al., 2006). Testing sessions were filmed using a digital camera (Sony HDR-AS100V) and later analyzed with ANY-maze video-tracking software (version 5.29). Rearing and grooming were registered by blind evaluation and time was quantified using the key resource of ANY-maze.

Inhibitory Avoidance (IA) Task

The IA task measures memory of an aversive experience through the avoidance of a location where an unpleasant experience occurred (Molina et al., 2016; Saraceno et al., 2016). This parameter constitutes an indicator of associative memory, which is hippocampal-dependent (Ennaceur and Delacour, 1988; Lorenzini et al., 1996; Izquierdo and Medina, 1997; Molina et al., 2016). The apparatus consisted of a black melamine square (60 × 60 × 40 cm) divided into two compartments: one was illuminated and the other was dark. A removable cover was used for this purpose. The floor of the dark compartment was made of a stainless steel grid, which could deliver an electric shock when pressing a button. Procedure: The test was divided in three sessions. The habituation session consisted of placing the rat in the illuminated compartment. After entering the dark section three times, the rat was removed from the apparatus. If the rat did not enter the dark side three times, it was removed after the three-minute trial was completed. The next trial was performed after 10 min by placing the rat again in the lit compartment. When it entered the dark section, doors were closed retaining the rat for 10 s inside this compartment. The training session took place after an hour. The rat was placed in the illuminated section and the latency to enter the dark side was registered. As soon as the rat entered the dark compartment, an electric shock (1.2 mA, 2s) was delivered. Then, the rat was returned to its home cage. One

hour later, the rat was placed in the lit compartment but this time the electric shock was not delivered. This is the retention session, when the latency to enter the dark side is again measured. The ratio between the latency to enter the dark compartment in the retention (T2) and the training session (T1), which is expressed as T2/T1, constitutes an indicator of aversive associative memory (Molina et al., 2016).

Order of Testing

Tests were performed in the order they were described above. As the EPM test is specific for measuring anxiety, it was performed firstly, in order to avoid the confounding effect of previous tests. Inversely, the IA task was administered at the end because the aversive effect of the electric shock could constitute a confounding variable for following tests.

Tissue Fixation and Immunohistochemistry

At P30, intracardiac perfusion and coronal hippocampal sections were performed as described previously (Saraceno et al., 2016). Four animals per treatment (four replicates) were used. Three sections were immunostained for each analyzed brain. Animals were anesthetized with intraperitoneal administration of ketamine 40 mg/kg and xylazine 5 mg/kg, and perfused intracardiacally with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and post-fixed in the same fixative solution for 2 h at room temperature, and then immersed overnight at 4°C in 0.1 M phosphate buffer, pH 7.4. Coronal hippocampal sections (50 μm thickness) were obtained using a Vibratome (VT 1000 S, Leyca Microsystems, Wetzlar, Germany). Immunohistochemistry was performed on free-floating sections under moderate shaking. Endogenous peroxidase was quenched (3% H_2O_2, 70% methanol, 30% H_2O_2 30 vol) and non-specific labeling was blocked using 0.3% normal goat serum. Free-floating sections were incubated overnight at 4°C with anti-neuron-specific nuclear protein (NeuN; 1:1,000, mouse-IgG; Millipore), anti-microtubule-associated protein 2 (MAP-2; 1:250, polyclonal rabbit-IgG; Abcam), anti-phosphorylated high and medium molecular weight neurofilaments (pNF H/M; 1:500, monoclonal mouse-IgG; Millipore) or anti-glial fibrillary acidic protein (GFAP; monoclonal rabbit IgG, 1:200, Cell Marque, a Sigma-Aldrich Company). Then sections were incubated for 2 h at room temperature (RT) with secondary antibodies (Biotinylated anti-mouse-IgG, 1:500, Vector; Biotinylated anti-rabbit-IgG, 1:500, Vector). Amplification was done using avidin-biotinylated horseradish peroxidase complex (1:500, Dako) in PBS for 1 h, followed by washing in PBS before chromogen development (DAB; Cell Marque, a Sigma-Aldrich Company). Light microscopic images were obtained (using a Leyca microscopy).

Electron Microscopy Procedure

Coronal brain sections of four animals were cut at a thickness of 40 μm with a vibratome through the level of the dorsal *neostriatum* and post-fixed for 1 h with 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. Then, tissue sections from hypoxic and control animals were stained by conventional osmium–uranium–lead method, following the protocol described in Capani et al. (2001). Thin sections (50-80 nm) were cut with Reichert Ultracut E by using glass knives. Sections were counterstained with a combination of lead citrate. Thin sections were examined using a JEOL 200CX electron microscope at 80–100 keV.

Morphometric Analysis

One hundred fifty by 150 μm was sampled in each photo in order to estimate the percentage of reactive area for pNF-H/M and MAP-2 using Image J Program (Image J 1.41o, NIH, USA). We focused our analysis on CA1 area of hippocampus since it is one of the most affected areas by PA insult (Petito and Pulsinelli, 1984; Pulsinelli, 1985; Van de Berg et al., 2000). The number of GFAP immunoreactive astrocytes was estimated manually in the *stratum radiatum* of CA1 hippocampal area. A total of 80 counting frames was assessed per animal. A blind observer selected 5 fields for each sector from 4 sections of *striatum* and 10 fields of cortex from 10 sections (two hundred fields of *striatum* and 200 fields of cortex). To estimate the percentage of reactive area the experiments were repeated at least 3 times.

Western Blot

Western blot analysis was performed using four animals per treatment (four replicates). Experiments were repeated three times for each brain. Animals were euthanized by decapitation, brains were dissected, homogenized in ice-cold lysis buffer (10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.1% Triton X-100, protease inhibitors). Tissues were thawed on ice and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatants were analyzed for total protein concentration using Bradford solution (Bio-Rad, Richmond CA, USA) in 96-well plates using bovine serum albumin (BSA) as standard. 90 μg of total protein were diluted in sample buffer (0.3 M Tris/HCl, pH 7, 50% glycerol, 5%SDS, 1 mM EDTA, 0.1% bromophenol blue). The samples were subjected to SDS-PAGE using the Mini-protein II cell (Bio-Rad, Richmond CA, USA) with precast 4–20% Precise gels (Bio-Rad, Richmond CA, USA). Proteins were transferred to PVDF membranes (MACHEREY-NAGEL, Germany) using the semi-dry transfer unit Hoefer TE 70 (Amersham Biosciences). Membranes were blocked with 5% non-fat milk powder and 1% BSA in Tris-buffered saline containing 0.05% Tween 20 and incubated overnight at 4°C with anti-microtubule-associated protein 2 (MAP-2; 1:1,000, polyclonal rabbit-IgG; Abcam), anti-phosphorylated high and medium molecular weight neurofilaments (pNF-H/M; 1:500, monoclonal mouse-IgG; Millipore) and anti-glial fibrillary acidic protein (GFAP; monoclonal mouse-IgG, 1:1,000; Santa Cruz Biotechnology). We used anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1,000, rabbit-IgG, Sigma-Aldrich) as loading control. Blots were rinsed three times in PBS with 0.5% Tween-20 buffer (PBST), and then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (1:3,000,Bio-Rad, Richmond CA, USA) for 1 h at RT. Immunoreactive bands were detected using an ECL western blotting analysis system (clarity western ECL substrate, Bio-Rad). Films were scanned and the optical density of protein

bands was quantified using Gel Pro Analyzer 3.1.00.00 (Media Cybernetics).

Statistical Analysis

Results were expressed as means ± SEM. Normal distribution and equality of variances were checked using Shapiro-Wilk test and Levene's test, respectively. Statistical analyses were conducted by two-way analysis of variances (ANOVAs) with birth condition (CTL and PA) and treatment (VHI and PEA) as the main factors. When interaction effects were significant, analyses were performed by *post hoc* comparisons using Student's *t*-test (two-tailed) adjusted by Bonferroni correction. Differences with a probability of 5% or less were considered to be significant. Statistical analysis was carried out using Graph pad Prism 5 program.

RESULTS

Vulnerability of CA1 Hippocampal Neurons to PA. Effects of PEA Treatment

Nuclear morphology was analyzed to evaluate the vulnerability of CA1 neurons at the *stratum radiatum* of rat hippocampus at P30 (**Figure 1**; **Table 1**). We observed a significant increase ($F = 2142$, $p < 0.05$) in the number of pyknotic nucleus in the hippocampal CA1 neurons layer of asphyctic animals (**Table 1**). In addition, the number of pyknotic nucleus was significantly reduced in the PA+PEA group ($F = 1978$, $p < 0.05$) (**Table 1**). Then we analyzed NeuN+ neurons and classified them in two categories: (a) normal neurons, characterized by an intense NeuN+ nucleus and (b) abnormal neurons, with NeuN fragmentation of the nucleus, or cytoplasmic staining without nuclear staining (Robertson et al., 2006). We observed a significant increase in the number of abnormal neurons in the hippocampal CA1 layer of asphyctic animals ($F = 2036$, $p < 0.05$) (**Table 1**; **Figures 1A,B**). PEA treatment improved significantly nucleus alterations ($F = 1936$, $p < 0.05$) (**Table 1**; **Figures 1B,D**). Electron microscopy analyses showed clear degeneration of CA1 neurons after PA (**Figures 1E,F**), which was reduced after treatment with PEA (**Figures 1F–H**). Altogether, these results indicate that major PA effects occurred on CA1 neurons and could be ameliorated by PEA treatment.

PA-Induced Alterations in pNF-H/M. Protective Effect of PEA Treatment

The accumulation of pNF-H/M as a measure of axonal dysfunction and degeneration was analyzed by immunostaining. **Figure 2A** shows a representative example of the *stratum radiatum* of CA1 hippocampal section immunostained for pNF-H/M. The two-way ANOVA revealed that the main factors of birth condition and treatment were both significant when analyzing the percentage of reactive area for pNF-H/M ($F = 2033$, $p < 0.0001$; $F = 509.8$, $p < 0.0001$, respectively), and the

TABLE 1 | Damage of CA1 hippocampal neurons after PA and effect of PEA treatment.

Groups	Pyknotic nuclei	NeuN + neurons	Normal neurons	Abnormal neurons
CTL	13.24 ± 1.42	76.35 ± 1.56	71.24 ± 0.33	5.11 ± 0.51
PA	20.96 ± 0.21*	64.24 ± 3.54	53.44 ± 0.82	10.8 ± 0.71*
CTL+PEA	12.34 ± 1.33	73.23 ± 2.32	69.25 ± 0.93	5.98 ± 0.43
PA+PEA	15.36 ± 1.97	67.26 ± 2.48	63.35 ± 0.72	7.91 ± 0.64

*Data expressed as mean ± SD. Statistical differences were assessed by two-way ANOVA. *P < 0.05.*

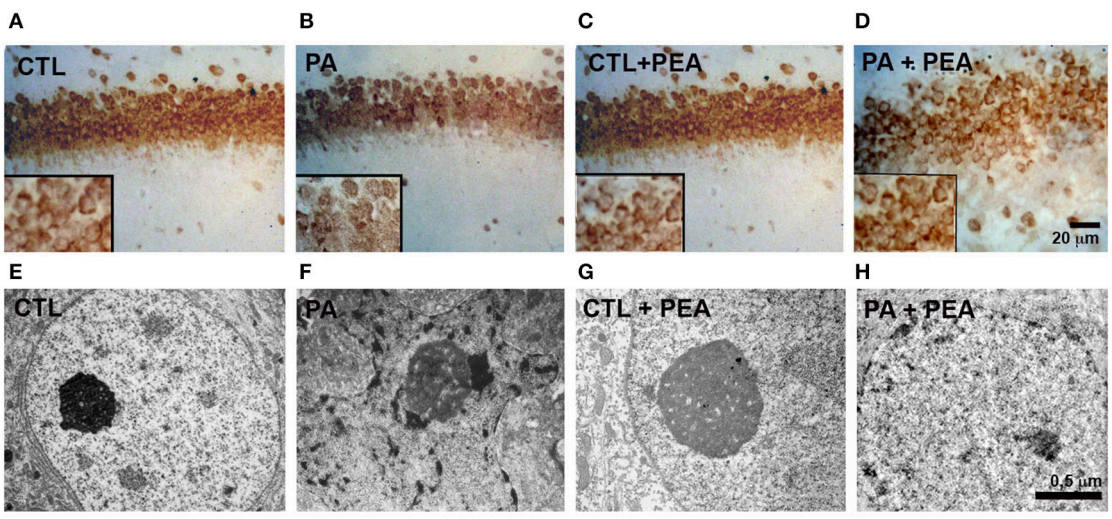

FIGURE 1 | Neuronal alterations induced by PA and recovery after PEA treatment. **(A-D)** Micrographs of Stratum radiatum of CA1 hippocampal area in the different groups analyzed by NeuN Immunostaining. **(E–H)**. Electron microscopy (EM) of CA1 neurons showing that most of the condensed cells correspond to neurons in degeneration. Statistical analysis of different parameters was performed. Significant differences were calculated by two-way analysis of variances (ANOVAs) tests followed by *post hoc* comparisons using Student's *t*-test (two-tailed) adjusted by Bonferroni correction.

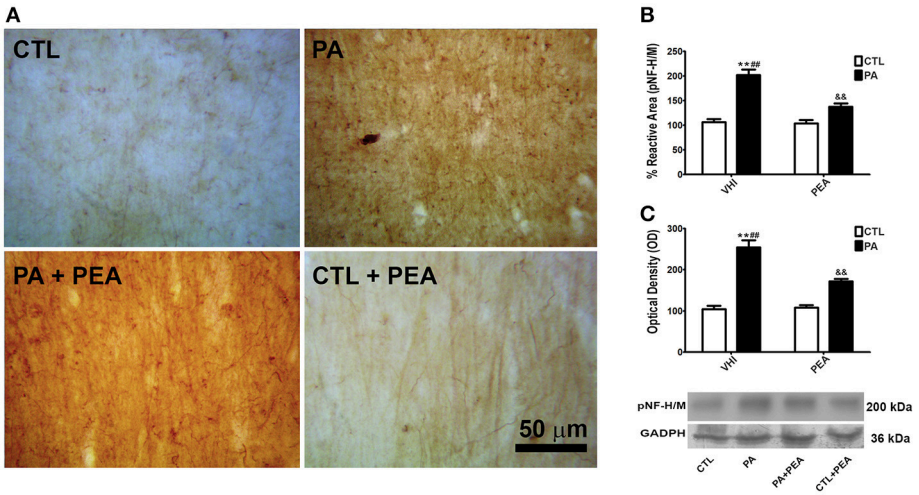

FIGURE 2 | pNF-H/M immunostaining and protein expression levels in the rat hippocampus. PEA treatment attenuated increased pNF-H/M expression after PA. **(A)** Representative image of the striatum radiatum of CA1 hippocampal area immunostained for pNF-H/M in the different groups. **(B)** The statistical analysis revealed a significant increase in the percentage of reactive area of pNF-H/M inmunostaining in PA rats treated with vehicle (PA) in comparison to CTL rats treated with vehicle (CTL), and a significant decrease in the percentage of reactive area of pNF-H/M inmunostaining in PA rats treated with PEA (PA+PEA) in comparison to PA rats treated with vehicule (PA), suggesting a partial reversion of axonal dysfunction and degeneration associated with perinatal asphyxia, although PA+PEA group did not reach values similar to controls. PEA by itself did not produce any differences in the percentage of reactive area of pNF-H/M inmunostaining, since CTL+PEA rats did not differ from CTL rats. **(C)** Consistent with these morphological observations, results similar to those of the percentage of reactive area were observed by western blot in protein expression levels of pNF-H/M. Bars and error bars represent mean + SEM calculated by two-way analysis of variances (ANOVAs) tests followed by *post hoc* comparisons using Student's *t*-test (two-tailed) adjusted by Bonferroni correction. $^{**}P < 0.001$, PA vs. CTL; $^{\#\#}P < 0.001$, PA vs. PA+PEA; $^{\&\&}P < 0.001$, PA+PEA vs. CTL.CTL, Control group; PA, rats subjected to PA; PA+PEA, rats subjected to PA and PEA treatment; CTL+PEA, control group subjected to PEA treatment.

interaction was also significant ($F = 604.8$, $p < 0.0001$). *Post hoc* analysis of the simple effects indicated that the reactive area for pNF-H/M at P30 showed a significant increase as a consequence of PA ($t = 49.27$, $p < 0.001$) (**Figure 2B**). This alteration was significantly attenuated in PA+PEA group ($t = 31.54$, $p < 0.001$), although this group did not reach values similar to controls ($t = 14.49$, $p < 0.001$) (**Figure 2B**). Consistent with these morphological observations, birth condition and treatment were significant with respect to protein expression for pNF-H/M ($F = 2494$, $p < 0.0001$; $F = 517.9$, $p < 0.0001$, respectively). The interaction was also significant ($F = 477.1$, $p < 0.0001$). An increase in pNF-H/M expression was observed in PA group ($t = 50.76$, $p < 0.001$), and reduced after PEA treatment ($t = 13.66$, $p < 0.001$) (**Figure 2C**). However, PA+PEA group still presented significant differences with controls ($t = 19.87$, $p < 0.001$) (**Figure 2C**). Treatment with PEA in CTL rats had no significant effect on both reactive area and protein expression for pNF-H/M in comparison to CTL rats injected with VHI ($t = 0.6472$, $p > 0.05$; $t = 0.09113$, $p > 0.05$, respectively) (**Figures 2B,C**, respectively).

Dendritic Cytoskeleton Alteration and Its Attenuation by PEA Treatment

Since PA affected neuronal population, we also studied cytoskeleton organization of neural processes. Changes in dendrite morphology were analyzed through immunostaining of a dendrite-specific marker, MAP-2 (**Figure 3A**). According to two-way ANOVA results for MAP-2 reactive area, both birth condition and treatment were significant ($F = 1342$, $p < 0.0001$; $F = 72.89$, $p < 0.0001$, respectively). The interaction was also significant ($F = 59.01$, $p < 0.0001$). *Post hoc* analysis revealed the PA group presented a significant decrease in the percentage of MAP-2 reactive area in comparison to the CTL group ($t = 31.33$, $p < 0.001$) (**Figure 3B**). This reduction in MAP-2 reactive area was partially reversed after PEA treatment ($t = 11.47$, $p < 0.001$), without reaching values similar to controls ($t = 20.47$, $p < 0.001$) (**Figure 3B**). These morphological findings were confirmed by western blot. Birth condition and treatment were significant factors for MAP-2 protein expression ($F = 5181$, $p < 0.0001$; $F = 316.7$, $p < 0.0001$, respectively), and the interaction was significant ($F = 289.7$, $p < 0.0001$). A significant reduction in MAP-2 expression was observed as a consequence of PA ($t = 62.93$, $p < 0.001$) (**Figure 3C**). This modification was attenuated in the PA+PEA group ($t = 24.62$, $p < 0.001$). However, this group still presented significant differences with controls ($t = 38.86$, $p < 0.001$) (**Figure 3C**). Finally, treatment with PEA did not exert a significant effect either in MAP-2 reactive area or in protein expression when comparing CTL and CTL+PEA groups ($t = 0.6053$, $p > 0.05$; $t = 0.5479$, $p > 0.05$, respectively) (**Figures 3B,C**, respectively).

GFAP Immunostaining and Protein Expression at P30 After PA

We used immunohistochemistry and western blot to analyze glial response to PA injury and PEA treatment. As regards the number of GFAP positive astrocytes, the two-way ANOVA

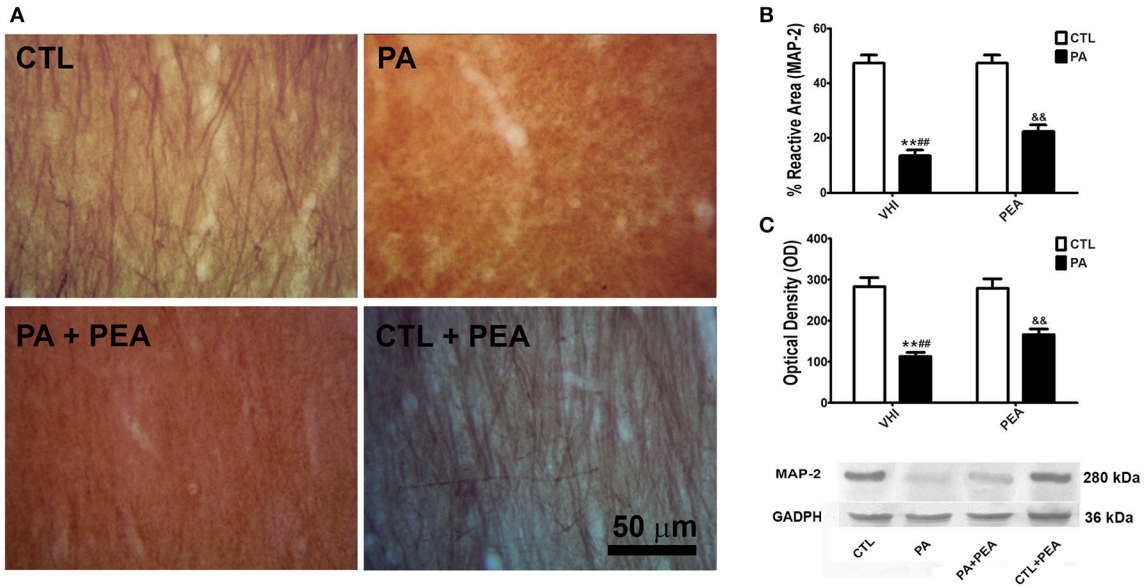

FIGURE 3 | MAP-2 immunostaining and protein expression levels in the rat hippocampus. **(A)** Representative image of the striatum radiatum of CA1 hippocampal area immunostained for MAP-2 in the different groups. **(B)** The statistical analysis revealed a significant decrease in the percentage of reactive area of MAP-2 positive dendrites in PA rats treated with vehicle (PA) in comparison to CTL rats treated with vehicle (CTL), and a significant increase in the percentage of reactive area of MAP-2 positive dendrites in PA rats treated with PEA (PA+PEA) in comparison to PA rats treated with vehicule (PA), suggesting a partial reversion of dendritic alterations associated with perinatal asphyxia, although PA+PEA group did not reach values similar to controls. PEA by itself did not produce any differences in the percentage of reactive area of MAP-2 positive dendrites, since CTL+PEA rats did not differ from CTL rats. **(C)** Consistent with these morphological observations, results similar to those of the percentage of reactive area were observed by western blot in protein expression levels of MAP-2. Bars and error bars represent mean + SEM calculated by two-way analysis of variances (ANOVAs) tests followed by *post hoc* comparisons using Student's *t*-test (two-tailed) adjusted by Bonferroni correction. $^{**}P < 0.001$, PA vs. CTL; $\#\#P < 0.001$, PA vs. PA+PEA; $\&\&P < 0.001$, PA+PEA vs. CTL.CTL, Control group; PA, rats subjected to PA; PA+PEA, rats subjected to PA and PEA treatment; CTL+PEA, control group subjected to PEA treatment.

indicated that birth condition and treatment were not significant ($F = 0.0009796$, $p = 0.9752$; $F = 0.02149$, $p = 0.884$). These results were confirmed when analyzing the expression of GFAP. Birth condition and treatment were not significant either ($F = 1.194$, $p = 0.2853$; $F = 0.9972$, $p = 0.328$, respectively).

Behavioral Modifications Induced by PA at P30. Protective Role of PEA

Locomotion and general activity were not affected by PA at P30. With respect to the number of line crossings in the OF test, the two-way ANOVA revealed neither birth condition ($F = 1.93$, $p = 0.1716$), nor treatment ($F = 0.6706$, $p = 0.4172$) were significant. Similarly, birth condition and treatment were not significant for time spent in closed arms in the EPM test ($F = 0.03662$, $p = 0.8493$; $F = 3.624$, $p = 0.0646$, respectively). In addition, birth condition and treatment were not significant for total distance traveled in the OF ($F = 0.8553$, $p = 0.3592$; $F = 0.005146$, $p = 0.9431$, respectively) and EPM test ($F = 0.1234$, $p = 0.7272$; $F = 2.645$, $p = 0.1119$, respectively).

In contraposition, vertical exploration might be altered at P30 as a consequence of PA, as it can be inferred from results in rearing, a prototypical behavior in rats. As for time spent rearing in the OF test, the two-way ANOVA revealed that the main factors of birth condition and treatment were significant ($F = 5.51$, $p = 0.0226$; $F = 5.779$, $p = 0.0197$, respectively). However, the interaction was not significant ($F = 2.888$, $p = 0.095$). *Post hoc* analysis indicated PA rats showed a significant decrease in time spent rearing with respect to the CTL group ($t = 2.838$, $p < 0.05$). This behavioral alteration was reversed in the PA+PEA group ($t = 3.119$, $p < 0.01$). This group did not present significant differences with controls ($t = 0.462$, $p > 0.05$) (**Figure 4A**). Similar results were found in the EPM test as regards time spent rearing. Birth condition and treatment were significant factors ($F = 41.26$, $p < 0.0001$; $F = 10.69$, $p = 0.0024$, respectively), and the interaction was also significant ($F = 11.67$, $p = 0.0016$). According to *post hoc* analysis of the simple effects, a significant reduction in time spent rearing was registered as a consequence of PA ($t = 6.958$, $p < 0.001$), and reversed after PEA treatment ($t = 4.728$, $p < 0.001$). PA+PEA group did not show significant differences with respect to controls ($t = 2.126$, $p > 0.05$) (**Figure 5A**). Both in OF and EPM test, treatment with PEA in CTL rats had no significant effect on time spent rearing in comparison to CTL rats injected with VHI ($t = 0.4677$, $p > 0.05$; $t = 0.1042$, $p > 0.05$, respectively) (**Figures 4A**, **5A**, respectively).

Although birth condition was not significant for time spent in open arms in the EPM test ($F = 3.203$, $p = 0.0813$), other anxiety-related behaviors were encountered as a consequence of PA at P30. With regard to time spent grooming in the OF test, two-way ANOVA revealed birth condition and treatment were significant ($F = 6.015$, $p = 0.0172$; $F = 16.64$, $p = 0.0001$, respectively), but

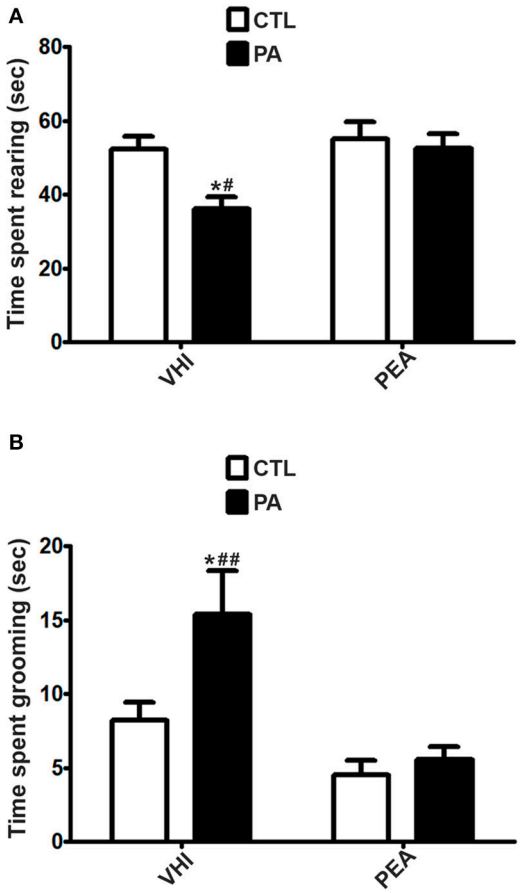

FIGURE 4 | Behavioral differences in time spent rearing and time spent grooming between the 4 groups in the OF test. **(A)**. The statistical analysis revealed a significant reduction in time spent rearing in PA rats treated with vehicle (PA) in comparison to CTL rats treated with vehicle (CTL) and PA rats treated with PEA (PA+PEA). In addition, PA+PEA rats did not show significant differences on time spent rearing in comparison to CTL rats, suggesting a reversion of this behavioral alteration associated with perinatal asphyxia. PEA by itself had no significant effect on time spent rearing, since CTL+PEA rats did not differ in time spent rearing from CTL rats. **(B)** The statistical analysis indicated time spent grooming was significantly augmented in PA rats treated with vehicle (PA) in comparison to CTL rats treated with vehicle (CTL) and PA rats treated with PEA (PA+PEA). In addition, PA+PEA rats did not show significant differences on time spent grooming in comparison to CTL rats, suggesting a reversion of this behavioral alteration associated with perinatal asphyxia. PEA by itself had no significant effect on time spent grooming, since CTL+PEA rats did not differ in time spent grooming from CTL rats. Bars and error bars represent mean + SEM calculated by two-way analysis of variances (ANOVAs) tests followed by *post hoc* comparisons using Student's *t*-test (two-tailed) adjusted by Bonferroni correction. $^{*}P < 0.05$, PA vs. CTL; $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$, PA vs. PA+PEA.CTL, Control group; PA, rats subjected to PA; PA+PEA, rats subjected to PA and PEA treatment; CTL+PEA, control group subjected to PEA treatment.

not the interaction ($F = 3.405$, $p = 0.8767$). *Post hoc* analysis indicated time spent grooming was significantly augmented as a result of PA ($t = 2.88, p < 0.05$), and reversed after PEA treatment ($t = 4303, p < 0.001$). PA+PEA group did not present significant differences with controls ($t = 0.4561, p > 0.05$) (**Figure 4B**). Time spent grooming was also altered in the EPM test as a consequence

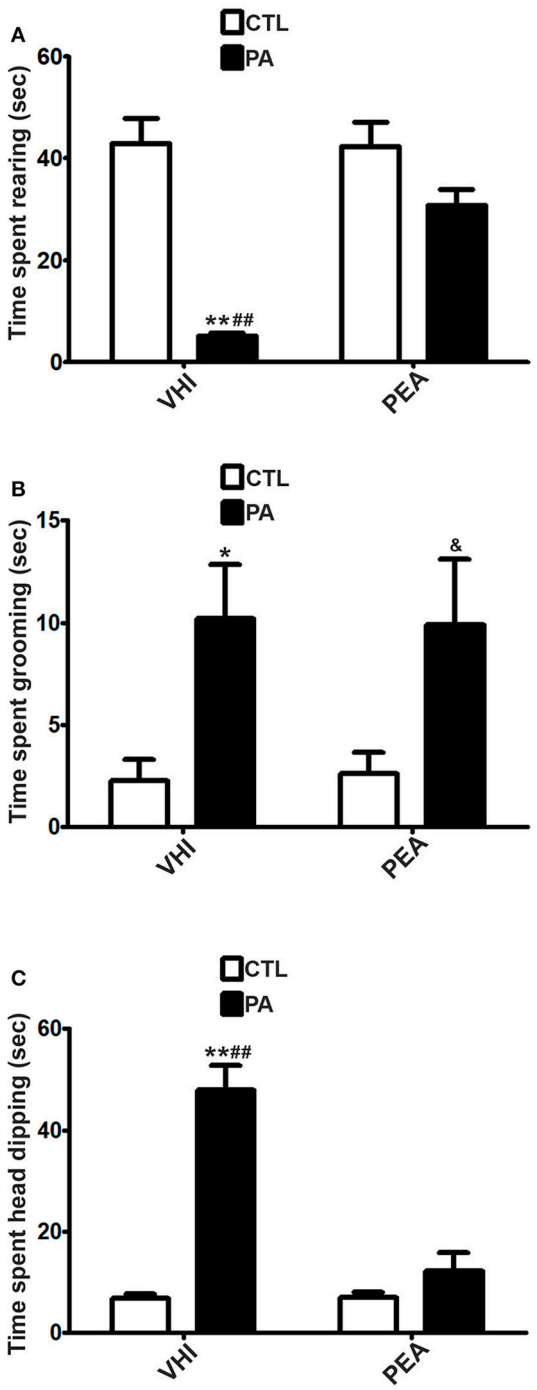

FIGURE 5 | Behavioral differences in time spent rearing, time spent grooming and time spent head dipping between the 4 groups in the EPM test. **(A)** The statistical analysis revealed a significant reduction in time spent rearing in PA rats treated with vehicle (PA) in comparison to CTL rats treated with vehicle (CTL) and PA rats treated with PEA (PA+PEA). In addition, PA+PEA rats did not show significant differences on time spent rearing in comparison to CTL rats, suggesting a reversion of this behavioral alteration associated with perinatal asphyxia. PEA by itself had no significant effect on time spent rearing, since CTL+PEA rats did not differ in time spent rearing from CTL rats. **(B)** The statistical analysis indicated time spent grooming was significantly augmented

(Continued)

FIGURE 5 | in PA rats treated with vehicle (PA) in comparison to CTL rats treated with vehicle (CTL), but PA rats treated with PEA (PA+PEA) did not differ on time spent grooming in comparison to PA rats treated with vehicule (PA), suggesting that PEA did not exert a protective effect in PA rats. In addition, PA+PEA rats presented significant differences on time spent grooming in comparison to CTL rats. PEA by itself had no significant effect on time spent grooming, since CTL+PEA rats did not differ in time spent grooming from CTL rats. **(C)** The statistical analysis indicated time spent head dipping was significantly augmented in PA rats treated with vehicle (PA) in comparison to CTL rats treated with vehicle (CTL) and PA rats treated with PEA (PA+PEA). In addition, PA+PEA rats did not show significant differences on time spent head dipping in comparison to CTL rats, suggesting a reversion of this behavioral alteration associated with perinatal asphyxia. PEA by itself had no significant effect on time spent head dipping, since CTL+PEA rats did not differ in time spent head dipping from CTL rats. Bars and error bars represent mean + SEM calculated by two-way analysis of variances (ANOVAs) tests followed by *post hoc* comparisons using Student's *t*-test (two-tailed) adjusted by Bonferroni correction. $^{*}P < 0.05$ and $^{**}P < 0.001$, PA vs. CTL; $^{\#\#}P < 0.001$, PA vs. PA+PEA; $^{\&}P < 0.05$, PA+PEA vs. CTL. CTL, Control group; PA, rats subjected to PA; PA+PEA, rats subjected to PA and PEA treatment; CTL+PEA, control group subjected to PEA treatment.

of PA, but PEA treatment did not exert a protective effect in this case. Two-way ANOVA indicated birth condition was significant ($F = 14.15, p = 0.0007$), in contraposition to treatment ($F = 0.0001407$, $p = 0.9906$) and the interaction ($F = 0.02445$, $p = 0.8767$). *Post hoc* analysis revealed time spent grooming was significantly increased in the PA group in comparison to the CTL group ($t = 2.873$, $p < 0.05$), but not reversed in the PA+PEA group ($t = 0.09661$, $p > 0.05$). This group presented significant differences with controls ($t = 2.464$, $p < 0.05$) (**Figure 5B**). In both OF and EPM tests, treatment with PEA in CTL rats had no significant effect on time spent grooming in comparison to CTL rats injected with VHI ($t = 1.54$, $p > 0.05$; $t = 0.1267$, $p > 0.05$, respectively) (**Figures 4B**, **5B**, respectively). As for time spent HD (in the EPM test), two-way ANOVA indicated birth condition, treatment and the interaction were significant ($F = 53.33$, $p < 0.0001$; $F = 33.4$, $p < 0.0001$; $F = 34.19$, $p < 0.0001$, respectively). *Post-hoc* analysis of the simple effects revealed time spent HD was significantly augmented in the PA group ($t = 9.781$, $p < 0.001$), and reversed in the PA+PEA group ($t = 7.99$, $p < 0.001$). This group did not show significant differences with controls ($t = 1.185$, $p > 0.05$). No significant differences were found between CTL and CTL+PEA groups either ($t = 0.04995$, $p > 0.05$) (**Figure 5C**).

Finally, aversive associative memory might not be affected at P30 as a consequence of PA. Two-way ANOVA revealed birth condition was not significant with respect to T2/T1 ratio from the IA task ($F = 0.854$, $p = 0.361$). Treatment was not significant either ($F = 0.0061$, $p = 0.931$).

DISCUSSION

In the present work we investigated biochemical, morphological and behavioral alterations induced by the PA rat model at P30, since this time point corresponds to the age of NDDs onset in humans (Herrera-Marschitz et al., 2014, 2017). We also examined the effect that the neuroprotective treatment with PEA exerted at this sensitive time-window when deficits become evident (Meredith et al., 2012). Interestingly, our findings show vulnerability of CA1 hippocampal neurons to PA at P30. These neurons showed clear signs of degeneration, including NeuN fragmentation of the nucleus, cytoplasmic staining without nuclear staining, and nuclear pyknosis. Neuronal cytoskeleton was affected as it can be inferred from an excessive accumulation of pNF-H/M and a significant reduction in MAP-2 marker. The functional correlate was associated with changes in prototypical behaviors, which indicate vertical exploration impairments and altered anxiety levels. PEA treatment (10 mg/kg) within the first hour of life could attenuate these alterations at P30, which precede well-known long-term impairments induced in the PA rat model (Capani et al., 2009; Saraceno et al., 2010, 2012; Galeano et al., 2011, 2015; Muñiz et al., 2014).

In previous works from our laboratory, the effect of PA was evaluated using both cesarean and vaginal fostered controls. Blanco et al. (2015) reported no differences in most of the behavioral, cellular and molecular parameters assessed using cesarean and vaginal controls. The effect of PA at P30 was also assessed using vaginal fostered control pups and major differences were found between both groups (Saraceno et al., 2016). Therefore, considering these results and the fact that we subject animals to a severe PA (19 min), we consider that differences between groups can be attributed mainly to the effect of PA insult. Additionally, in the present work, we mixed asphyctic rats with control rats in order to provide the same mother and postnatal environment, and asphyctic rats were well accepted by these surrogate mothers (see Materials and Methods section).

Neuronal Cytoskeleton Modifications Induced by PA at P30

Previous studies have shown that neurofilaments change their degree of phosphorylation after cerebral hypoxia-ischemia (Mink and Johnston, 2000) and serve as clinical biomarkers of hypoxic-ischemic encephalopathy (Douglas-Escobar et al., 2010). Dendritic alterations are also a common finding under glucose/oxygen deprivation (Park et al., 2008) and hypoxic-ischemic injury (Zhu et al., 2003; Takita et al., 2004). A 40% decrease of MAP-2-positive cells/mm^3 was observed in organotypic hippocampal cultures from asphyxia-exposed animals (Morales et al., 2007). Since MAP-2 is a cytoskeletal protein, its expression depends on ATP concentration, which is reduced as a result of hypoxia (Ashworth et al., 2003). Calpain-induced proteolysis of MAP-2 may be an initial response to a hypoxic insult (Johnson and Jope, 1992). In agreement with this background, our results reveal a significant increase of pNF-H/M and a reduction of MAP-2 as a result of PA at P30. These findings suggest pNF-H/M and MAP-2 constitute promising biomarkers of short-term PA-induced damage in CA1, which can be considered as an initial degenerative process of neurons, as it can be inferred from our electron microscopy analyses. In addition, dendritic alterations (Matesic and Lin, 1994) and aberrant accumulation of pNF-H/M (Dale and Garcia, 2012) are well-known hallmarks of neurodegeneration in several diseases.

Functional Correlate of Neuronal Cytoskeletal Alterations at P30

Recent evidence suggests hyperphosphorylation of neurofilaments (Chen et al., 2017) and decreased MAP-2 levels (Soares et al., 2016) in the hippocampus are correlated with cognitive and anxiety dysfunctions in murine models of neurodegeneration and global cerebral ischemia, respectively. In the present study, PA-induced hippocampal accumulation of pNF-H/M and reduction of MAP-2 at P30 exhibited a similar behavioral correlate. PA induced a significant decrement in time spent rearing, a hippocampal-dependent behavior which accounts for vertical exploration in response to novelty (Lever et al., 2006). We hypothesize exploration could have been modulated by excessive grooming displayed by PA rats. Long time spent grooming has been associated with increased anxiety levels (Kalueff and Tuohimaa, 2005), which tend to interfere on free exploration (Lever et al.). In addition, the significant augmentation encountered in time spent HD suggests PA induced an increase in risk assessment behaviors (RAB), which might also alter exploratory activity.

Alterations in rearing, grooming and HD at P30 might precede long-term impairments in locomotion and horizontal exploration (Galeano et al., 2011). In fact, these PA-induced modifications in prototypical behaviors at P30 could not be attributed to impairments in locomotion or general activity, since no differences were observed in number of line crossings, time spent in closed arms, and total distance traveled in OF and EPM tests. Unlike the OF and the EPM tests, the IA task might not be sensitive to early changes induced by PA. Aversive associative memory was not impaired at P30, reproducing previous findings from our laboratory (Saraceno et al., 2016).

Glial Response to PA at P30

Unlike pNF-H/M and MAP-2, GFAP might not be an appropriate marker at P30. No significant differences were observed in the number of GFAP astrocytes or in GFAP expression 1 month after PA. Similar results were reported in previous studies from our laboratory (Saraceno et al., 2016), where we suggested PA-induced modifications in astrocyte population might be progressive and sustained long after PA. Astrogliosis is generally reported in a tertiary phase post-insult, occurring months after PA (Douglas-Escobar and Weiss, 2015). In this sense, we have previously observed a markedly significant increase in the number of GFAP immunoreactive astrocytes at P120 (Saraceno et al., 2010). Consistent with our current and previous findings, recent clinical evidence suggests GFAP is not an early marker of injury in PA (Looney et al., 2015).

Neuroprotective Effect of PEA Treatment

PEA treatment (10 mg/kg) displayed protective effects by attenuating PA-induced degeneration and cytoskeletal alterations of CA1 neurons. As for microglia, even when PEA is produced by microglial cells and has a great effect on their modulation (Walter et al., 2003; Guida et al., 2017b), rodents subjected to hypoxic-ischemic injury present low microglial activity at P30. Therefore, the pharmacological inhibition of microglia at P30 could worsen the outcome after PA (Ferrazzano et al., 2013). PEA could also reverse exploration deficits and modulate anxiety levels at P30. *In vitro* studies in organotypic hippocampal slices have shown neuroprotective effects of PEA via PPAR-α (Koch et al., 2011; Scuderi et al., 2012). This receptor and its endogenous ligand, PEA, are involved in several cognitive and emotional processes (Fidaleo et al., 2014).

Although PEA endogenous content tends to increase around P30 in physiological conditions (Lee et al., 2013), a dysregulation of NAEs signaling system is induced by PA at this time point (Blanco et al., 2015; Holubiec et al., 2017). One month after PA, the expression of enzymes responsible for synthesis (diacylglycerol lipase-α–DAGL-α, and *N*-acyl-phosphatidylethanolamine-NAPE-hydrolyzing phospholipase D—NAPE-PLD) and degradation (fatty acid amide hydrolase—FAAH) of PEA, and its receptor PPAR-α, is decreased in hippocampal CA1 (Blanco et al.). Therefore, the neuroprotective effects of PEA on PA we observed in CA1 at this time point might be attributable to a counter regulation of this signaling system through the exogenous administration of PEA. In addition, hippocampal sensitivity to NAEs signaling might also contribute to explain the neuroprotective action of PEA treatment in this brain area and its dependent behaviors. Recent evidence has supported the deleterious effects of NAEs dysregulationon control of neurogenesis, neuronal death and gliosis, in a regional-dependent manner, presenting the hippocampus specific vulnerability (Rivera et al., 2015).

CONCLUSION

PEA treatment (10 mg/kg) within the first hour of life could attenuate PA-induced alterations in CA1 neurons at P30, including nuclear pyknosis, aberrant accumulation of pNF-H/M in axons, and reductions in MAP-2 dendritic protein. These effects were associated with significant improvements in exploratory activity and a regulation of anxiety levels. Therefore, PEA represents a putative neuroprotective agent for PA-induced hippocampal alterations at P30. Future studies should focus on the protective effect of PEA treatment on PA at earlier and later developmental stages.

AUTHOR CONTRIBUTIONS

MIH: Writing and Investigation. LDU, NT-U, CFK, and JPL: Investigation. FC: Supervision.

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Protective Effects of L-902,688, a Prostanoid EP4 Receptor Agonist, against Acute Blood-Brain Barrier Damage in Experimental Ischemic Stroke

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Ischemic stroke occurs when a clot forms in the brain vasculature that starves downstream tissue of oxygen and nutrients resulting in cell death. The tissue immediately downstream of the blockage, the core, dies within minutes, but the surrounding tissue, the penumbra is potentially salvageable. Prostaglandin E_2 binds to four different G-protein coupled membrane receptors EP1–EP4 mediating different and sometimes opposing responses. Pharmacological activation of the EP4 receptor has already been established as neuroprotective in stroke, but the mechanism(s) of protection are not well-characterized. In this study, we hypothesized that EP4 receptor activation reduces ischemic brain injury by reducing matrix metalloproteinase (MMP)-3/-9 production and blood-brain barrier (BBB) damage. Rats underwent transient ischemic stroke for 90 min. Animals received an intravenous injection of either the vehicle or L-902,688, a highly specific EP4 agonist, at the onset of reperfusion. Brain tissue was harvested at 24 h. We established a dose-response curve and used the optimal dose that resulted in the greatest infarct reduction to analyze BBB integrity compared to vehicle-treated rats. The presence of IgG, a blood protein, in the brain parenchyma is a marker of BBB damage, and L-902,688 (1 mg/kg; i.v.) dramatically reduced IgG extravasation ($P < 0.05$). Consistent with these data, we assessed zona occludens-1 and occludin, tight junction proteins integral to the maintenance of the BBB, and found reduced degradation with L-902,688 administration. With immunoblotting, qRT-PCR, and/or a fluorescence resonance energy transfer (FRET)-based activity assay, we next measured MMP-3/-9 since they are key effectors of BBB breakdown in stroke. In the cerebral cortex, not only was MMP-3 activity significantly decreased ($P < 0.05$), but L-902,688 treatment also reduced MMP-9 mRNA, protein, and enzymatic activity ($P < 0.001$). In addition, post-ischemic administration of the EP4 agonist significantly reduced

pro-inflammatory cytokines IL-1β ($P < 0.05$) and IL-6 ($P < 0.01$) in the ischemic cerebral cortex. Most importantly, one injection of L-902,688 (1 mg/kg; i.v) at the onset of reperfusion significantly reduces neurological deficits up to 3 weeks later ($P < 0.05$). Our data show for the first time that pharmacological activation of EP4 with L-902,688 is neuroprotective in ischemic stroke by reducing MMP-3/-9 and BBB damage.

Keywords: prostaglandin E_2, EP4 receptor, ischemia, stroke, blood-brain barrier, matrix metalloproteinase-9, neuroinflammation

INTRODUCTION

Stroke is listed as the fifth leading cause of death in the USA and about 87% of strokes are ischemic (Mozaffarian et al., 2016). Recombinant tissue plasminogen activator (rtPA) is the only FDA-approved drug for ischemic stroke. Only a small proportion of stroke patients are eligible to receive rtPA because it carries a high risk of bleeding/hemorrhagic transformation in addition to direct neurotoxicity (Kaur et al., 2004) and has a short effective time window of only 4.5 h after stroke onset (Del Zoppo et al., 2009). It is therefore essential to search for alternative pharmaceutical interventions to reach a larger percentage of ischemic stroke patients.

An ischemic stroke occurs when a major cerebral artery is occluded, and cells just downstream in the core of the stroke necrotically die within minutes. Cell death is perpetuated into the surrounding penumbra over the course of hours to days later. Reactive oxygen/nitrogen species (ROS) formation further compromises the integrity of an already degraded blood-brain barrier (BBB) by activating matrix metalloproteinases (MMP) i.e., MMP-3, MMP-9 that cleave the basement membrane of the neurovascular unit and the tight junction proteins (TJPs) between endothelial cells (Rosell et al., 2008; Sood et al., 2008; Candelario-Jalil et al., 2011; Turner and Sharp, 2016; Hafez et al., 2018). This triggers an inflammatory response and infiltration of immune cells which have been associated with increased cell death, formation of free radicals/ROS, and secondary injury (Yilmaz et al., 2006; Jin et al., 2010; Benakis et al., 2014).

Following an ischemic stroke, breakdown of the BBB, vasogenic edema, and hemorrhagic conversion are mainly mediated by MMPs, in particular MMP-3 and MMP-9, which have been shown to be critical in inflammation-mediated neurovascular damage (Asahi et al., 2000; Candelario-Jalil et al., 2009; Stanimirovic and Friedman, 2012; Lakhan et al., 2013). Genetic knockout or inhibition of MMP-3 or MMP-9 dramatically reduces neurovascular injury following focal cerebral ischemia in rodents (Asahi et al., 2000; Harris et al., 2005; Suzuki et al., 2007; Dejonckheere et al., 2011; Hafez et al., 2016, 2018). Neuroinflammation-mediated BBB disruption significantly contributes to the progression of brain injury in the penumbra after stroke. Therefore, understanding mechanisms of BBB damage could lead to the identification of novel targets for therapeutic intervention.

As part of the neuroinflammatory response to stroke, a large quantity of arachidonic acid released from the membrane by phospholipases is metabolized into prostaglandin H_2 mainly by cyclooxygenase-2 (COX-2), and then further metabolized into several prostanoids. Prostaglandin E_2 (PGE_2) is one of the major prostanoids formed after ischemic stroke by increased COX-2 activity (Nogawa et al., 1997; Manabe et al., 2004; Kawano et al., 2006; Candelario-Jalil et al., 2007). Prostaglandins are short-lived, lipid mediators that are essential to inflammatory signaling. PGE_2 can have paracrine or autocrine effects and is the endogenous ligand for four G-protein coupled receptors EP1-EP4. PGE_2 can have opposing effects depending on which receptor is activated (Sugimoto and Narumiya, 2007).

In ischemic stroke, the increase in COX-2-derived PGE_2 formation correlates with BBB opening and infiltration of peripheral immune cells (Candelario-Jalil et al., 2007). Moreover, *in vivo* data show that direct injection of PGE_2 into the rat brain leads to increased permeability of the BBB (Schmidley et al., 1992; Messripour et al., 2015). In the context of focal cerebral ischemia, previous studies have shown that activation of EP1 and EP3 PGE_2 receptors significantly exacerbate stroke injury (Manabe et al., 2004; Kawano et al., 2006; Ahmad et al., 2007, 2008; Abe et al., 2009; Fukumoto et al., 2010; Zhen et al., 2012; Shimamura et al., 2013). We recently showed that genetic deletion or pharmacological blockade of the EP1 receptor results in a dramatic reduction in stroke injury and BBB permeability, which correlated with reduced levels of MMP-3 and MMP-9 (Frankowski et al., 2015). Stroke-induced BBB damage is significantly reduced in EP3 deficient mice or in wild-type animals treated with an EP3 receptor antagonist (Ikeda-Matsuo et al., 2011).

Unlike EP1 and EP3 receptors, activation of EP2 and EP4 receptors has previously been shown to be neuroprotective in stroke (McCullough et al., 2004; Ahmad et al., 2005; Liang et al., 2011; Akram et al., 2013). Although several studies have provided strong evidence of a protective role of EP4 in neuroinflammation and cerebral ischemia, nothing is known of the effects of EP4 activation on BBB permeability after stroke. In this study, our objective was to investigate whether EP4 receptor activation would impact BBB permeability and neurobehavioral outcomes in a clinically relevant animal model of transient focal cerebral ischemia. We hypothesized that EP4 receptor agonism with L-902,688 reduces infarct size and neurological deficits by reducing MMP-3, MMP-9, and BBB damage.

Abbreviations: PGE_2, prostaglandin E_2; EP4, Prostaglandin E_2 receptor 4; MMP, Matrix metalloproteinase; tPA, Tissue plasminogen activator; BBB, Blood-brain barrier; ZO-1, Zonula occludens-1; MCAO, Middle cerebral artery occlusion; CCA, Common carotid artery; TBS, Tris-buffered saline; TBST, Tris-buffered saline with 0.1% Tween 20.

MATERIALS AND METHODS

Animals

Adult male rats (10–12 weeks, ∼280–320 g, Sprague Dawley from Charles River Laboratories International, Wilmington, MA, US) were allowed to acclimatize for 1 week before experiments in housing facilities on a 12 h light/dark cycle with free access to food and water with two rats per cage. All animal procedures were performed in accordance with approved guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals, the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines), and the guidelines approved by the Institutional Animal Care and Use Committee at the University of Florida (protocol #201406503). Experiments were planned to reduce the total number of animals used and to reduce potential pain and suffering.

Intraluminal Filament Model of Transient Focal Ischemia and Drug Treatment

To mimic ischemic stroke, rats were subjected to 90 min of transient middle cerebral artery occlusion (MCAO) using the intraluminal filament model of focal ischemia, described in detail in our previous publications (Candelario-Jalil et al., 2007; Hawkins et al., 2013, 2017). Rats were deeply anesthetized with 2–2.5% isoflurane in medical grade oxygen and maintained at a constant 37°C throughout surgery on a heated platform (Cat # TP-700 T/Pump; Stryker Global Industries, Kalamazoo, MI, USA). A midline ventral cut was made, and the common carotid artery (CCA) was separated from the vagus nerve and ligated with a 4-0 silk suture (Cat # SP116; Harvard Apparatus, Holliston, MA, USA). The external carotid artery (ECA) and pterygopalatine arteries were temporarily clipped with a microvascular clip to prevent incorrect placement of the occluding filament. An arteriotomy was performed on the CCA a few millimeters above the ligation to allow for a 4-0 silicone-coated filament (Cat # 403523PK10; Doccol Corporation, Sharon, MA, USA) insertion through the internal carotid artery up into the middle cerebral artery until detection of a slight resistance. After temporarily closing the ventral incision, rats were allowed to recover in a temperature controlled heated chamber (Cat # ICS DW-1 Warmer, Thermo-Care, Paso Robles, CA, USA) for about 80 min to prevent hypothermia before re-anesthetizing the animal to remove the filament. At the onset of reperfusion, animals randomly received an intravenous injection of vehicle (saline; $n = 10$), 0.3 mg/kg L-902,688 ($n = 8$), or 1.0 mg/kg L-902,688 ($n = 8$). L-902,688 (5-[(1E,3R)-4,4-difluoro-3-hydroxy-4-phenyl-1-buten-1-yl]-1-[6-(2H-tetrazol-5R-yl)hexyl]-2-pyrrolidinone) was obtained from Cayman Chemical (Ann Arbor, MI, USA; Cat # 10007712). L-902,688 is a potent EP4 agonist with a K_i value of 0.38 nM and an EC_{50} value of 0.6 nM. It displays >4,000-fold selectivity for EP4 over other prostanoid receptors and has a half-life *in vivo* of ∼12 h in rats (Young et al., 2004). Treatment schedule was determined by simple randomization using a coin flip to determine the initial treatment and then treatment was alternated. Visual confirmation of occlusion was demonstrated by curling and circling behavior during the 90-min occlusion period. In this stroke model, the core of the stroke is represented by subcortical cell death, and the potentially viable penumbra is represented by the cortex in which cell death occurs mainly by apoptosis at later time points.

Tissue Collection and Homogenization

Rats were deeply anesthetized with 150 mg/kg i.p. pentobarbital and perfused with ice-cold physiological saline. Brains were extracted and sliced at 2 mm intervals in a rat brain matrix (Zivic Instruments, Pittsburgh, PA, USA). The fourth slice (anterior to posterior), which roughly corresponds to bregma and represents the core of the stroke in this model, was dissected into ipsilateral and contralateral cortex and striatum/subcortex, and immediately frozen on dry ice for molecular analyses. The remaining slices were used for infarct calculation. Tissue was weighed and homogenized with a Tissue-Tearor in radioimmunoprecipitation buffer containing 1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl pH 7.6, and 1% IGEPAL® CA-630 at 10 μL/mg of tissue and HALT Protease Inhibitor Cocktail, HALT Phosphatase Inhibitor Cocktail and 0.5 M EDTA (Cat. No. 78430; Cat. No. 78428; and Cat. No. 1860851, respectively; Thermo Fisher Scientific, Waltham, MA, USA) at 10 μL/mL of total volume. Samples were sonicated with a Vibra-Cell™ sonicator (Sonics & Materials Inc., Newtown, CT, USA) twice for 15 s separated by 15-min incubations on ice before centrifugation at 14,000× g for 20 min at 4°C. The resulting supernatants were stored at −80°C until use.

Infarct Calculation

To measure the infarct size, brain slices 1–3 and 5–6 were incubated in the dark in 2% 2,3,5-triphenyltetrazolium chloride in phosphate-buffered solution (PBS) for 30 min at room temperature, and placed in 4% paraformaldehyde. Live tissue stains red, and dead tissue remains white. Sections were scanned with an HP Scanjet 8300 (Palo Alto, CA, USA) at 600 dpi rostral side down except for the 3rd slice which was also scanned caudal side down to represent the rostral side of the 4th slice. Due to the significant edema produced by this stroke model, infarcts were calculated indirectly (Swanson et al., 1990; Frankowski et al., 2015). Using Adobe Photoshop CS5, the red tissue was delineated for each slice and the stroke surface area (mm^2) was calculated by subtracting live, red tissue on the ipsilateral side from the red tissue on the contralateral side. To calculate total infarct volume, the surface area (mm^2) of dead tissue was summed for each slice and multiplied by the thickness of the slice (2 mm).

ELISA and MMP Activity Assay

To measure BBB permeability, we performed ELISA analyses for immunoglobulin G (IgG). Blood proteins like IgG are not present in the brain parenchyma unless the BBB was compromised, providing an indirect method of BBB permeability; we therefore measured IgG in 100 μg protein from brain lysates prepared from thoroughly perfused rat brains. We used a commercially available rat IgG ELISA kit (Cat# E101, Bethyl Laboratories, Inc., Montgomery, TX, USA).

Two large contributors of BBB degradation in stroke are MMP-3 and MMP-9 which proteolytically cleave tight-junction proteins between endothelial cells and collagen IV in the basement membrane along the endothelium (Candelario-Jalil et al., 2009). Using a fluorometric immunocapture assay that our team developed (Hawkins et al., 2013), we measured MMP-3 and MMP-9 activity in 50 μg of brain lysate. Briefly, 96-well plates were coated with Protein A/G to stably orient and immunocapture antibodies, coated with either an MMP-3 antibody (Cat # SC-6839-R, Santa Cruz Biotechnology, Dallas, TX, USA) or an MMP-9 antibody (Cat # SC-6841-R, Santa Cruz Biotechnology), and was incubated with 50 μg of total protein, then probed with a specific FRET peptide substrate (For MMP3: Substrate XIII, Cat # 60580-01 or MMP-9: Substrate III, Cat # 60570-01). The substrate (5-FAM/QXL™ 520) is bound to a quencher molecule that can be cleaved by either MMP-9 or MMP-3 to allow fluorescence. Values were normalized to 1 ng of recombinant rat MMP-9 or MMP-3.

Immunoblotting

We probed 40 μg of total protein for the tight junction proteins occludin (Cat # ab167161; AbCam) at 1:1,000 reduced in 5% β-mercaptoethanol and denatured with 10 min of boiling, and zonula occludens-1 (Cat # 61-7300; Life Technologies, Carlsbad, CA, USA) (ZO-1) at 1:500 reduced in 2% β-mercaptoethanol without boiling. Because it is known to degrade tight-junction proteins, so we also probed 50 μg of total protein for MMP-9 (Cat # ab76003, Abcam) at 1:5,000 reduced in 5% β-mercaptoethanol and denatured with 10 min of boiling. To separate proteins, we ran samples through 4–20% Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA, USA) at 200 V for 45 min in 0.1% SDS Tris-glycine buffer. Gels were equilibrated in Tris-glycine buffer containing 10% methanol for 10 min, then transferred at 25 V for 30 min onto either a nitrocellulose (Cat # 926-31092, Li-Cor, Lincoln, NE, USA) or PVDF (Immobilon-FL, Millipore, Billerica, MA, USA) membrane using the semi-dry Trans-Blot Turbo transfer apparatus. Membranes were blocked in 5% milk in TBS for 1 h at room temperature, then incubated overnight at 4°C with primary antibody in 5% milk in TBST. Membranes were washed 4 times with TBST, and incubated with goat anti-rabbit IRDye 800CW (1:30,000; Li-Cor) in 5% milk in TBST containing 0.01% SDS for 1 h at room temperature. Excess antibody was removed with four more TBST washes, and incubated with primary antibody against β-actin (1:10,000, Cat # A1978, Sigma-Aldrich, Saint Louis, MO, USA) for 1 h at room temperature to ensure equal protein loading. Membranes were washed four times with TBST, incubated with donkey anti-mouse IRDye 680LT (1:40,000; Li-Cor) in 5% milk in TBST containing 0.01% SDS for 1 h at room temperature, and scanned with an Odyssey infrared scanning system (Li-Cor). Target protein signal was divided by actin signal to obtain densitometric values, and normalized across blots by dividing by a control sample.

qRT-PCR

Tissue (3 mm) corresponding to the core of the stroke near bregma was dissected into ipsilateral and contralateral hemispheres and further divided into striatal and cortical sections and placed in RNAlater RNA Stabilization Reagent (Cat. No. 76106, Qiagen, Germany) at 10 μL/ mg of tissue. RNA was isolated with the Aurum Total RNA Fatty and Fibrous Tissue kit (Cat No. 732-6830; Bio-Rad) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed into cDNA with iScript™ Reverse Transcription Supermix (Cat # 1708841, BioRad), and diluted to 10 ng/μL with IDTE buffer pH 8.0 (Cat #11-05-01-13; Integrated DNA Technologies, Coralville, IA, USA). Twenty nanograms of cDNA from ipsilateral and contralateral cortical and subcortical tissue were run in triplicate, probed with exon-exon spanning primers (500 nM, Integrated DNA Technologies) for *IL-1*β, *IL-6*, *Mmp-9*, or *Mmp-3,* and normalized to the housekeeping gene *Ywhaz* (Frankowski et al., 2015; **Table 1** for primer sequences) with PerfeCTa® SYBR® Green Fastmix® (Cat # 95072-012; Quanta Biosciences, Beverly, MA, USA) using a Bio-Rad CFX96 Touch Real-Time PCR Detection System with the following procedure: polymerase activation/DNA denaturation phase at 95°C for 30 s, then 40 cycles of denaturing at 95°C for 5 s and annealing at 60°C for 30 s. Specificity of each primer was confirmed using non-template controls and melt curves. The normalized expression shown in the bar graphs (**Figure 4**) was calculated using the CFX Manager™ software (Bio-Rad) and represent the relative quantity of the target gene normalized to the reference gene (*Ywhaz*), and further normalized to the biological control (contralateral sample of the vehicle-treated group).

Assessment of Long-Term Neurological Deficits

Rats were trained on tasks 24 and 48 h before inducing MCAO and tested 48 h, and at 1, 2, and 3 weeks post-ischemia. To measure long-term sensory and fine motor control deficits with the adhesive removal test, rats were trained to remove a small

TABLE 1 | Primer sequences for qRT-PCR experiments.

Gene	Accession number	Forward	Reverse
IL-1B	NM_031512	5′-GTGCTGTCTGACCCATGT-3′	5′-TTGTCGTTGCTTGTCTCTCC-3′
IL-6	NM_012589	5′-CAGAGCAATACTGAAACCCTAGT-3′	5′-CCTTCTGTGACTCTAACTTCTCC-3′
Mmp9	NM_031055	5′-GAACTCACACAACGTCTTTCAC-3′	5′-GGAGGTCATAGGTCACGTAGG-3′
Mmp3	NM_133523	5′-CTATTCCTGGTTGCTGCTCAT-3′	5′-CTGTGGAGGACTTGTAGACTG–3′
Ywhaz	NM_013011	5′-GAAGAGTCGTACAAAGACAGCA-3′	5′-GCTTCTGCTTCGTCTCCTTG-3′

IL, interleukin; Mmp9, matrix metalloproteinase-9; Mmp3, matrix metalloproteinase-3; Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

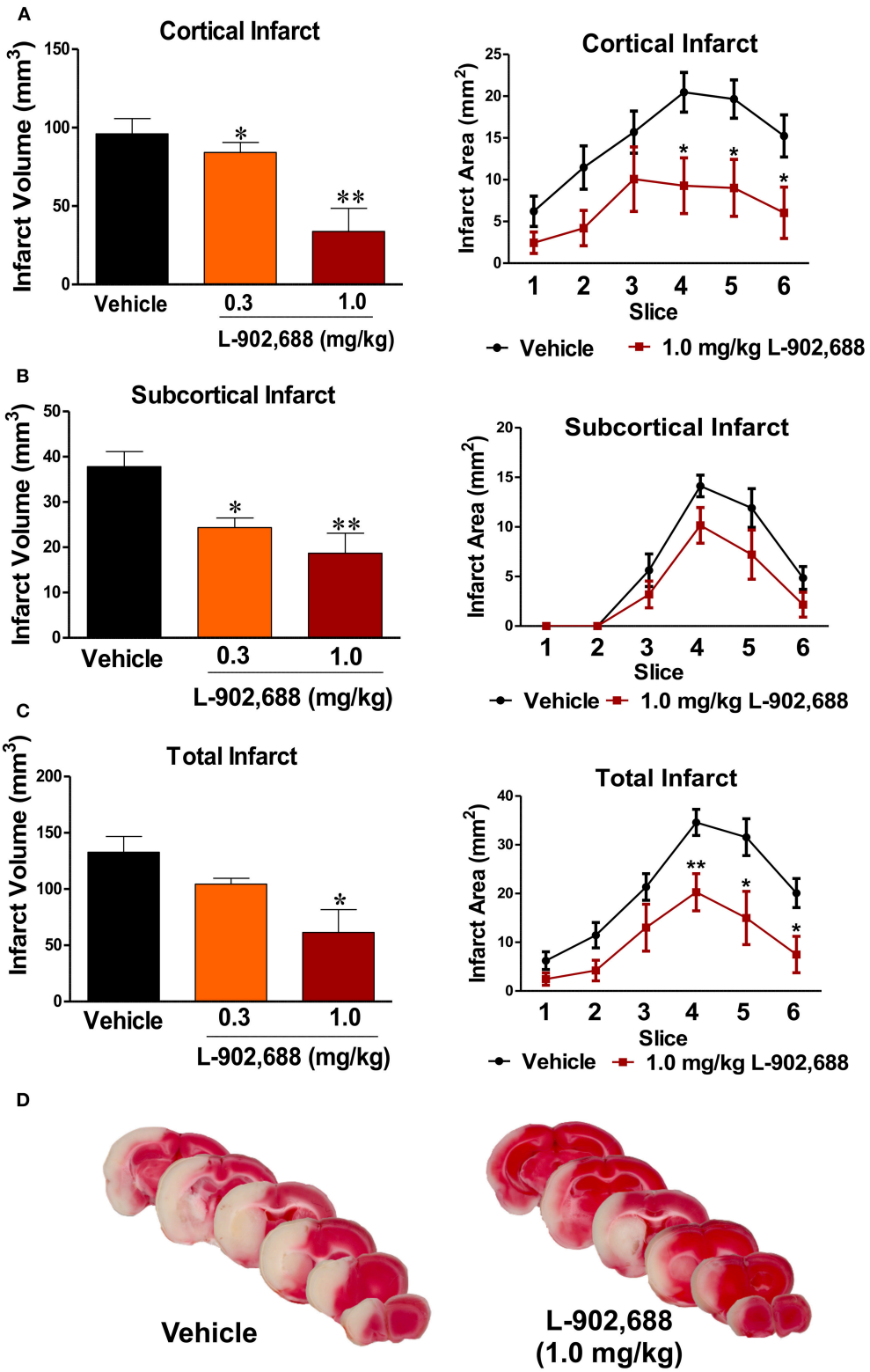

FIGURE 1 | Reduced Infarct with EP4 Agonist L-902,688. Using a one-way ANOVA with a Dunnett's Multiple Comparison posttest, we found that 1.0 mg/kg significantly reduced infarct size in both the **(A)** cortex ($p = 0.0012$) and the **(B)** subcortex ($p = 0.0018$). **(C)** Total infarct volume is reduced with 1.0 mg/kg L-902,688 (Students' *t*-test $^*P = 0.0123$) **(D)** Representative TTC-stained slices from a vehicle- and L-902,688-treated brains after 24 h of reperfusion following 90 min of MCAO. Vehicle ($n = 10$), 0.3 mg/kg ($n = 8$), and 1.0 mg/kg ($n = 8$) L-902,688. $^*P < 0.05$ and $^{**}P < 0.01$ compared with vehicle.

sticker placed on the front, contralateral paw, and the latency to remove was recorded for 3 trials, and the average of the two lowest values was chosen for analysis. To measure motor deficits, rats were trained to stay on a rotarod that accelerated from 4 to 40 rpm. Latency to fall off was measured for 5 trials and the average of the two highest values were normalized to each animal's baseline values. Experimental details of the adhesive removal and accelerating rotarod tests have been described by our group in recent publications (Hawkins et al., 2017; Yang et al., 2017).

Statistics

Infarct measurement was performed with a one-way ANOVA with a Dunnett's multiple comparison post-test. PCR data was analyzed with a Student's *t*-test comparing vehicle-treated ipsilateral data to L-902,688-treated ipsilateral data. Behavioral performance was analyzed using a *t*-test between vehicle-treated rats and L-902,688-treated rats at each time point. Statistics were analyzed with GraphPad Prism version 6.0, and a *p*-value of less than 0.05 was considered statistically significant. Data are reported as mean ± SEM.

RESULTS

After 24 h of reperfusion, infarct volume was significantly reduced in the cortex ($^*P < 0.05$, $^{**}P < 0.01$, **Figure 1A**) and subcortex ($^*P < 0.05$, $^{**}P < 0.01$, **Figure 1B**) with 0.3 and 1.0 mg/kg L-902,688, respectively compared to the vehicle-treated group. Because only 1.0 mg/kg L-902,688 significantly reduced total infarct volume ($^*P < 0.05$, $n = 8$–10, **Figure 1C**), this dose was used for the rest of the study. Representative TTC-stained brain sections are shown in **Figure 1D** for both treatment groups, which help to better appreciate the reduction in infarct size in stroked rats receiving the EP4 agonist, L-902,688, at the onset of reperfusion (after 1.5 h of stroke onset).

To elucidate the mechanism of protection with 1.0 mg/kg L-902,688, IgG extravasation into the brain parenchyma was measured with an ELISA. Because IgG is a blood protein, there is minimal amount detected in a thoroughly perfused brain unless the BBB integrity is compromised. With 1.0 mg/kg L-902,688, we found significant reduction of IgG in the ipsilateral cortex ($^*P < 0.05$, **Figure 2A**) and the ipsilateral subcortex ($^*P < 0.05$, **Figure 2B**) compared to the ipsilateral vehicle cortex and subcortex.

Because MMP-3 and MMP-9 are major contributors to BBB damage after stroke, we wanted to see if the reduced BBB damage evidenced by reduced IgG extravasation was associated with reduced MMP-3/MMP-9 activity and protein levels. Densitometric analysis of immunoblots showed reduced MMP-9 levels in the ipsilateral cortex ($^*P < 0.05$, **Figure 3A**) and a non-significant reduction in the ipsilateral subcortex ($p = 0.0792$, **Figure 3B**) compared to ipsilateral vehicle values. This effect was mirrored in our MMP-9 activity assay data in the cortical ($^*P < 0.05$, **Figure 3C**), but not subcortical (**Figure 3D**) tissue. Because MMP-3 can activate MMP-9, we also measured MMP-3 activity and found 1.0 mg/kg L-902,688 reduced MMP-3 activity in the ipsilateral cortex ($^*P < 0.05$, **Figure 3E**), but not in the subcortex (**Figure 3F**).

We also measured reduced expression of cortical and subcortical IL-1β and IL-6 expression ($^*P < 0.05$, $^{**}P < 0.01$, **Figures 4A–D**). Treatment with 1.0 mg/kg L-902,688 also significantly reduced MMP-9 ($^{***}P < 0.001$) and MMP-3 expression ($^{**}P < 0.01$) in the cortex (**Figures 4E,G**) and non-significantly reduced mRNA levels of MMP-9 and MMP-3 in the subcortex ($p = 0.2335$, **Figure 4F;** $p = 0.5104$, **Figure 4G**).

Reduced IgG extravasation and reduced MMP-9 and MMP-3 activity suggested that 1.0 mg/kg L-902,688 reduced stroke-induced BBB damage. We therefore measured levels of the tight junction proteins ZO-1 and occludin in cortical tissue with immunoblotting. We found a non-significant preservation of ZO-1 in the ipsilateral cortex in L-902,688-treated rats compared to vehicle-treated rats (**Figure 5A**). Additionally, degradation of the 125-kDa occludin dimer was significantly reduced in the ipsilateral cortex with

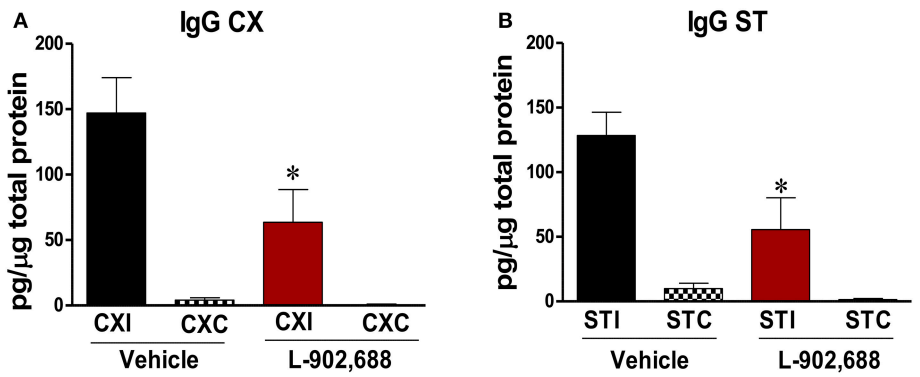

FIGURE 2 | Reduced IgG in the brain in stroked rats treated with the EP4 Agonist L-902,688. **(A)** There is significantly reduced IgG after 24 h of reperfusion in the 1.0 mg/kg L-902,688 CXI group compared to the vehicle CXI group. ($^*P = 0.0413$). **(B)** In animals receiving 1.0 mg/kg L-902,688, the ipsilateral subcortical IgG levels are also significantly reduced. (Student's *t*-test $^*P = 0.0254$). CXI, ipsilateral cortex; CXC, contralateral cortex; STI, ipsilateral subcortex; STC, contralateral subcortex. Vehicle ($n = 10$) and 1.0 mg/kg L-902,688 ($n = 8$).

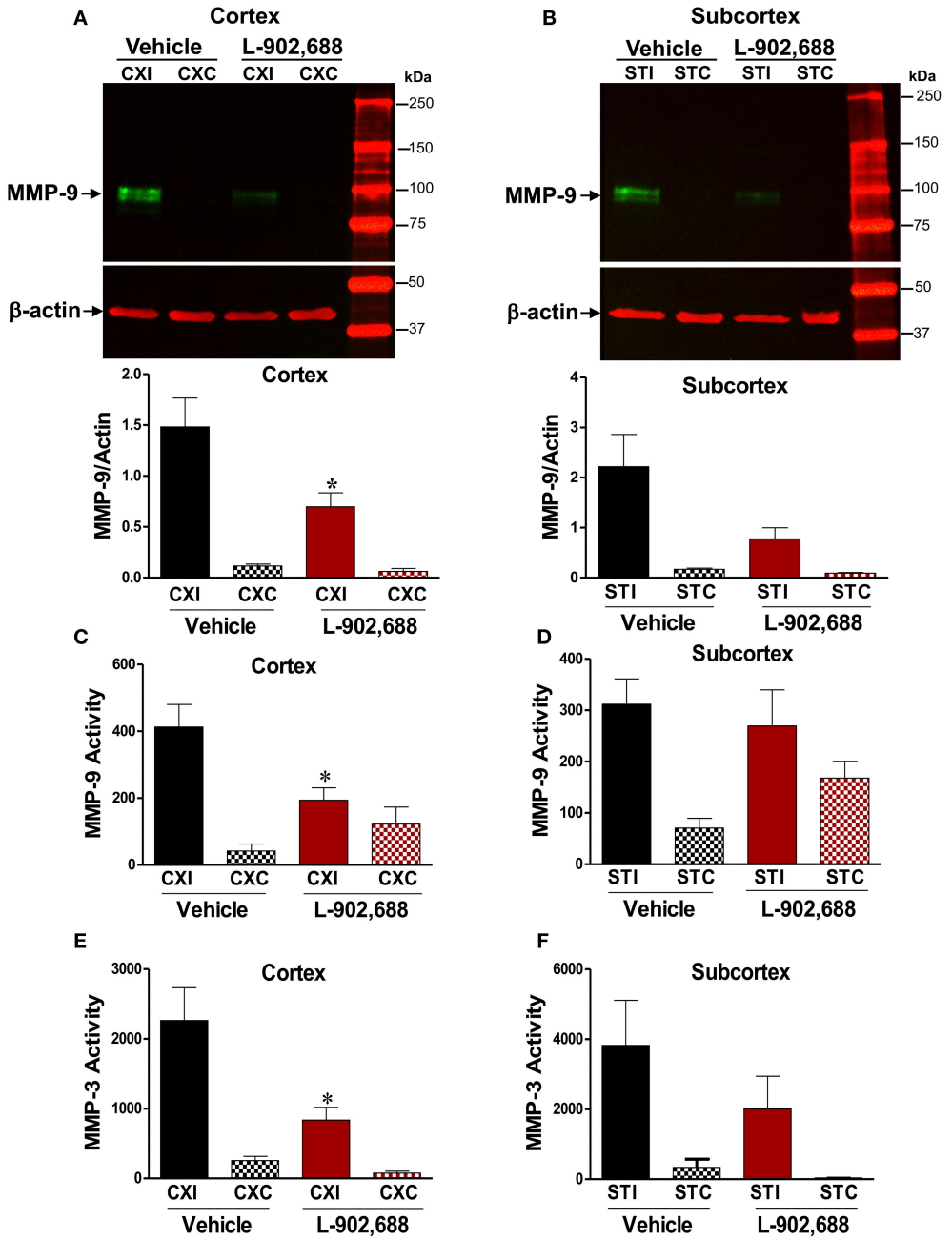

FIGURE 3 | Reduced MMP-9 and MMP-3 Activity in ischemic rats given the EP4 Agonist L-902,688. We measured reduced MMP-9 protein with immunoblotting after 24 h of reperfusion in the **(A)** cortex (*$P < 0.05$) and **(B)** the subcortex ($P = 0.0792$). There was reduced MMP-9 activity with L-902,688 treatment in the cortex **(C)**, but not in the subcortex **(D). (E)** In the cerebral cortex, 1.0 mg/kg L-902,688 significantly reduces MMP-3 activity in the ipsilateral side of the treated group vs. the vehicle group. *$P = 0.0215$. **(F)** In the subcortex, the effect of L-902,688 in the ipsilateral hemisphere is less pronounced ($P = 0.1304$). CXI, ipsilateral cortex; CXC, contralateral cortex; STI, ipsilateral subcortex; STC, contralateral subcortex. Vehicle ($n = 10$) and 1.0 mg/kg L-902,688 ($n = 8$).

EP4 receptor activation. This is associated with reduced injury-induced low molecular weight 65-kDa occludin (**Figure 5B**).

Finally, we wanted to confirm that the reduction in infarct size was associated with reduced neurological deficits long-term. EP4 activation with one intravenous injection of 1.0 mg/kg L-902,688 at the onset of reperfusion showed sustained improvement in neurological function. Animals receiving 1.0 mg/kg L-902,688 performed better at the adhesive removal test at 1, 2, and 3 weeks after stroke (*$P < 0.05$, **$P < 0.01$, **Figure 6A**) and were also able to stay on the rotarod longer than vehicle-treated rats at 1, 2, and 3 weeks after stroke (*$P < 0.05$, **Figure 6B**).

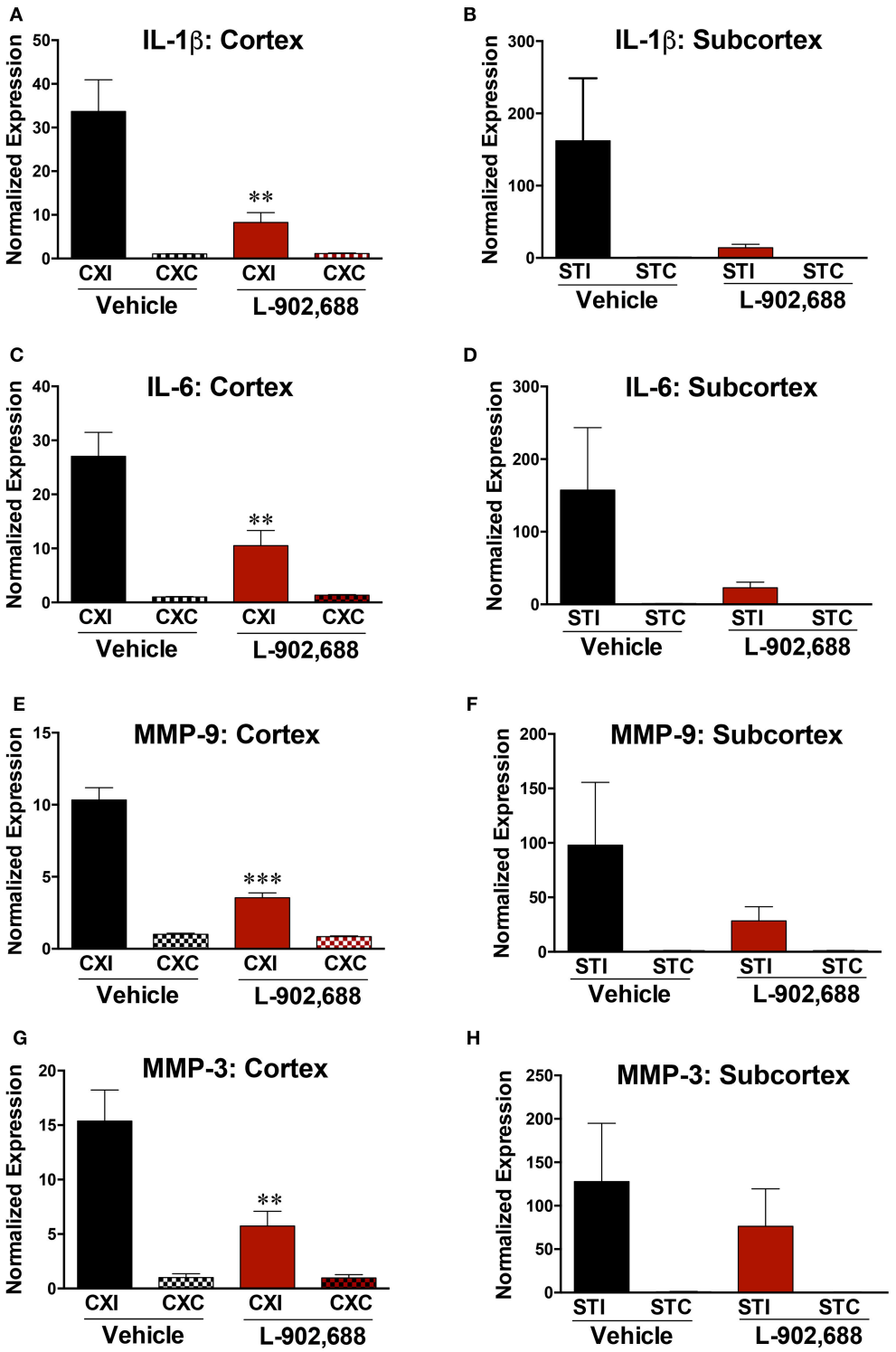

FIGURE 4 | Reduced IL-1β, IL-6, MMP-9, and MMP-3 expression with L-902,688 treatment. **(A)** IL-1β is decreased in the ipsilateral cortex (**$P = 0.0082$) in rats given L-902,688. **(B)** There was a non-significant ($P = 0.0888$), but substantial decrease in IL-1β in the ipsilateral subcortex. **(C)** IL-6 is significantly downregulated in the ipsilateral cortex (**$P = 0.0089$) and slightly reduced in the subcortex **(D)** ($P = 0.1186$). **(E)** L-902,688 decreased MMP-9 in the ipsilateral cortex (***$P < 0.001$) **(F)** There was a trend toward MMP-9 downregulation with treatment in the ipsilateral subcortex, but it did not reach significance ($P = 0.2335$). **(G)** EP4 receptor activation with L-902,688 potently reduces MMP-3 mRNA expression in the ischemic cerebral cortex (**$P = 0.0083$). **(H)** No significant reduction in MMP-3 expression was found in the ischemic subcortical region between treatment groups ($P = 0.5104$). Student's *t*-test comparing vehicle and L-902,688 ipsilateral groups. CXI, ipsilateral cortex; CXC, contralateral cortex; STI, ipsilateral subcortex; STC, contralateral subcortex. Vehicle ($n = 10$) and 1.0 mg/kg L-902,688 ($n = 8$).

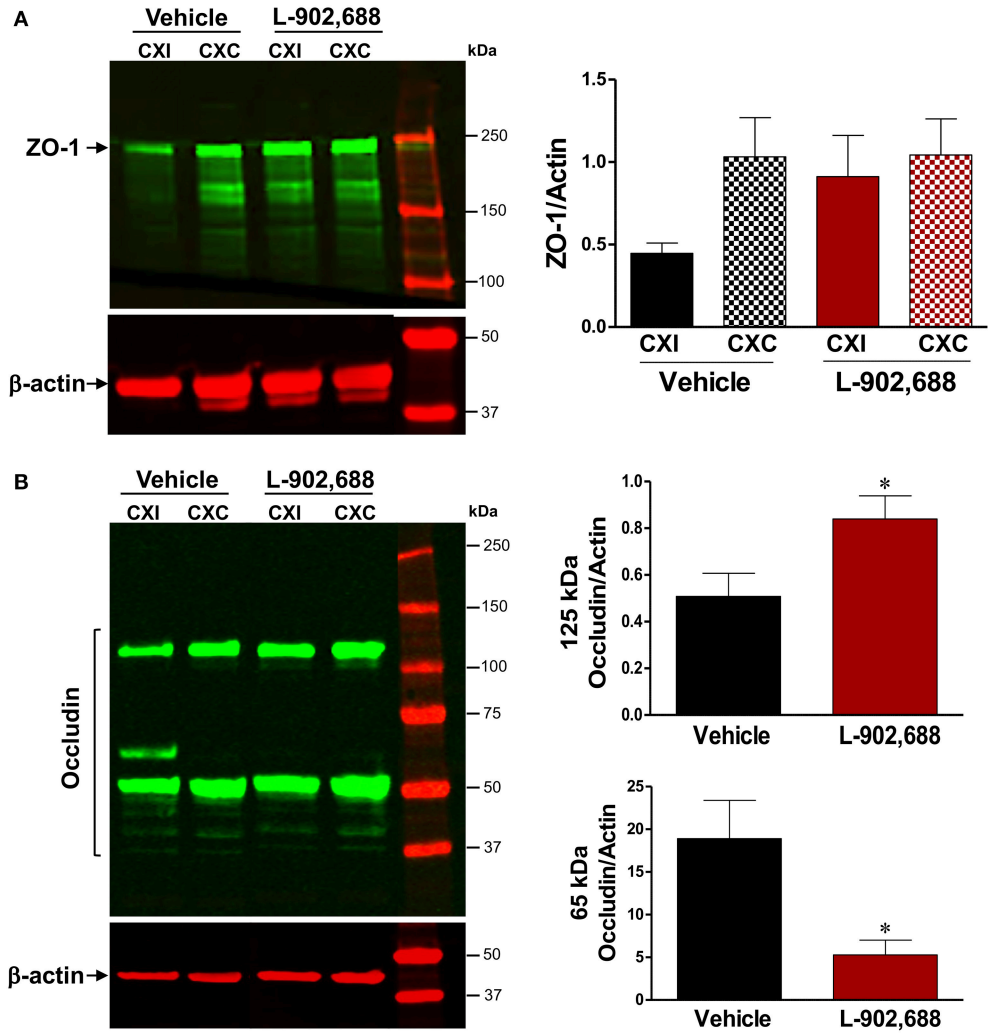

FIGURE 5 | Effect of L-902,688 on Tight Junction Proteins. We performed immunoblots to measure **(A)** ZO-1 in vehicle ($n = 10$) and L-902,688 ($n = 8$) groups and found a non-significant trend toward ZO-1 preservation in the ipsilateral hemisphere of the treated group compared to the vehicle in cortical samples. **(B)** Degradation of the dimeric form of occludin (~125 kDa) was reduced in the ipsilateral cortex of L-902,688 and this was associated with decreased induction of the low molecular weight occludin (~65 kDa). Data are reported as ipsilateral divided by contralateral due to the wide variability in the occludin content in both the contralateral and the ipsilateral hemispheres of vehicle-treated rats. CXI, ipsilateral cortex; CXC, contralateral cortex; STI, ipsilateral subcortex; STC, contralateral subcortex. *$P < 0.05$ compared with vehicle-treated animals.

DISCUSSION

Here, we show for the first time that activation of the EP4 receptor with L-902,688 given at the onset of reperfusion significantly reduces infarct size, blood-brain barrier (BBB) breakdown, MMP-3 and MMP-9 levels, degradation of tight junction proteins, and stroke-induced increase in the expression of the pro-inflammatory cytokines IL-1β and IL-6. More importantly, post-ischemic treatment with L-902,688 resulted in an improved long-term neurological recovery as assessed using the adhesive removal and accelerating rotarod tests.

Expressed in cardiovascular, neuronal, and immune cells (Sando et al., 1994; Hata and Breyer, 2004; Yokoyama et al., 2013) the EP4 receptor is uniquely suited to influence infarct outcome in ischemic stroke. The BBB can be conceptually dissected into neurovascular units comprised of neurons, astrocytic endfeet, pericytes, and endothelial cells. Typically, the neurovascular unit synergistically maintains homeostatic levels of permeability for vasculature to parenchyma substance exchange. During an ischemic event, the BBB undergoes biphasic opening at 3 and 48 h after reperfusion which is correlated with MMP levels in this stroke model (Rosenberg et al., 1998). This is relevant because MMP-9-induced BBB opening is detrimental in the acute phase of stroke (Sood et al., 2008; Yang et al., 2010).

Because endothelial cells are on the front line, they are the first cell type to be affected by hypoxia. One mechanism of neuroprotection may be via EP4 receptor-induced vasodilation, altering cerebral blood flow in response to ischemia (Taniguchi

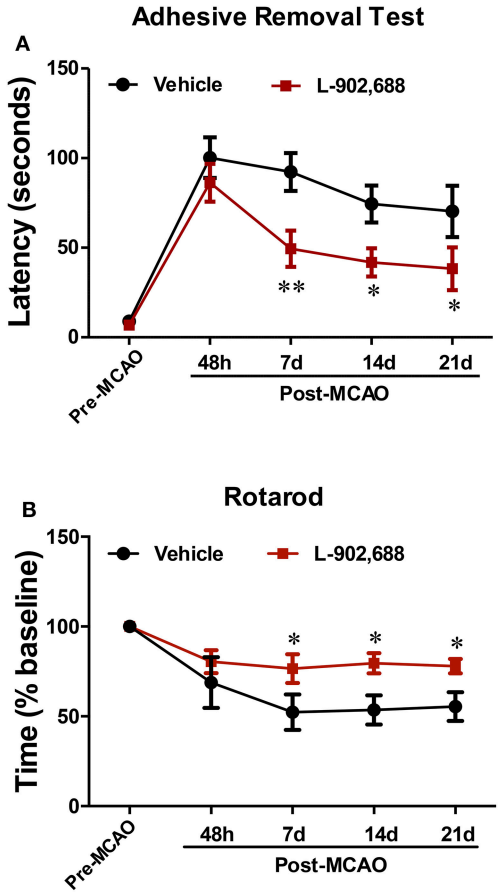

FIGURE 6 | Post-ischemic treatment with L-902,688 Reduces Stroke-Induced Neurological Deficits. **(A)** L-902,688 administration reduces the latency to contralateral adhesive removal in rats subjected to stroke. (**$P < 0.01$, *$P < 0.05$). **(B)** L-902,688 increases the time spent on the rotarod compared to vehicle-treated rats (*$P < 0.05$). Data analyzed with multiple *t*-tests; $n = 10$ in each treatment group.

et al., 2014). This makes sense in light of the fact that EP4 receptor activation increases eNOS and phospho-Ser^{1177} eNOS, increasing local NO levels. Direct protection may also shield neurons from against stroke insult, as well because neuronal EP4 receptor activation reduces cell death *in vitro* and *ex vivo* after excitotoxic challenge and *in vitro* after hypoxic/hypoglycemic challenge (Liang et al., 2011). These effects likely translate into reduced infarct size with a single bolus of EP4 receptor agonist L-902,688 at the onset of reperfusion in our stroke model of transient focal ischemia. This reduction is correlated with decreases in several measures of BBB permeability. EP4 receptor activation reduces IgG extravasation in both the core of the stroke represented by the ipsilateral subcortex and in the penumbra represented by the ipsilateral cortex. EP4 receptor activation similarly reduced stroke-induced MMP-9 and MMP-3 activity, particularly in the cortex, and stroke-induced MMP-9 mRNA levels. MMP-9 and MMP-3 are key contributors to BBB disruption in the context of ischemic stroke since these proteases degrade the basal lamina and tight junction proteins essential to the barrier function of the neurovascular unit (Rosenberg et al., 1998; Asahi et al., 2001; Rosell et al., 2008; Candelario-Jalil et al., 2009; Turner and Sharp, 2016).

Not surprisingly, EP4 receptor agonism reduced pro-inflammatory gene transcription along with reduced infarct and BBB damage. IL-1β activates microglia/macrophages, stimulating more IL-1β release and triggering immune cell infiltration that contribute to increased BBB permeability and apoptotic death in penumbral neurons (Yamasaki et al., 1995; Hawkins and Davis, 2005; McColl et al., 2007; Clausen et al., 2008; Sandoval and Witt, 2008). We found robust reductions in IL-1β gene expression in the cortex and the subcortex with EP4 receptor activation. L-902,688 further reduced acute phase IL-6 expression, and increased IL-6 is correlated with larger stroke volume and worse outcome (Waje-Andreassen et al., 2005). These data are interesting because EP4 activation has shown to upregulate IL-6 expression in some cell types (Hata and Breyer, 2004; Zhou et al., 2016). L-902,688-dependent IL-6 reductions are likely reflective of reduced cell damage/infarction (Tarkowski et al., 1995; Suzuki et al., 1999, 2009; Smith et al., 2004). In a model of subarachnoid hemorrhage in rats, a very recent study found that AE1-329, an EP4 receptor agonist, significantly reduced BBB damage, edema, and expression of IL-1β, IL-6, and TNF-α (Xu et al., 2017).

EP4 agonism protects the tight junction proteins between endothelial cells that are vital determinants of BBB permeability. In the cortex, we found a non-significant trend toward ZO-1 preservation with EP4 receptor activation, and significant preservation of dimeric occludin (125 kDa) in the ipsilateral cortex which was associated with reduced stroke-induced 65-kDa occludin, a likely phosphorylated form of occludin. Increased levels of the 65-kDa band of occludin have been detected in models of ischemic stroke and are associated with BBB disruption (Kago et al., 2006; Takenaga et al., 2009; Fukumoto et al., 2010; Muthusamy et al., 2014; Frankowski et al., 2015). No change in the lower molecular weight occludin band (50 kDa) was observed between ipsilateral and contralateral hemispheres irrespective of the treatment group. Our model of focal ischemia typically induces such occludin alterations (Frankowski et al., 2015).

Oxidative stress is a key mechanism of BBB disruption and neuronal death in ischemic stroke (Chan, 2001; Li et al., 2017). Free radicals/ROS directly damage endothelial cells composing the BBB and indirectly activate MMPs, which lead to proteolytic breakdown of basal lamina proteins and TJPs resulting in injury to the neurovascular unit (Gürsoy-Ozdemir et al., 2004; Kahles et al., 2007; Hafez et al., 2018). One potential mechanism through which EP4 receptor activation could reduce stroke-induced BBB opening is reduction of oxidative damage during the reperfusion phase. This notion is supported by previous studies showing that EP4 agonists reduce free radical formation in neurons and microglia exposed to amyloid β (Echeverria et al., 2005) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) (Pradhan et al., 2017). It remains to be determined whether treatment with L-902,688 or other EP4 agonists reduces oxidative stress *in vivo* after stroke.

The EP4 receptor is expressed in many cell types including endothelium, neurons, microglia, astrocytes, and peripheral

immune cells (Sando et al., 1994; Hata and Breyer, 2004; Yokoyama et al., 2013; Bonfill-Teixidor et al., 2017). Permeability of the BBB after stroke can be altered by complex cellular and molecular interactions between cells of the neurovascular unit and the peripheral immune system. We found that EP4 receptor activation with L-902,688 potently reduces levels of some of the key mediators of stroke-induced BBB damage including IL-1β, IL-6, MMP-3, and MMP-9. The main cellular sources of these pro-inflammatory mediators after stroke include activated microglia and astrocytes, as well as infiltrating neutrophils and macrophages (Benakis et al., 2014; Amantea et al., 2015). It has been shown that EP4 signaling decreases the activation of nuclear factor-kB (NF-kB), a master regulator of pro-inflammatory gene transcription, in activated microglia (Shi et al., 2010; Woodling et al., 2014), as well as in peripheral immune cells (Takayama et al., 2002, 2006; Minami et al., 2008). Based on our data and previous reports, suppression of immune cell activation and production of pro-inflammatory mediators are suggested as mechanisms by which EP4 agonism confers neurovascular protection in ischemic stroke.

Most importantly, we found that a single administration of L-902,688 at the onset of reperfusion reduced sensorimotor deficits in the adhesive removal test and the rotarod assessment up to 3 weeks after stroke. Although infarct size is not always a consistent indicator of stroke outcome, sensitive behavioral assessments like the adhesive removal and rotarod tests are relatively reliable indicators of functional neurological deficits that can detect changes at least up to 3 weeks after injury (Yang et al., 2017).

Limitations of our study include the utilization of one type of transient stroke model that includes reperfusion injury, and these data would be strengthened by confirming neuroprotection in other stroke models. We only used young healthy male rats, which is another limitation of our study. Since age, diabetes, hypertension, and hypercholesterolemia are among the most important risk factors for stroke, future studies should investigate the effects of EP4 agonism in animals of both sexes with these comorbid conditions. Furthermore, because we were limited to pharmacological intervention in rats, it would be of interest to subject transgenic conditional knockout mice lacking EP4 specifically in myeloid cells, endothelial cells, or neurons to stroke to determine the relative contribution of EP4 activation from different cells of the neurovascular unit. Future studies will determine whether delayed administration of L-902,688 (several hours after stroke onset) also confers sustained, long-term neuroprotection as was found with a different EP4 receptor agonist in mice (Liang et al., 2011). This will provide further support that EP4 receptor activation has clinical relevance if it proves to be effective up to 4.5 h after stroke, the current therapeutic window for tPA.

To our knowledge, we are the first group to establish that EP4 agonism with a single administration of L-902,688 at the onset of reperfusion is neuroprotective in a transient MCAO stroke model in rats up to 3 weeks after ischemia. This neuroprotection is due to the dynamic crosstalk between inflammation and BBB degradation. EP4 activation reduces pro-inflammatory IL-1β gene transcription and matrix metalloproteinases MMP-3 and MMP-9, major contributors of BBB damage. These effects culminate in reduced tight junction protein degradation that maintain the integrity of the BBB and reduced long-term neurological deficits.

AUTHOR CONTRIBUTIONS

KD, AM, DS, BS, CY, and EC-J: Performed experimental procedures; KD and EC-J: Designed research and planned all the experiments; KD, AM, and EC-J: Analyzed the data and prepared the figures; KD and EC-J: Wrote the article; EC-J: Conceived and led the project; All the authors read and approved the final version of the manuscript.

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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.

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Role of mTORC1 Controlling Proteostasis after Brain Ischemia

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Intense efforts are being undertaken to understand the pathophysiological mechanisms triggered after brain ischemia and to develop effective pharmacological treatments. However, the underlying molecular mechanisms are complex and not completely understood. One of the main problems is the fact that the ischemic damage is time-dependent and ranges from negligible to massive, involving different cell types such as neurons, astrocytes, microglia, endothelial cells, and some blood-derived cells (neutrophils, lymphocytes, etc.). Thus, approaching such a complicated cellular response generates a more complex combination of molecular mechanisms, in which cell death, cellular damage, stress and repair are intermixed. For this reason, animal and cellular model systems are needed in order to dissect and clarify which molecular mechanisms have to be promoted and/or blocked. Brain ischemia may be analyzed from two different perspectives: that of oxygen deprivation (hypoxic damage *per se*) and that of deprivation of glucose/serum factors. For investigations of ischemic stroke, middle cerebral artery occlusion (MCAO) is the preferred *in vivo* model, and uses two different approaches: transient (tMCAO), where reperfusion is permitted; or permanent (pMCAO). As a complement to this model, many laboratories expose different primary cortical neuron or neuronal cell lines to oxygen-glucose deprivation (OGD). This *ex vivo* model permits the analysis of the impact of hypoxic damage and the specific response of different cell types implicated *in vivo*, such as neurons, glia or endothelial cells. Using *in vivo* and neuronal OGD models, it was recently established that mTORC1 (mammalian Target of Rapamycin Complex-1), a protein complex downstream of PI3K-Akt pathway, is one of the players deregulated after ischemia and OGD. In addition, neuroprotective intervention either by estradiol or by specific AT2R agonists shows an important regulatory role for the mTORC1 activity, for instance regulating vascular endothelial growth factor (VEGF) levels. This evidence highlights the importance of understanding the role of mTORC1 in neuronal death/survival processes, as it could be a potential therapeutic target.This review summarizes the state-of-the-art of the complex kinase mTORC1 focusing in upstream and downstream pathways, their role in central nervous system and their relationship with autophagy, apoptosis and neuroprotection/neurodegeneration after ischemia/hypoxia.

Keywords: signaling, PI3K-Akt, autophagy, stroke, neuroinflammation, neuronal stress response, glia stress response, MCAO

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mTORC: ROLE IN ADULT CENTRAL NERVOUS SYSTEM (CNS)

As mentioned previously, in this review we try to summarize the state-of-the-art of the complex kinase mTORC1 in the brain. We will present some general structural features, and some of elements defined, so far, as belonging to this complex.

We will recapitulate some of the most representative upstream and downstream pathways and their role in central nervous system. We will revise their connexion of mTORC1 with autophagy, apoptosis and neuroprotection/neurodegeneration, mostly focused on ischemia/hypoxia. We are aware that we can only mention some of the published works, due to the lack of space.

mTORC1/2 Structure and Function

Mammalian target of rapamycin (mTOR) is a protein with serine-threonine kinase activity that is part of two different multiprotein complexes which differ in some of their components and their upstream and downstream signaling (Takei and Nawa, 2014; Bockaert and Marin, 2015; Switon et al., 2017). This kinase regulates essential aspects of cell function including growth, differentiation, survival and energy homeostasis by harmonizing anabolic and catabolic processes, with the aim of maintaining cellular physiology.

Structurally, mTOR is a multi-domain protein comprised of a C-terminal FAT domain (FATC), a C-terminal kinase domain (KD), a rapamycin binding domain (FRB), a transactivation/transformation-associated domain (FAT) and an N-terminal domain which provides a site of regulatory protein interaction (HEAT repeats) (Hwang and Kim, 2011; Yang et al., 2013; Switon et al., 2017). This HEAT repeats domain allows interaction between mTOR and regulatory-associated proteins, forming two complexes known as mTORC1 and mTORC2 which function as homodimers (Yip et al., 2010; Aylett et al., 2016), and include common and specific proteins. The common regulatory components of mTORC1/2 are mammalian lethal with SEC13 protein 8 (mLST8), Tel two-interacting protein 1 (Tti1), telomere maintenance 2 (Tel2), DEP domain-containing mTOR-interacting protein (DEPTOR) and the kinase mTOR (Yip et al., 2010; Switon et al., 2017). There are two regulatory-associated proteins specific to mTORC1 [regulatory associated protein of Tor (Raptor) and proline-rich AKT1 substrate 40 kDa (PRAS40)] whereas the characteristic proteins of mTORC2 are rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase-interacting protein 1 (mSin1), and protein observed with Rictor (PROTOR).

Consequently, mTORC1 and mTORC2 differ not only in the composition of their specific regulatory proteins but also in their rapamycin sensitivity, mediated by FK506-binding protein (FKPB12). In fact, FKPB12 is another mTOR-interacting protein that, in presence of rapamycin, blocks the catalytic domain of mTORC1, reducing its kinase activity. In contrast, mTORC2 does not interact with FKBP12-rapamycin, and is thus rapamycin-insensitive. Nevertheless, sustained treatment with rapamycin inhibits mTORC2 activity, probably by sequestration of mTOR protein or uncoupling of mTORC2 components such as SIN1 (Sarbassov et al., 2006; Chen and Sarbassov, 2011; Switon et al., 2017).

The role of each regulatory protein in the activity of mTORC1/2 is not completely understood. Studies using mLST8-deficient mice have revealed that mLST8 is necessary to maintain Rictor-mTOR but not Raptor-mTOR interactions, and consequently perturbs mTORC2 but not mTORC1 activity (Guertin et al., 2006). Tti1 and Tel2 are important components for assembly and maintenance of mTORC1/2 activity, and are also critical for mTOR stability (Kaizuka et al., 2010). As DEPTOR is an endogenous inhibitor of mTOR, its abundance modulates activity of mTORC1/2, and it is found to be overexpressed in some cancer types (Peterson et al., 2009; Liu et al., 2010; Catena and Fanciulli, 2017). PRAS40 is a negative regulator of mTOR, preventing binding of mTORC1/2 to its substrate (Wang et al., 2007). Raptor is important for mTORC1 assembly, regulates mTORC1 activity and localization, and plays a key role in the recruitment of mTORC1 substrates, the best-characterized of which are translational regulators (Hara et al., 2002; Yonezawa et al., 2004; Aylett et al., 2016). Whereas Rictor and mSin1 stabilize each other and constitute the structural basis of mTORC2, Rictor also gives mTORC2 substrate specificity (Dos et al., 2004). PROTOR doesn't affect mTORC2 assembly, but plays a role modulating mTORC2 kinase efficiency (Pearce et al., 2007, 2011; see **Figure 1**).

In addition to these regulatory proteins, mTORC1 activity is regulated by phosphorylation of specific C-terminal Ser/Thr residues, performed by different kinases such as: Akt, p70 ribosomal S6 kinase (p70S6K), AMP-activated protein kinase (AMPK), and also possesses an autophosphorylation site (Chong et al., 2013). Therefore, the activity of mTORC1 is highly regulated indicating the importance of maintaining mTORC1 activity levels within a physiological range. Indeed, mTORC1 is considered to be a nutrient-sensitive complex that regulates cellular growth, protein synthesis and autophagy, whereas mTORC2 has been defined as a regulatory kinase of Akt/PKB and thus a component of growth factor signaling, although mTORC2 has not been characterized as fully as mTORC1.

From a functional viewpoint, mTORC1 is localized at a strategic position in signaling pathways. On one hand, its activity is modulated upstream by several pivotal intra- and extracellular metabolic and energetic factors including growth factors, amino acids, cell energetic status and oxygen availability, through different pathways (Weichhart, 2012; Dibble and Cantley, 2015; Chen et al., 2017; Switon et al., 2017). Thus, mTORC1 can be considered to be a molecular sensor for essential information related to nutrient availability, growth factors and energetic balance. Additionally, mTORC1 modulates important cellular processes such as translation, transcription, lipid synthesis, autophagy and the cell cycle (Pla et al., 2016; Ben-Sahra and Manning, 2017; Hong et al., 2017; Liu et al., 2017).

It is currently accepted that activation of the kinase activity of mTOR increases the rate of protein synthesis by both activating the processes of translation initiation and elongation in cells. In

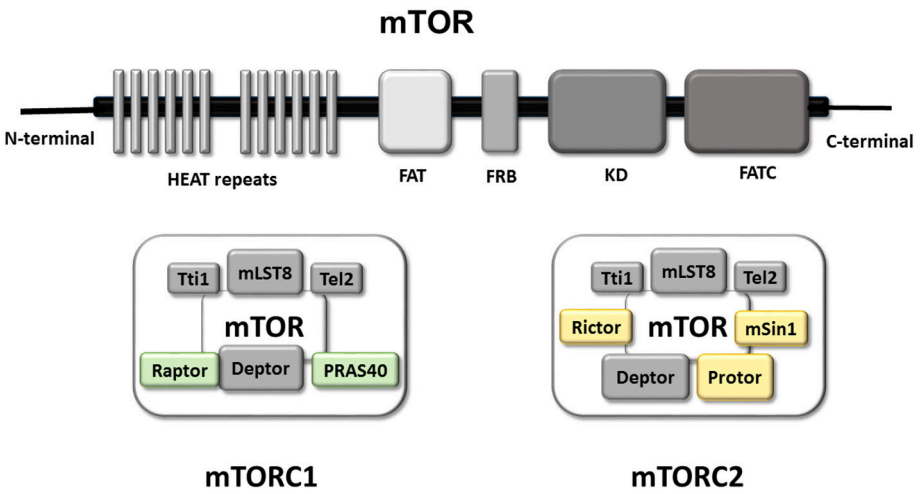

FIGURE 1 | mTOR Functional domains and composition of mTORC1 and mTORC2. Graphical representation of the main functional domains of mTOR protein. mTOR contains at C-terminal a FAT domain (FATC), the kinase domain (KD), a rapamycin binding domain (FRB) and a transactivation domain-associated protein domain (FAT). The N-terminal domain is composed by a HEAT repeats site with a key role in the interaction with regulatory proteins permitting the formation of mTOR complex (mTORC1 right and mTORC2 left). Representation of the mTORC1 and mTORC2 complex Both complex are formed, so far, by five common proteins and two/three specific proteins. The common components of both complexes are in gray (Tti1; mLST8; Tel2; Deptor in gray). Whereas Raptor and PRAS40 are present in mTORC1 (green), and Rictor, Protor and mSin1 are part of mTORC2 (yellow).

fact, two of the best know targets of mTORC1, p70S6K and 4E-binding proteins (4EBPs), have important roles in two phases of the translation machinery: initiation and elongation (Chong et al., 2013).

mTORC1/2 and CNS

Expression of mTOR in the adult brain is high, predominantly in neurons but also in glial cells (Perluigi et al., 2015). However, most of the knowledge regarding the neurologic role of mTORC1 comes from studies using neurons, and only limited information related to glial cells is available. In neurons, mTORC1 activity is modulated by neuron-specific molecules including neurotransmitters (e.g., glutamate, dopamine, serotonin, GABA, cannabinoids), and neurotrophic factors [e.g., brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1)] (Bermudez-Silva et al., 2016; Chen et al., 2017). Some important processes related to development and maturation of the CNS are controlled by mTORC1 including neurogenesis, axonogenesis, axon guidance, dendritogenesis, and growth of dendritic spines (Jaworski and Sheng, 2006). In addition, mTORC1 plays a key role in adult brain physiology and pathology, influencing higher brain functions such as learning, memory (Jaworski and Sheng, 2006), feeding behavior (review in: Cota et al., 2006; Woods et al., 2008), synaptic plasticity (Bockaert and Marin, 2015), cognition (Burket et al., 2014), circadian rhythm (Jouffe et al., 2013) and sensorial perception by modulation of sensitivity to peripheral sensory afferents (Obara and Hunt, 2014).

Similarly, mTORC1 plays a role in gliogenesis during brain development (Cloëtta et al., 2013), and has also been implicated in differentiation and maturation of several glial cell types in the adult brain, especially in oligodendrocytes and microglia (Tyler et al., 2009). Recent data emphasizes the role of mTORC1 in the control of lipid biosynthesis in oligodendrocytes and Schwann cells, through sterol regulatory element-binding proteins (SREBPs). Consequently, its contribution to correct peripheral nerve function is related to velocity of action potential conduction and precise brain myelination (Norrmén et al., 2014; Zou et al., 2014). The role of mTORC1 in the balance of M1/M2 microglia phenotype has been reported (Chen et al., 2016), and reduction of mTORC1 activity decreased pro-inflammatory cytokines and chemokine synthesis, reducing the M1 microglia phenotype (Li et al., 2016). Also, mTORC1 has been found to be important for survival and size preservation of astrocytes (Pastor et al., 2009).

Taking into account the broad cerebral functions regulated by mTORC1 and its central position in cellular energetic/metabolic homeostasis, dysregulation of its activity (increasing or decreasing) would be detrimental for normal brain physiology. Recent evidence supports that modification of mTORC1 activity can trigger diseases of the nervous system, since disruption in mTORC1 signaling affects multiple pathways including energy production, mitochondrial function, cell growth, glucose/lipid metabolism and autophagy.

Mutations in the genes encoding different molecular components of mTORC1 or its related signaling pathways result in diseases characterized by severe neurological symptoms including epilepsy, tumor growth, autism and cognitive disability. Use of transgenic animals has enabled correlation of mTORC1 dysregulation with neurological indicators (for a revision see Switon et al., 2017). Some human genetic disorders have been associated with disturbed mTORC1 activity, including Tuberous Sclerosis complex, currently considered mTORopathy. It is a multi-system autosomal-dominant disorder characterized

by benign tumors in some systemic organs, cortical and cerebellar tuber, subependymal nodules, retinal hamartomas, and hypomyelination (Carson et al., 2015; Mühlebner et al., 2016; Hodgson et al., 2017). Clinical manifestations include epilepsy, mental disability and autism (Zaroff et al., 2004; Mühlebner et al., 2016). This disorder is caused by mutations in TSC1 and TSC2 that encode Tuberous Sclerosis Complexes (Tsc1 and Tsc2) that belong to the canonical pathway of mTORC1 activation by trophic factors (see below), whereas these Tsc1/2 mutations induce mTORC1 hyperactivation.

Neurofibromatosis is caused by mutations in the gene that encodes neurofibromin, a modulator of mTORC1 activity (Giovannini et al., 2014). It is characterized by the appearance of tumors in the central and peripheral nervous system and anomalies in other tissues (skin, kidney, and bone). Clinical symptoms consist of learning disabilities, epilepsy and anxiety among other. Analysis of human samples has demonstrated that mTORC1 is over-activated, though administration of rapamycin in *in vitro* and *in vivo* models reduced the severity of tumors without toxicity (Giovannini et al., 2014).

In the adult brain, maintaining a precise balance of protein translation and degradation (proteostasis) is fundamental to avoid accumulation of toxic protein aggregates and oxidized proteins that might trigger brain degeneration, such as in Alzheimer's disease (AD), Parkinson's (PD), or Huntington's (Ht). Indeed, mTORC1 is an important player in proteostasis, through its capacity to control translation and autophagy.

In some neurodegenerative disorders such as AD or PD, characterized by the anomalous accumulation of aggregated misfolded proteins, data strongly suggests an anomalous level of mTOR-dependent autophagy. In fact, the reduction of autophagy with aging is a key mechanism that may contribute to the accumulation of protein aggregates in neurons (for a review see: Dazert and Hall, 2011; Perluigi et al., 2015). Following this hypothesis, it has been demonstrated in some animal models of AD or Ht that administration of rapamycin promotes elimination of toxic, misfolded proteins through induction of autophagy, reducing disease severity (Spilman et al., 2010; Kiriyama and Nochi, 2015; Benito-Cuesta et al., 2017).

Cerebral ischemia is an unexpected injury that may trigger, among other things, neuronal and glial cell death, and later neuronal degeneration (explained in detail below).

Our "working hypothesis" is that mTORC1 may play an important role in limiting ischemic damage. Several evidences sustain this assumption: (i) mTORC1 has the capacity to control anabolic/catabolic cell processes; (ii) mTORC1 potentially modulate neuronal apoptosis and autophagy; and (iii) considering the role of mTORC1 promoting neurogenesis and improving angiogenesis. Consequently, we propose mTORC1 as a potential therapeutic target for ischemic stroke.

In this review, we discuss the role of mTORC1 with an emphasis on the current knowledge about its impact in brain ischemia, analyzing recent evidence from both *in vivo* and *in vitro* models of cerebral ischemia.

SIGNALING PATHWAYS UPSTREAM OF mTORC1

The level of activation of mTORC1 depends on integration of extracellular signals through different pathways, permitting the combination of multiple events to generate a suitable response in order to maintain cellular homeostasis. In this section we explain the key players in signaling upstream of mTORC1.

The Canonical Pathway: PI3K/Akt/mTOR

As a general rule, growth factors bind to two types of receptors, Tyrosine kinase receptors (RTKs) or G-protein-coupled receptors (GPCRs). Downstream of both, activation of class I PI3-kinase is an essential step for initiating the signaling cascades. The activation of this lipid kinase generate a plethora of responses such as PDK1/Akt pathway, among others.

It is well known that complete activation of Akt requires the action of two different kinases (pyruvate dehydrogenase kinase 1, PDK1 and mTORC2) at two respective amino acid residues: Thr308 and Ser473 (Zhao et al., 2006; Dibble and Cantley, 2015). Downstream, Tsc has been identified as the connection between Akt and mTORC1, and is formed by three proteins: Tsc1, Tsc2, and TBC1D7 (Dibble and Cantley, 2015). Tsc1 (also called Hamartin) is a stabilizing factor of Tsc2. While Tsc2 (named Tuberin) is a guanosine triphosphatase (GTPase)-activating protein (GAP) toward Ras homolog enriched in brain protein (Rheb).

Akt phosphorylates Tsc2 and induces its dissociation from Tsc1 (Dibble and Cantley, 2015). Activation of Tsc1/2 inactivates the GTPase Rheb, which functions as a molecular switch by alternating between GTP and GDP-bound forms (Parmar and Tamanoi, 2010). GTP-Rheb directly activates mTORC1 (Malik et al., 2013), but Tsc1/2 action converts GTP-Rheb into GDP-Rheb, inactivating mTORC1. The loss of Tsc1/2 function is a determinant of mTORC1 over-activation that results in brain disease independent of growth factors (Frindlay et al., 2005; Hwang and Kim, 2011). Tsc2 activity can also be regulated by kinases such as mitogen-activated protein kinase (MAPK) and AMP-activated protein kinase (AMPK). In fact, AMPK activated by low cellular energy status (increased ratio of AMP/ATP) can phosphorylate Tsc2 at different residues than Akt; whereas phosphorylation of Thr1462 or Ser664 by Akt or MAPK, respectively, inhibits Tsc and activates mTORC1 (Ma et al., 2007), phosphorylation of Ser1345 or Ser1337/Ser1341 by AMPK or GSK3β, respectively, increases Tsc1/2 activity and consequently inhibits mTORC1 (Inoki et al., 2006; Takei and Nawa, 2014).

Thus, the AMPK-dependent pathway counterbalances the effects of trophic factors on mTORC1 activity as long as cellular energy is below an "optimal level" for survival.

Hormones, Growth Factors and Neurotransmitters

Hormones and growth factors like insulin, IGF-1, epidermal growth factor (EGF), neurotrophins [NGF, BDNF and neurotrophin 3/4 (NT-3; NT-4)], or some neurotransmitters, induce activation of downstream effectors of PI3K, Akt and

subsequently mTORC1, through binding of specific membrane receptors (Switon et al., 2017; see scheme in **Figure 2**). Whereas, insulin/IGF-1 and neurotrophins are the best characterized growth factors that activate mTORC1 complexes by the PI3K/Akt pathway (Takei and Nawa, 2014; Chen et al., 2017).

In the nervous system, axonal guidance molecules such as Reelin, Semaphorin, Ephrins, and Netrin-1 have been shown to activate mTORC1 through PI3K/Akt, and also some neuronal receptors like Glu receptors, cannabinoid receptors (CBRs), angiotensin receptors (AT2R), and μ-opioids, among others (Polakiewicz et al., 1998; Page et al., 2006; Puighermanal et al., 2009; Mateos et al., 2016). In some cases, this activation mediates important brain processes such as synaptic plasticity.

Amino Acids

Besides growth factors, some amino acids (not only glutamate and aspartate as neurotransmitters) have pivotal roles in the regulation of mTORC1 activity. Experimental evidence suggests that amino acids, particularly leucine, serve as extracellular stimuli that modulate mTORC1. The connection between mTORC1 and amino acid levels is the Rag family of small G-proteins (Rag A-D) (Kim et al., 2008; Sancak et al., 2008). RagA-D proteins are heterodimers that locate to the lysosome surface, an important site of mTORC1 activation. The active conformations of this GTP/GDP binding protein class are GTP-RagA/B and GDP-RagC/D (Dibble and Manning, 2013; see scheme in **Figure 2**).

A reduction of amino acid availability induces the GDP-RagA/B form, which triggers detachment of Rag from the lysosomal surface and consequently inactivates mTORC1. In contrast, when amino acid supply is sufficient, Rag binds GTP and undergoes conformational change, directly binding to Raptor and recruiting mTORC1 to the lysosomal membrane (Jewell et al., 2013). This situation allows close proximity between mTORC1 and Rheb on the lysosome surface, which can activate mTORC1. However, the mechanism by which other amino acids may modulate mTORC1 activity is not completely understood (Saxton and Sabatini, 2017).

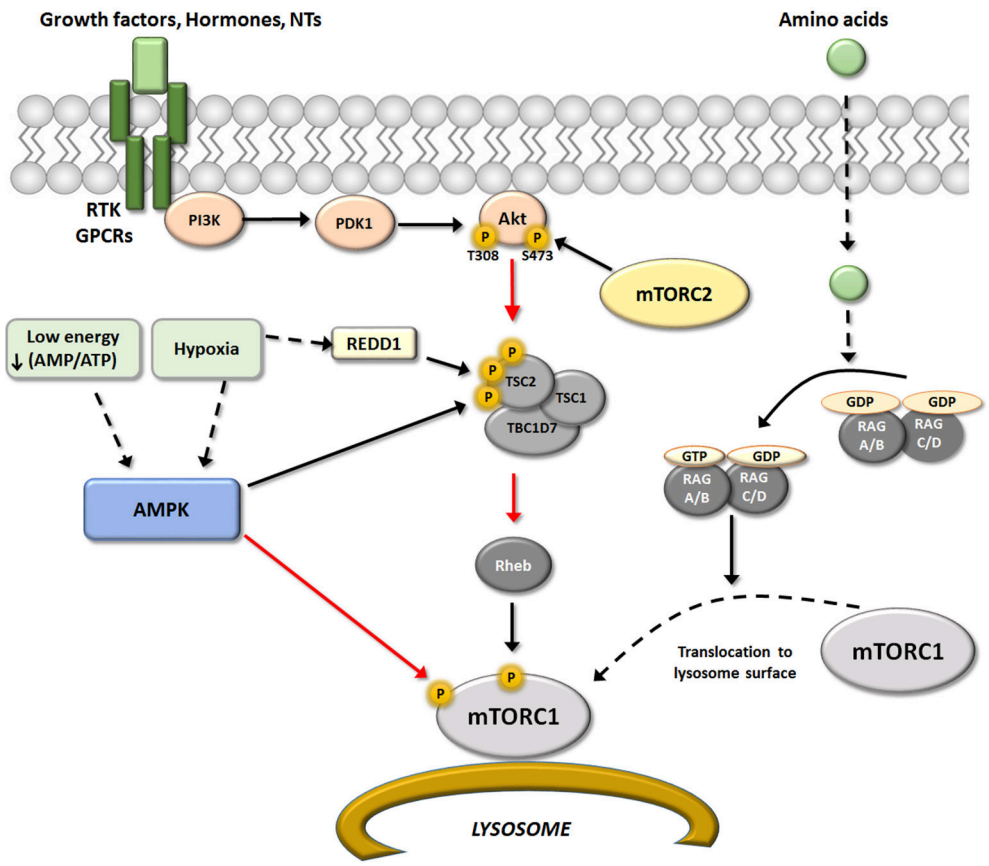

FIGURE 2 | Upstream regulators of mTORC1. Schematic representation of the different upstream pathways of mTORC1. The canonical pathway regulated mTORC1 activity through RTK/GPCRs receptors via modulation of PI3K/PDK1/Akt/TSC/Reb. This pathway may be positively regulated by growth factors, hormones and some neurotransmitters, and negatively regulated by AMPK. AMPK is activated in situations of low energy and/or hypoxia resulting in an inhibition of mTORC1 activity that may occurs at two levels. One by direct Raptor phosphorylation, that result in an inhibition of mTOR activity (red arrow); and second modifying TSC/REDD1 complex by phosphorylation. In addition, amino acids levels could modulated mTORC1 activity directly at the lysosome surface (an important place for activation of mTORC1) by modulation of heterodimers Rag A-D. Black arrows represent activation and red arrows represent inhibition.

Nutrients, Energy Status and Stressful Conditions

Energetic status, nutrient availability, and related stress conditions such as glucose deprivation, hypoxia, and DNA damage can modify mTORC1 activity (Shimobayashi and Hall, 2014). In the brain, both glia and neurons use glucose as a primary energy source, and are thus highly sensitive to fluctuations in blood glucose levels (Poels et al., 2009). Hypoglycemia may diminish ATP levels in glia/neurons and increase reactive oxygen species (ROS), triggering AMPK and promoting metabolic reprogramming. As discussed before, AMPK is sensitive to cellular energy status, sensing the AMP/ATP ratio and directing mTORC1 to modulate Tsc1/2 as a compensatory mechanism to preserve cellular energy pools. Activation of AMPK inhibits mTORC1 activity by two pathways, each of which involves phosphorylation of different substrates: Tsc2 and Raptor (Agarwal et al., 2015).

Similarly, a reduction in oxygen availability (hypoxia) inhibits mTORC1 through multiple pathways, especially when hypoxic conditions are sustained over time. Hypoxic activation of Tsc complex occurs by two pathways, one being AMPK-dependent, and the other acting via REDD 1. The increment in REDD1 after hypoxia is enough to liberate Tsc2 from chaperone 14-3-3 and permit its association with Tsc1. Hypoxic conditions may also reduce mTORC1 activity through other proteins that interfere with Rheb/mTORC interaction, including promyelocytic leukemia tumor suppressor (PML) and hypoxia-inducible proapoptotic protein BNIP3 (Brugarolas et al., 2004; Wouters and Koritzinsky, 2008; see scheme in **Figure 2**).

In summary, the precise impact of amino acids, glucose, growth factors, and neurotransmitters on mTORC1 activity in both physiological and pathological brain situations is not fully understand. This is partly because there isn't much information about the effects of selective elimination of each factor; thus, more work must be performed in cellular and animal models to clarify this issue.

SIGNALING PATHWAYS DOWNSTREAM OF mTOR

The ability of mTORC1 sensing energy and nutrient levels makes it a central node in regulation of metabolism. In nutrient-rich conditions, mTORC1 kinase activity promotes anabolic pathways (translation, transcription and lipid synthesis) and downregulates cellular catabolic processes (protein degradation).

Anabolic Metabolism Regulated by mTORC1

The best-identified anabolic process mediated by mTORC1 is protein synthesis, which consumes large amounts of energy and consequently is highly regulated. Activation of mTORC1 (when cellular nutrient and energy levels are optimal) favors protein synthesis. As mentioned previously, p70S6K and 4EBPs are key targets of mTORC1, and are phosphorylated at various sites (p70S6K1 at Thr389; 4EBP at several sites), favoring 5′ cap-dependent mRNA translation. In addition, mTORC1 upregulates several steps of ribosome biogenesis including transcription of ribosomal RNA and ribosomal protein synthesis (Iadevaia et al., 2014). Correct generation of ribosomes is necessary for cellular protein synthesis in general, which is critical for cell survival.

Recently, several studies have focused on the role of mTORC1 in mRNAs translation location-dependent. As neurons are long cells, some proteins must be produced locally on demand at synapses, sometimes far away from cellular soma. This is an important process that contributes to synaptic plasticity (Jaworski and Sheng, 2006; Urbanska et al., 2012). Transcripts activated by mTORC1 in response to BDNF have been described in synaptoneurosomes prepared from cortical neurons, some encoding proteins with important roles in dendritic spines (Panja and Bramham, 2014).

Stress factors such as glucose reduction and insufficient amino acids induce inhibition of mTORC1, promoting diminished phosphorylation of 4EBP1. In such conditions, 4EBP1 associates with the cap-binding eukaryotic translation initiation factor 4E (EIF4E), which prevents EIF4E/EIF4G interaction and therefore prevents cap-dependent translation (Wouters and Koritzinsky, 2008). In conclusion, mTORC1 has a pivotal role modulating 5′ cap-dependent translation, but also mediates translation independently of this canonical pathway. A role has been described for mTORC1 in cap-independent translational control of some critical factors in the brain such HIF-1α, VEGF, and IGF2 (Dai et al., 2011), a mechanism that would maintain expression of important factors during stressful situations. This cap-independent translation happens despite the absence of mTORC1 phosphorylation of signal transducer and activator of transcription 3 (STAT3), which diminishes the transcription of some proteins that use the cap-independent translation mechanism (Dodd et al., 2015).

In addition to its effect on protein synthesis, mTORC1 induces *de novo* nucleotide synthesis through different mechanisms (for a review, see: Ben-Sahra and Manning, 2017). Nucleotide synthesis is not only necessary for ribosome biogenesis but also for cell growth; several reports have indicated that the impact of mTORC1 on nucleotide synthesis is mediated by p70S6K1-dependent phosphorylation (Ben-Sahra et al., 2016). mTORC1 also activates SREBP, a transcription factor that induces expression of genes involved in fatty acid and cholesterol biosynthesis. This is essential to supply lipids for membrane elongation in growing cells, not only in neurons but also in glial cells and oligodendrocytes for myelination of the nervous system (Norrmén et al., 2014).

All of this evidence shows that mTORC1 tightly coordinates the biosynthesis of the three key macromolecular components of the cell through parallel pathways (see scheme in **Figure 3**).

Catabolic Metabolism Regulated by mTORC1

Autophagy is the catabolic mechanism by which dysfunctional or unnecessary cellular components are degraded through the action of lysosomes (Mizushima and Komatsu, 2011; Boya et al., 2013), and three major subtypes have been described: chaperone-mediated autophagy, microautophagy and macroautophagy.

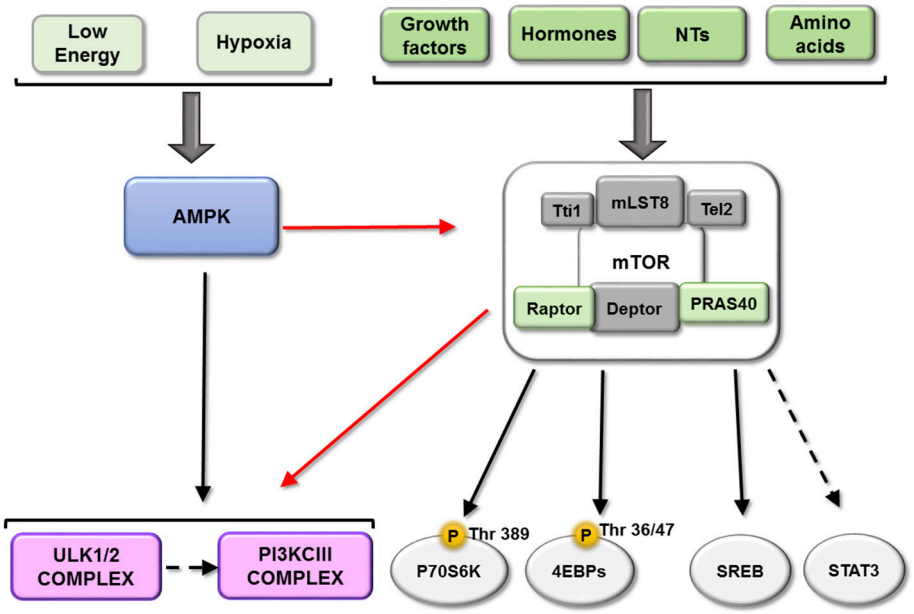

FIGURE 3 | Downstream of mTORC1. mTORC1 play a key role integrating information about the availability of some important metabolic factors. This figure represents the main mTORC1substrates described until now: P70S6K, EBPs, ULK complex and PI3KIII and their relationship. Black arrows represent activation and red arrows represent a inhibition.

Macroautophagy consists of the formation of an isolated membrane to sequester a portion of cytoplasm within a double membrane vesicle or autophagosome, which subsequently fuses with the lysosome to degrade its content. Apart from the upregulation of macromolecule synthesis, mTORC1 is also able to suppress catabolism, mostly via macroautophagy (hereafter referred to simply as autophagy).

mTORC1 inhibits autophagy through several mechanisms, the first of which involves its phosphorylation of ULK1/2 and ATG13, dissociating and inactivating the pro-autophagy complex ULK [composed of ULK1/2, ATG13, ATG101 and RB1-inducible coiled-coil protein 1 (RB1CC1)/FIP200]. Secondly, mTORC1 can phosphorylate ATG14 and UVRAG, which inactivates phosphatidylinositol 3-kinase class III (PI3KCIII) complex during initial (composed of PIK3C3/Vps34, Beclin-1, PIK3R4/Vps15, ATG14 and AMBRA1) and maturing stages (composed of PIK3C3/Vps34, Beclin-1, PIK3R4/Vps15 and UVRAG) of autophagosomes (Nakamura and Yoshimori, 2017). Conversely, both ULK and PI3KCIII complexes are activated by AMPK-dependent phosphorylation (Kim et al., 2011, 2013; see scheme in **Figure 3**). A third mechanism involves mTORC1 phosphorylation of transcription factor TFEB, which prevents its translocation to the nucleus and therefore the transcription of several ATGs and lysosomal proteins (see scheme in **Figure 4**).

ROLE OF mTOR IN ISCHEMIA/HYPOXIA

Different Approaches *in Vitro* and *in Vivo*

Cerebral ischemia, also known as ischemic stroke, is a sudden vascular accident that occurs when blood flow is reduced (transiently or permanently) to any cerebral artery by a clot, thrombus or atherosclerotic plaque. Globally, it is the second leading cause of death and the leading cause of adult long-term disability, and thus has a great social and economic impact (Thrift et al., 2014). Age is one of the most influencing factors related to incidence of stroke (Benjamin et al., 2017), and due to longer life expectancy, the incidence of ischemic stroke will increase over time. There does not currently exist any pharmacological treatment to reduce brain damage after stroke; the only therapeutic approaches are reperfusion by thrombolytic administration (only rtPA is approved) or surgery. However, not all stroke patients are appropriate for reperfusion, and only 10-20% of stroke patients receive rtPA (Lees et al., 2010), mainly because the window to restore blood flow to a cerebral artery is short (about 3–4 h from first symptoms) and the risk of cerebral hemorrhage is high after this time (Lenglet et al., 2014). The scientific community is making an intense effort to identify possible therapeutic targets to reduce ischemic damage and improves patient quality of life and to potentiate the effects of reperfusion in suitable patients. However, to date the pharmacological approaches that have been shown to be neuroprotective, reducing damage after stroke in animal models, have failed in humans (Cheng et al., 2004).

There are two different approaches to analyze the effects of ischemia and test potential neuroprotective compounds. The most commonly used *in vitro* model depends on oxygen and glucose deprivation (OGD) of primary cultured neurons or glial cells. This allows for precise control of duration of OGD exposure, permitting the study (even at molecular levels) of ischemia in each neural cell type at different times of injury. The

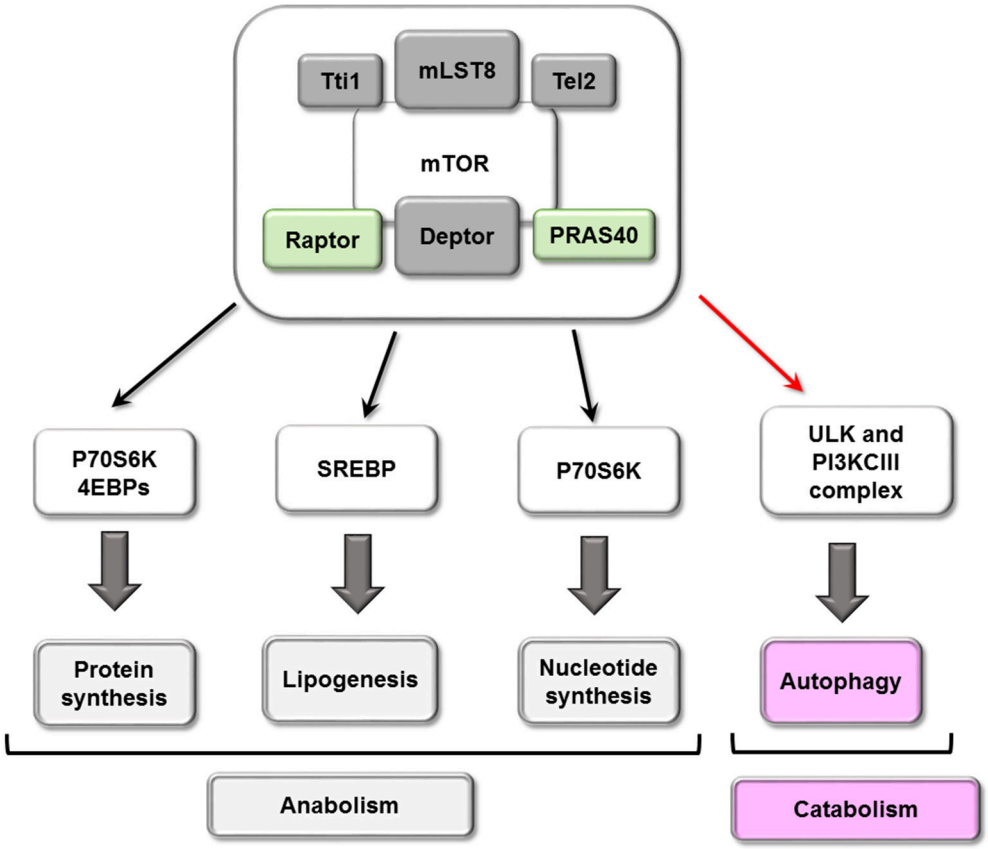

FIGURE 4 | Cellular processes mTORC1 mediated. mTORC1 play a central role in cellular metabolism controlling some anabolic (synthesis of proteins and nucleotide, lipogenesis) and catabolic (autophagy) processes.

most common *in vivo* model used is occlusion of middle cerebral artery (MCAO), which can mimic patients that have been treated after stroke (transient MCAO; tMCAO), or those who were not treated (permanent MCAO; pMCAO).

After hypoxia or ischemic conditions, the brain suffers a series of events collectively called "ischemic cascade," including energy failure, excitotoxicity, neuroinflammation and delayed neuronal death by apoptosis (Pérez-Alvarez and Wandosell, 2013; Perez-Alvarez and Wandosell, 2016). The reduction in blood flow to the brain decreases availability of nutrients, oxygen, and energy, compromising neural cell viability, and the degree of cell damage depends on the duration and severity of ischemia. Accordingly, the brain is especially sensitive to energy and nutrient fluctuations for several reasons: (1) the brain is highly dependent on glucose and oxygen from the blood (it is estimated that it consumes more than 50% of total glucose in the body); (2) neurons use glucose as a primary energy source (although astrocytes can partially supplement nutrient requirements for neurons); and (3) release of energy from glucose relies on oxidative metabolism. Thus, it is reasonable to conclude that mTORC1 may play a key role after ischemia, in the balance between anabolic and catabolic processes needed to protect neurons from death.

Some studies have confirmed that after brain ischemia, the reduction (via blockage) of oxygen, glucose and growth factors triggers a decrease in mTORC1 activity. A dramatic inhibition of neuronal mTORC1 has been described after ischemia, through diminished activity of the PI3K/Akt pathway in both *in vitro* and *in vivo* models (Dutta et al., 2015; Mateos et al., 2016). Consequently, evidence supports lower mTORC1 phosphorylation of p70S6K, with a subsequent inhibition of its activity (Hwang and Kim, 2011; Dutta et al., 2015; Mateos et al., 2016). Studies with p70S6K1/2$^{-/-}$ mice confirmed a role for this protein in brain protection after pMCAO (Pastor et al., 2009).

Glial cells are also affected by cerebral ischemia; after OGD/reperfusion, activation of the mTOR pathway is key to restore proliferation, migration and production of inflammatory mediators by astrocytes and microglia (Chong et al., 2007; Li et al., 2015). In pathological situations, a decrease in mTORC1 activity using rapamycin reduced glia scar formation after traumatic spinal cord injury, preventing astrocytes growth and proliferation (Goldshmit et al., 2015). Anoxic conditions reduce p70S6K1 mRNAs in astrocyte cultures, leading to cell death after OGD by an unbalance of pro- and anti-apoptotic factors and increased

ROS (Pastor et al., 2009). Therefore, some negative aspects of ischemic injury related to cellular death are triggered by deregulation of PI3K/Akt/mTORC, and increased activity of this pathway has proven to ameliorate the damage (see scheme in **Figure 5**).

It is well known that ischemic conditions induce transcription of HIF-1, a transcriptional factor that is a heterodimer composed of HIF-1α and HIF-1β. HIF translocates to the nucleus and can reprogram gene expression to facilitate cell survival in hypoxic conditions. The main process triggered by HIF consists of a shift from oxygen-consuming oxidative phosphorylation to oxygen-independent glycolysis, ensuring continued generation of ATP during hypoxia (reviewed in Chen and Sang, 2016). HIF-1 activity is determined by HIF-1α, thus the amounts of available HIF-1α are precisely regulated by a nutrient- and oxygen-dependent mechanism. In normal conditions, physiological oxygen levels induce HIF-1α ubiquitination and proteasomic degradation. In contrast, the low levels of oxygen, glucose and amino acids during hypoxia induce AMPK activity, stabilizing HIF-1α in combination with chaperones (such as Hsp90), increasing HIF-1 activity. A connection between HIF-1 and mTORC1 has been reported: Isoflurane preconditioning, which reduces neurological deficits, infarct volume, brain edema and apoptosis after ischemia induction, up-regulated HIF-1α expression via Akt/mTOR/p70S6K activation (Yan et al., 2016). Although translation of HIF-1α is not affected by inhibition of mTORC1, some reports have described an increment of HIF-1α synthesis when mTORC1 activity was increased (Hudson et al., 2002; Brugarolas et al., 2004; Bernardi et al., 2006; see scheme in **Figure 5**).

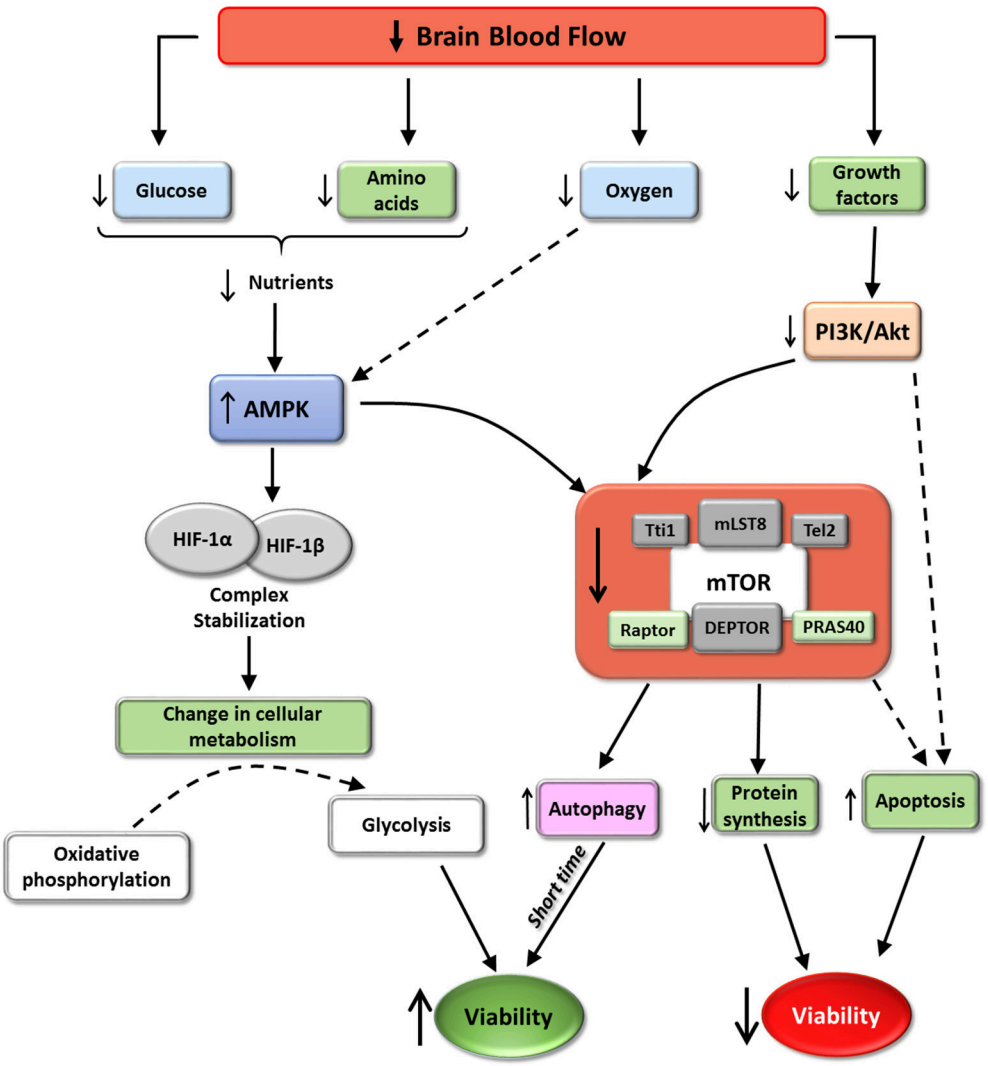

FIGURE 5 | mTORC1 and cerebral ischemia. The scheme represent some of the effects of cerebral ischemia on mTORC1 activity according with the available data. Ischemia induces mTORC1 inhibition. These situation promotes a reduction in protein synthesis and an increase autophagy and in some situations apoptosis. The increment in autophagy, almost at short time, may induce an increment in cell viability. However, autophagy imbalance or ischemic persistence may trigger apoptosis. The balance of this process via mTORC1, determinates the final destiny of hypoxia affected cells.

Some Neuroprotective Agents Regulate mTOR Activity

Many data support the hypothesis that PI3K-Akt-mTORC1 represents a major contribution, in almost all cell types, to controlling cell survival and proteostasis. In fact, in many neurodegenerative diseases, dysfunction of this pathway has been reported (Heras-Sandoval et al., 2014; Perluigi et al., 2015; Zhao et al., 2015). As a consequence, recovery or enhancement of this pathway has been proposed as a therapeutic strategy, with targets ranging from PI3K, Akt, and GSK3 to mTORC1 (Shimobayashi and Hall, 2014).

Considering the diversity of pathologies affecting the CNS and the broad processes regulated by mTORC, below is presented an analysis of the role of mTORC1 as a regulator of macro-autophagy and neuroprotection, using an acute damage disease model, such as ischemic stroke. The role of mTORC1 as a neuroprotective target in ischemia models is still a controversial matter. Some contradictory data have been reported in relation to beneficial or detrimental effects of autophagy induction in the context of cerebral ischemia (Buckley et al., 2014; Chauhan et al., 2015; Luo et al., 2015; Zhang et al., 2017). Indeed, beneficial effects have been described after ischemic brain injury by reducing autophagy (Guo et al., 2014; Wang et al., 2016; Jiang et al., 2017; Zhang et al., 2017). Moreover, have been reports some improvement in pathophysiological parameters by increasing autophagy before ischemic damage (Yin et al., 2012; Chauhan et al., 2015; Luo et al., 2015). The use of rapamycin and chloroquine in mouse models to induce or block autophagy, respectively, immediately after stroke indicated that both increase and decrease of autophagy reduced the size of the infarct area (Buckley et al., 2014). However, only increased autophagy before damage or at the same time, improved neurological score, and reduced neuronal death to a greater extent (Buckley et al., 2014; Chauhan et al., 2015).

Some data using an "ischemic tolerance" or "ischemic preconditioning" paradigm in the heart indicate that sublethal ischemic insults prior to real damage may be protective, permitting the hypothesis that a protective endogenous mechanism triggered after a moderate brain ischemia may make tissue resistant to a more severe ischemic insult (Lee et al., 2000). Some evidence supports that the mTORC1 pathway is a mediator of this neuroprotective process by inducing autophagy, and Tsc1 has been revealed as a key player. Overexpression of Tsc1 conferred protection to hippocampal neurons after OGD by inducing efficient autophagic flux. However, downregulation of TSC1 triggered autophagosome accumulation, compromising autophagy (Papadakis et al., 2013). However, not only has the role of mTORC1 in autophagy been reported to be neuroprotective in the paradigm of ischemic tolerance, but its inhibition promoted this protective mechanism in part through changes in translation (Wouters and Koritzinsky, 2008).

Treatment with rapamycin after 1 h of hypoxic or ischemic conditions in an *in vitro* model of OGD improved neuronal survival after damage (Fletcher et al., 2013), and rapamycin preconditioning before tMCAO improved neurological deficits and reduced infarct area and brain edema (Yin et al., 2012). Reports on the effects of pre-ischemic rapamycin administration have revealed a parallel between increased autophagy and decreased mTORC1 activity, diminishing apoptosis, significantly reducing infarct area and oxidative stress, and improving behavioral outcomes after ischemia (Chauhan et al., 2011; Chong et al., 2013).

Nevertheless, as we have commented previously, some reports demonstrated detrimental effects of autophagy induction in rodents models of cerebral ischemia (Guo et al., 2014; Jiang et al., 2017; Zhang et al., 2017). These controversial results must be analyzed carefully taking into account the moment in which autophagy is induced or blocked in animal models. Autophagy induction before or just after ischemic damage, result in beneficial effects measured as improvement of neurological score, reduction of infarct area or diminution of brain edema (Yin et al., 2012; Chauhan et al., 2015; Luo et al., 2015). Opposite results have been obtained when autophagy induction was later (after almost 1 h of ischemic damage) (Guo et al., 2014; Jiang et al., 2017; Zhang et al., 2017).

In some mouse models, treatment with agents that increase the activity of the PI3K/Akt/mTORC pathway (before/after induction of ischemia) has been shown to be neuroprotective, reducing ischemic damage and improving neurological score (Guo et al., 2014; Zheng et al., 2014; Lisi et al., 2015; Mateos et al., 2016; Chen et al., 2017).

Taking into account these data, we can conclude that after ischemic damage the accessibility of growth factors decrease, the canonical pathway PI3K/Akt/mTORC1 is downregulated and consequently autophagy may be enhanced.

However, authophagy induction before ischemia makes neural tissue more resistant to damage, while a sustained increment of autophagy or a greater inhibition of mTORC1 or lysosomal proteases activity after ischemia induce detrimental effects related with neuronal and/or glia, survival.

In conclusion autophagy could be considered an endogenous protective mechanism, at some extent, triggered after ischemic damage, because downregulation of PI3K/Akt/mTORC1 pathway. However, sustained increment of autophagy over the time or an excess of "levels" of autophagy, or sustained inhibition of PI3K/Akt/mTORC1 are detrimental for neuronal survival. This highlights the importance of a fine tune control of both: the autophagy process and mTORC1 activity, to maintain neuronal viability in ischemic conditions.

An important neuroprotective element used in ischemia models is estradiol, which has been reputed to reduce ischemic damage either pre- or post-insult. In general, estrogens function via different types of response: "genomic actions" regulated by classical estrogen receptors (ERs); "non-genomic" or "rapid actions" initiated by ERs and membrane-associated receptors; and ER-independent mechanisms, such as the antioxidant effects reported by estradiol (for a review see: Mann et al., 2007; Arevalo et al., 2015). In addition to the "classical" nuclear actions, rapid estrogen effects have been described that occur within minutes and thus cannot be attributed to genomic mechanisms (Toran-Allerand et al., 2002; Raz et al., 2008). In fact, estradiol may activate MAPK/Erk (Singh et al., 1999), PI3K (Honda et al., 2000) and Akt (Cardona-Gomez et al., 2004; Mendez et al., 2006), among other cytoplasmic elements, using either classical

or membrane-associated receptors (for review see: Arevalo et al., 2015). It has been reported that estradiol may have therapeutic effects in ischemia models, as injection (even post-ischemia) reduced the damage area, at least in part due to activation of the PI3K/Akt pathway (Koh, 2007; Pérez-Álvarez et al., 2012; Pérez-Alvarez and Wandosell, 2013). This strongly support the hypothesis that estradiol may control the activity of mTORC1 via cytoplasmic signaling (Varea et al., 2013).

Another important neuroprotective mechanism after cerebral ischemia is angiogenesis, the generation of new capillaries, which has been revealed to be a critical contributor to tissue recovery. Postmortem analysis of ischemic brain tissue revealed a significant increase in the number and size of microvessels in affected areas compared to the contralateral hemisphere, associated with longer patient survival (Krupinski et al., 1994). Angiogenesis after ischemic insult contributes to improved cerebral blood flow in the penumbra and reduces brain damage. mTORC1 has been identified as a potent mediator in this process, as mTORC1 activation increases VEGF levels (via the PI3K/Akt/mTORC1 pathway) by inducing its transcription in neurons (Chen et al., 2012; Mateos et al., 2016). This angiogenic enhancement reduces ischemic damage by decreasing the affected area and resulted in a higher neurological score in animal models of stroke (Mateos et al., 2016). Indeed, it has been reported that rapamycin administration reduced VEGF induction, preventing angiogenesis and increasing apoptotic cell death (Stahl et al., 2008).

At a pharmacological level, the role of statins must be mentioned, having been reported to have pleiotropic effects on neuroprotection. Statins inhibit HMG-CoA reductase, generating not only a reduction of cholesterol but also other intermediate metabolites essential for the formation of isoprenoids, such as geranyl pyrophosphate or farnesyl pyrophosphate, that are incorporated into such proteins as the Ras and RhoA families, among others, which play key roles in cellular signaling.

A second described effect for statins is enhancement of angiogenesis. Atorvastatin promoted angiogenesis, boosting functional recovery after stroke through a mechanism increasing VEGF, BDNF, eNOs, and some synaptic proteins such as glutamate receptor GluN1 or GluN2B (Cespedes-Rubio et al., 2010; Gutierrez-Vargas et al., 2014) in a mouse MCAO model (Brouet et al., 2001; Chen et al., 2003). Some of these actions are mediated by activation involving, directly or indirectly, the PI3K/Akt/mTORC1 pathway (Kureishi et al., 2000; Yang et al., 2015).

CONCLUSIONS

PI3K/Akt/mTORC1 is an important pathway that is implicated in some neurodegenerative diseases and also in recovery from ischemic stroke. Thus, genetic or pharmacologic enhancement of this pathway should be considered a potent neuroprotective tool, though the exact role of mTORC1 after stroke and its connection with regulation autophagy must be more carefully investigated to clarify controversial data. For instance, temporal studies are needed since it is very possible that the role of mTORC1 and autophagy depends on duration as well as the severity of nutrient/growth factor/oxygen deprivation. Accordingly, more work should be carried out to clarify whether alternative methods to enhance neuronal autophagy have additional therapeutic benefits after brain ischemia.

AUTHOR CONTRIBUTIONS

MP-A and FW, "The mTORC: role in adult CNS"; MP-A and MV, "Signaling pathways downstream of mTORC"; MP-A and IB-C, "Signaling pathways downstream of mTORC"; MP-A, "Role of mTOR in ischemia/Hypoxia"; FW and MP-A, "Some neuroprotective agents regulates mTOR Activity"; FW, "Conclusion." The figures has been designed and made by MV, FW, and MP-A.

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Antioxidative and Antiapoptotic Effects of Delta-Opioid Peptide [D-Ala^2, D-Leu^5] Enkephalin on Spinal Cord Ischemia-Reperfusion Injury in Rabbits

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Background: In our previous study, we found that regional administration of delta-opioid peptide [D-Ala^2, D-Leu^5] enkephalin (DADLE) could provide dose-dependent protection on spinal cord ischemia-reperfusion (I/R) injury in rabbits. However, the relative protective molecular mechanisms underlying this neuroprotection remain unclear. The purpose of this study was to investigate whether DADLE provided the protection in spinal cord I/R injury through its antioxidant property by decreasing malondialdehyde (MDA) and nitric oxide (NO) levels and increasing glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) levels and through its antiapoptotic capacity by inhibiting caspase-3 and p53 expression.

Methods: The rabbits were divided into three groups. The animals in Group NS and Group DADLE were administered with normal saline (NS) or DADLE via aorta during 30 min of ischemia respectively, while the one in Group Sham received no intervention. During the period of reperfusion, the rabbit's blood samples were collected for enzyme-linked immunoabsorbent assay (ELISA) examinations of MDA, NO, GSH-Px and SOD. At 48 h after reperfusion, the lumbar spinal cords were harvested for immunohistochemical, real-time polymerase chain reaction (PCR) and western blot studies to detect the caspase-3 and p53 expressions.

Results: The activities of serum MDA and NO showed significant reductions in the DADLE group as compared with the control group. By contrast, the levels of serum GSH-Px and SOD were significantly higher in the DADLE group than those in the NS group. In addition, caspase-3 and p53 expression were significantly increased in the NS group, while DADLE mitigated these changes.

Conclusions: The protective effects of DADLE at the dosage of 0.05 mg/kg may be related to its antioxidant and antiapoptosis properties in the rabbit model of spinal cord I/R injury.

Keywords: DADLE, spinal cord, ischemia-reperfusion injury, oxidative stress, apoptosis, rabbit models

INTRODUCTION

Spinal cord ischemia-reperfusion (I/R) is one of the most serious complication of thoracoabdominal aortic surgery, leading to postoperative paraplegia in 4 to 16% of patients (Zvara, 2002). Despite some efforts to develop neuroprotective agents in experimental spinal cord ischemia, few so far has been translated into clinical application. In our previous research, we revealed that the aorta-infused [D-Ala^2, D-Leu^5] enkephalin (DADLE), a synthetic delta-opioid peptide, could approximately reduce the rate of paraplegia by more than 50% and increase the surviving neurons number by 3 times in a rabbit model of spinal cord I/R (Liu et al., 2015a). In order to achieve more reasonable utilization of agent, it is essential to investigate the mechanisms underlying neuroprotective effects of DADLE in the spinal cord.

Oxidative stress reaction is now believed to play a major role in the progression of several organ I/R injury such as brain (Liu et al., 2015b), liver (Liu et al., 2014), and kidney (Nie et al., 2016), including spinal cord (Wang et al., 2017). During I/R, the oxidative processes resulting from the abundant free radicals may cause protein breakdown, lipid peroxidation and DNA damage. The endogenous antioxidant system alleviates these damages through enzymatic substances, such as glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD). In addition, several authors have demonstrated that the neuroprotective effects of various antioxidative therapies, for example, melatonin and oxytetracycline (Topsakal et al., 2003) were regarded as promising targets for therapeutic interventions in spinal cord I/R injury. DADLE has been demonstrated to promote the survival of neurons in the central nervous system including decreasing methamphetamine-induced dopaminergic transporter loss through suppressing reactive oxygen species formation and inhibiting brain lipid peroxidation (Tsao et al., 1998). However, it remains unknown whether DADLE can protect the I/R-induced spinal cords injury through antioxidant property.

Moreover, neuronal apoptosis induced by I/R damage has been reported as another mechanism for neurological deficits in the spinal cords (Jia et al., 2012). Spinal cord I/R injury causes disorders of energy metabolism and produces over-produced free radicals, which exacerbate mitochondrial membrane permeabilization and lead to activation of caspase-9 and caspase-3, subsequently trigger apoptosis (Hayashi et al., 1998). Thus, inhibition of apoptosis through pharmacological pathway might be a potent neuroprotective strategy. DADLE was shown to preserve serum-deprived rat pheochromocytoma cells through an antiapoptotic property (Hayashi et al., 2002). We therefore assessed the effects of DADLE on the neuronal apoptosis in the spinal cords.

The objective of the current study was to investigate the antioxidative effect of DADLE based on the detection of malondialdehyde (MDA), nitric oxide (NO), GSH-Px and SOD levels. Meanwhile, we also studied the antiapoptotic effect of DADLE by evaluating the expression of caspase-3 and p53. We hypothesized that DADLE provided the neuroprotection of spinal cords through its antioxidant activity by decreasing MDA and NO levels and increasing GSH-Px and SOD levels, and through its antiapoptosis property associated with caspase-3 and p53.

Abbreviations: [D-Ala^2, D-Leu^5], DADLE; I/R, ischemia-reperfusion; MDA, malondialdehyde; NO, nitric oxide; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; NS, normal saline; PCR, polymerase chain reaction.

METHODS AND MATERIALS

Animals Preparation

The experiment and animal care protocol were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University (Shanghai, P.R. China), and complied with the "Guide for the Care and Use of Laboratory Animals" published by National Institutes of Health (National Academy Press, Washington DC, revised 1996). Twenty-two New Zealand white rabbits (male and female) weighing 2.5 to 3.0 kg were randomly allocated to one of the following three groups: Group NS ($n = 8$), Group DADLE ($n = 8$) and Group Sham ($n = 6$). Spinal cord ischemia was induced by aortic clamping at infrarenal aorta. During the ischemic period, the rabbits in Group NS and Group DADLE received normal saline or DADLE 0.05 mg/kg through the clamped aorta, while the animals in Group Sham underwent the same surgical schedule but without aortic clamping.

Induction of Spinal Cord I/R Injury

Animals were anesthetized by the intramuscular administration of ketamine (25 mg/kg) and atropine (0.10 mg/kg). The right ear artery was catheterized for arterial blood pressure and heart rate monitoring and blood specimens sampling. Then they were given endotracheal intubation and mechanical ventilation according to the following parameters: tidal volume, 8 mL/kg; respiratory rate, 30 bpm; ratio of inspiratory time to expiratory time, 1: 1.5; and fraction of inspired oxygen, 1.0. General anesthesia was maintained by intravenous injection of midazolam 0.5 mg/kg, fentanyl 8–10 μg/kg, and vecuronium 0.2–0.25 mg/kg in interval. Core temperature was maintained between 37–38°C supported by a heating lamp throughout the procedure.

The model of spinal cord ischemia was established as described previously (Liu et al., 2016). In brief, an incision was made in the right iliac region and the right femoral artery was exposed. Sequentially, an abdominal incision was made and the retroperitoneal infrarenal aorta was exposed. At the time after heparinization for 5 min, a 22-gauge PA catheter was inserted from the right femoral artery incision to the descending aorta, which was used for monitoring the distal artery pressure and drug perfusion. The tip was advanced approximately 13–14 cm from the site of insertion, and located 1.5 cm distal to the left renal artery. Then aortic cross-clamping was performed by two artery clips, just inferior to left renal artery for 30 min. DADLE 0.05 mg/kg or NS at the same volume was administered into the clamped aortic segments respectively, at the same rate of 3 ml/kg/h during the ischemic period. Meanwhile, the bilateral common iliac arteries were clamped to increase the local drug concentration. The efficacy of the clamping was confirmed by the immediate and continual absence of pulsation and a rapid decrease to less than 20 mmHg in distal abdominal aortic

pressure. Immediately at the end of 30 min ischemia, drug infusion was stopped and the artery clips were removed. Then the catheter was removed and the incision were sutured layer-by-layer. The animals were tracheal extubated after they regained consciousness. In order to prevent infection, the animals were injected benzylpenicillin (300,000 U/kg) intramuscularly postoperation. Crede's maneuver was carried out on rabbits to avoid urinary retention three times a day.

Tissue Sampling

At the onset after reperfusion (T1), 1 h after reperfusion (T2), 6 h after reperfusion (T3), 24 h after reperfusion (T4) and 48 h after reperfusion (T5), arterial blood samples from the NS and DADLE groups were obtained for MDA, NO, GSH-Px and SOD analyses. However, the blood samples of the Sham group were just collected at 48 h after the sham operation because we did not perform the aortic clamping in this group. Following centrifugation of 3,000 rpm for 15 min, the supernatant was collected and stored at −80°C in refrigerator until measurement. After completing neurologic deficit score, the spinal cords segments of L4 and L5 were quickly harvested under the general anesthesia and the animals were sacrificed by intravenous injection of sodium pentobarbital (200 mg/kg). One part of the tissue sample was fixed in 10% formalin for 48 h, embedded in paraffin and cut into coronal 4 μm sections for caspase-3 immunohistochemistry examination. Another part of the sample was frozen using liquid nitrogen and then transferred to −80°C freezer for p53 mRNA and protein expression analysis.

Biochemical Analysis of Oxidative Stress Markers

To ascertain the effect of DADLE on antioxidant mechanism concerning spinal cord I/R injury, we measured the contents of serum MDA and NO, two markers of oxidative stress and the activities of GSH-Px and SOD, the most important enzymes to protect against oxidative stress.

MDA Activity

The level of serum MDA concentration was measured by the thiobarbituric acid method using an MDA kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. A total of 100 μL of serum sample was mixed with thiobarbituric acid, acetic acid and sodium dodecyl sulphate and incubated at 95°C for 40 min. After cooling with water, the sample was centrifuged at a rate of 4,000 rpm for 10 min. The absorbance values in all tubes were measured at the wavelength of 532 nm using a spectrophotometer. Concentrations of MDA were determined using a standard curve calculated from tetraethoxypropane. The content of MDA was expressed as nmol/L serum.

NO Activity

To determine the NO activity in serum, nitrite and nitrate were measured as indexes of NO production using an assay kit (Jiancheng Bioengineering Institute, China). In brief, the sample was mixed with the Griess reagent mixture in a 96-well microtiter plate at room temperature for 10 min (Cortas and Wakid, 1990). Nitrite products in the samples were calculated by measuring absorbance at 540 nm with a standard curve established sodium nitrite. The results were expressed as μmol/L serum.

GSH-Px Activity

The GSH-Px activity was estimated using an GSH-Px kit according to the manufacturer's instructions (Jiancheng Bioengineering Institute, China). In brief, the supernatant was mixed with phosphate buffer, sodium azide, glutathione reductase enzyme, NADPH and H_2O_2. The absorbance reduction of NADPH caused by enzymatic reaction at 340 nm were measured after initiation of the reaction. One GSH-Px unit was defined as the enzyme activity required conversion of NADPH to $NADP^+$. The GSH-Px activity was expressed as units per mg protein.

SOD Activity

The serum SOD activity was measured using a SOD kit (Nanjing Jiancheng Bioengineering Institute). It used a xanthine-xanthine oxidase system as a superoxide generator to conduct the inhibition of nitroblue tetrazolium reduction. In brief, 100 μL sample was mixed with the xanthine and xanthine oxidase in potassium phosphate buffer and incubated at 37°C for 20 min. The enzymatic reaction was stopped by $CuCl_2$. SOD activity was measured by detecting the absorbance at the wavelength of 450 nm. One unit of activity was defined as the enzyme that caused 50% inhibition rate. SOD activity was expressed as units per mL serum.

Caspase-3 Immunohistochemistry

Caspase-3 immunohistochemistry was processed according to the protocol provided by the manufacturer (Boster Biological Technology co. Ltd., Wuhan, China). In brief, spinal cords sections were deparaffinaged and rehydrated in ethanol and then radiated in sodium citrate buffer (pH 6.0) by microwave for 15 min to achieve antigen retrival. The sections were then incubated with 3% H_2O_2 to inactivate endogenous peroxidases for 10 min, following a rinse with phosphate-buffered saline for three times. Subsequently, these sections were incubated at 37°C for 2 h with an anti-caspase-3 primary antibody (1:100, Abcam, Cambridge, MA, USA) and then rinsed in phosphate-buffered saline three times. Next, the sections were blocked with goat anti-rabbit biotinylated secondary antibody (Boster Biological Technology co. Ltd., Wuhan, China) at 37°C for 30 min. After three additional washes in phosphate-buffered saline, caspase-3 signals were visualized by the 3,3′-diaminobenzidine tetrahydrochloride method. Finally, the sections were counterstained with hematoxyline and coverslipped.

Immunohistochemical images were taken with an optical microscope (Zeiss, AxioCam MRc, German). Cells with a blue color were regarded as neurons, while neurons presenting brown color were regarded as caspase-3 positive cells. The normal and caspase-3 positive neurons were counted by two researchers blinded to the experimental assignment. At least three high power fields were randomly selected on each section. The ratio of caspase-3 positive cells in each high power field was used for the statistical analyses.

Real-Time Polymerase Chain Reaction (PCR) Analysis of p53

Apoptosis was postulated to participate in neuron injury induced by I/R (Hayashi et al., 1998). To investigate whether DADLE modulated the process of apoptosis in ischemic spinal cords, we assayed the p53 mRNA expression, a reliable index for apoptosis. Total RNA was extracted from the spinal cord tissues using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out according to the manufacturer's instructions of the RNA real-time PCR kit (BioTNT Co., Ltd, Shanghai, China). The PCR reaction was performed by adding 2 μL cDNA template, 8.5 μL ddH_2O, 12.5 μL SYBR Green mix and 1 μL one of the following primers: p53, 5′-CCCGACAGCCAGGTCATC-3′ (forward), 5′-GTTGAAGGTGGTCTCGTGG-3′ (reverse); β-actin, 5′-TGGAGGAGTCGCAGTCGGA-3′ (forward), 5′-GAGGTGGTCAGCAGGTTGT-3′ (reverse). The primers were designed using the Primer5 program and synthesized from Invitrogen (Carlsbad, CA, USA). All samples were tested in triplicate. The real-time PCR was performed with an initial denaturation step at 95°C for 15 min, and a amplification step at 95°C for 15 s and 60°C for 30 s for 40 cycles. Real-time PCR products were separated with electrophoresis and visualized under ultraviolet light after ethidium bromide staining. The relative level of p53 mRNA was calculated by $2^{-\Delta\Delta CT}$ method. mRNA expression values were normalized using β-actin as an internal control.

Western Blot Analysis of p53

In order to further confirm the effect of DADLE on p53, western blot analysis was also performed. Hundred micro gram protein samples underwent 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% dry milk Tris-buffer and incubated overnight at 4°C with a monoclonal antibody to p53 (1:500; GeneTex, USA). After a rinse with Tris-buffered saline with Tween for three times, the corresponding secondary antibody (Boster Biotechnology Co., Ltd., Wuhan, China) was added and incubated for 1 h. The blots were detected by a chemiluminescence detection system. β-actin was used as an internal reference to correct the variations of different samples. The gray values of the target bands were quantified using an image analysis software (Image Pro Plus 6.0).

Statistical Analysis

All data were expressed as mean ± standard deviation. SPSS 17.0 statistical software package was used for statistical analysis (version 13.0, SPSS Inc., Chicago, IL, USA). The difference in means between two groups was assessed by the Student's *t*-test. Differences in means among three groups were analyzed using one-way ANOVAs followed by intergroup comparison using the Dunnett test. The difference between each time point in different groups was analyzed using two-way ANOVAs with time and drug treatment considered as factors followed by the Bonferroni *post hoc* test. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Assessment of the Level of Serum MDA

As reported in **Figure 1**, serum MDA level in group NS was significantly increased compared with the Sham group at T5 ($P < 0.05$, **Figure 1A**). In addition, significant differences were presented between the NS and DADLE groups from the time points of T1 to T4. Compared to the NS group, DADLE treatment remarkably decreased MDA activity in the blood ($P < 0.05$, **Figure 2A**).

Assessment of the Level of Serum NO

Accordingly, the data revealed that serum NO from ischemic animals that treated with NS significantly increased compared with the Sham group at T5 in rabbits ($P < 0.05$, **Figure 1B**). However, compared with the NS group, NO concentration was remarkably attenuated by DADLE treatment at all-time points ($P < 0.05$, **Figure 2B**).

Assessment of the Level of Serum GSH-PX

As shown in **Figure 1C**, ischemic spinal cords induced a significant reduction of GSH-Px activities compared with the Sham group at T5 ($P < 0.05$). However, our results showed that administration of DADLE significantly reversed such reduction compared with the NS treated group from T2 to T5, especially at T3 ($P < 0.05$, **Figure 2C**).

Assessment of the Level of Serum SOD

The analysis of data revealed that a lower level of SOD was observed in the NS group compared with the Sham group at T5 (**Figure 1D**). The change of SOD activity was similar to GSH-Px in response to NS and DADLE treatment. SOD level was significantly higher in the DADLE group compared with the NS group at the time points of T1 and from T3 to T5 ($P < 0.05$), and there was no significant change between two groups at T2 (**Figure 2D**).

Assessment of the Level of Caspase-3

To evaluate whether DADLE reduced caspase activity in the ischemic spinal cord, caspase-3 expression was examined using immunohistochemical methods. The results showed that caspase-3 immunoreactivity was barely detectable above background in the Sham group, while there was intense protein immunoreactivity in saline-treated ischemic animals. Moreover, DADLE treatment significantly decreased caspase-3 positive cells ($P < 0.05$, **Figure 3**). These results indicated that DADLE could protect the spinal cord against apoptotic cell death process associated with I/R injury.

Assessment of the Level of p53 Expression

We performed real-time PCR and western blot to evaluate whether DADLE affected the expression of p53 at 48 h after spinal cord I/R. The real-time PCR analysis showed a significant increase of p53 mRNA expression in saline-treated rabbits at 48 h after reperfusion compared with the Sham group ($P < 0.05$), while this increase was attenuated by DADLE ($P < 0.05$, **Figure 4**).

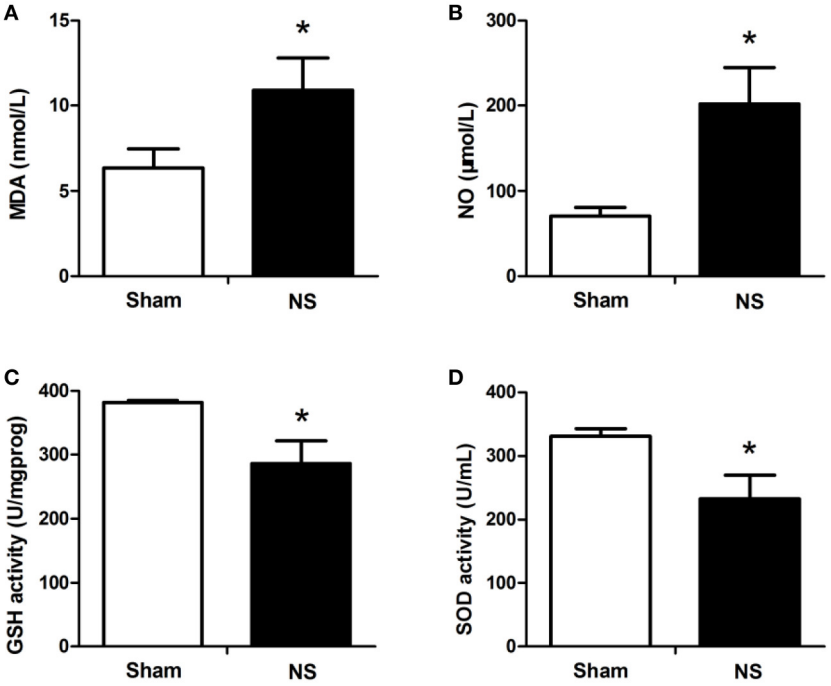

FIGURE 1 | ELISA results of MDA, NO, GSH-Px. and SOD expression in the spinal cords between the NS and Sham groups. Compared with the Sham group, I/R injury significantly increased the levels of MDA **(A)** and NO **(B)** and decreased the levels of GSH-Px **(C)** and SOD **(D)**. The blood samples were harvested from the spinal cord I/R and sham rabbits at 48 h after operation. The differences between two groups were analyzed using by the Student's *t*-test. Bars represent mean ± SD ($n = 6$–8). *$P < 0.05$ vs. the Sham group.

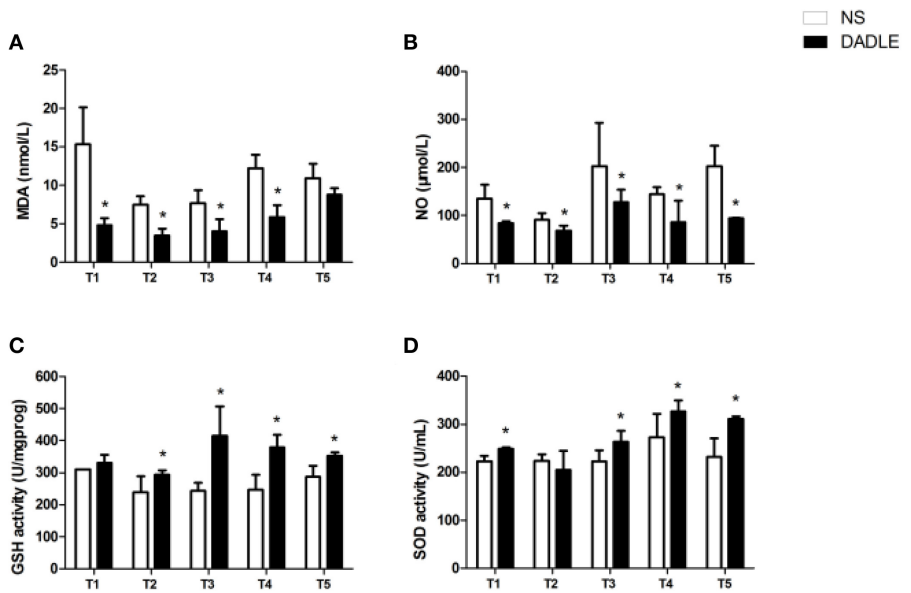

FIGURE 2 | DADLE-induced decrease in MDA and NO levels and increase in GSH-Px and SOD levels in spinal cord I/R injury. **(A)** The effects of DADLE on MDA activity. **(B)** The effects of DADLE on NO activity. **(C)** The effects of DADLE on GSH-Px activity. **(D)** The effects of DADLE on SOD activity. T1 = the onset of reperfusion; T2 = 1 h after reperfusion; T3 = 6 h after reperfusion; T4 = 24 h after reperfusion; T5 = 48 h after reperfusion. Differences in means in NS and DADLE groups at each time point were analyzed using two-way ANOVAs followed by the Bonferroni *post hoc* test. Bars represent mean ± SD ($n = 8$). *$P < 0.05$ vs. the NS group.

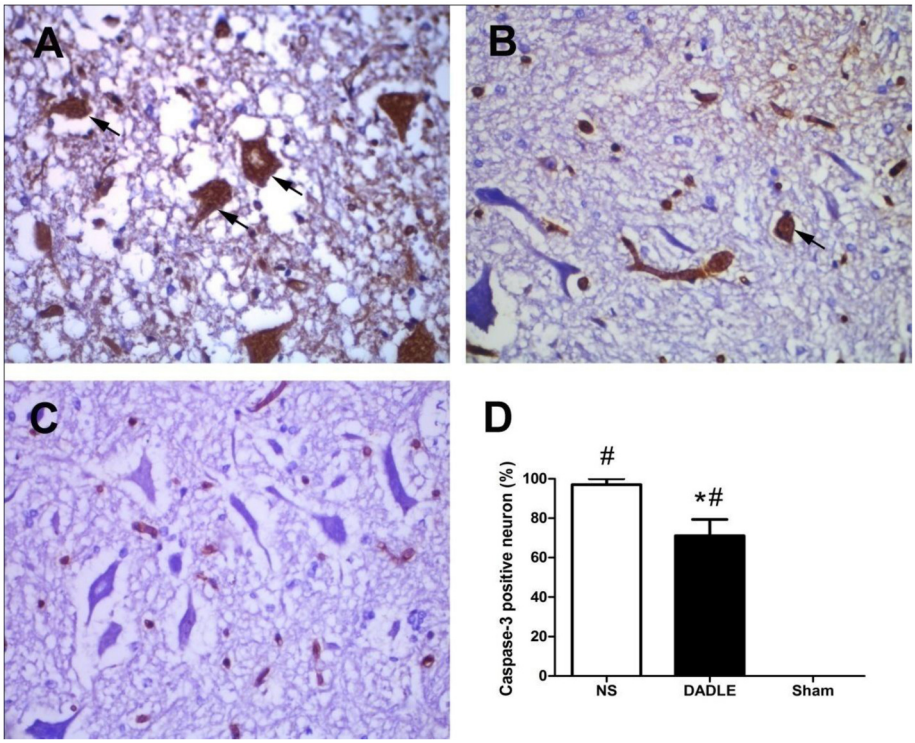

FIGURE 3 | Immunohistochemical staining of spinal cords using caspase-3 antibody at 48 h after reperfusion. The samples of spinal cords were harvested from the spinal cord I/R and sham rabbits at 48 h after operation. Neurons in Group NS contained intense caspase-3 immunoreactivity **(A)**, which was significantly attenuated in Group DADLE **(B)**, while normal spinal cords stained poorly with caspase-3 in Group Sham **(C)**. Comparison of percentage of caspase-3 positive neuron in each high power field **(D).** Bars represent mean ± SD ($n = 6$–8). $^{*}P < 0.05$ vs. the NS group. $^{\#}P < 0.05$ vs. the Sham group. The arrows indicate caspase-3 positive cells.

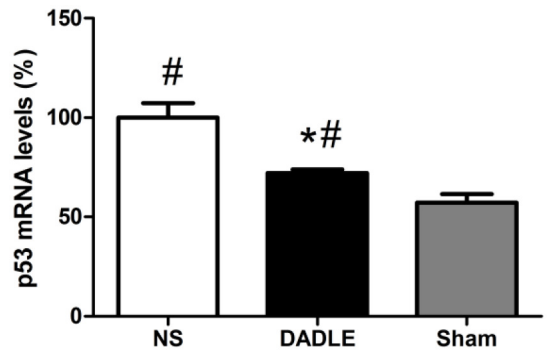

FIGURE 4 | Quantitative p53 mRNA expression through real-time PCR in the spinal cords at 48 h after reperfusion. The samples of spinal cords were harvested from the spinal cord I/R and sham rabbits at 48 h after operation. The transcript level of p53 were up-regulated in the normal saline-treated rabbits, while a marked reduction was shown in the DADLE group. Bars represent mean ± SD ($n = 6$–8). $^{*}P < 0.05$ vs. the NS group. $^{\#}P < 0.05$ vs. the Sham group.

The trend is also confirmed at protein level by western blot experiments (**Figure 5**). The results demonstrated that p53 protein level in the NS group had a significant increase compared with the Sham group at 48 h after reperfusion, which was remarkably reduced by DADLE ($P < 0.05$). These data can be visualized in the representative blot and the thickness of the bands reflects the changes as described above.

DISCUSSION

Spinal cord I/R injury comprises the most important complication of thoracic and thoracoabdominal aortic aneurysm repair surgery and is considered to be one of the major cause of neurological disability worldwidely. Despite intensive efforts to develop new neuroprotective agents for experimental central nervous system ischemia models, most so far have failed to show clinical effects. Our previous studies showed that DADLE could attenuate spinal cord I/R injury during the ischemic period and the neuroprotective effect appeared most significant at a dose of 0.05 mg/kg (Liu et al., 2016). Antioxidative and antiapoptotic properties of DADLE were only reported *in vitro* experiments or in the model of cerebral I/R injury (Tsao et al., 1998; Hayashi et al., 2002). However, the pathophysiology processes involving the I/R injury in the brain and spinal cord are not identical. In this study, we further investigated whether DADLE protected spinal cord against I/R injury through its antioxidant and antiapoptotic properties on the basis of previous studies.

Two major findings were made in this study. Firstly, our data showed that DADLE attenuated the production of MDA

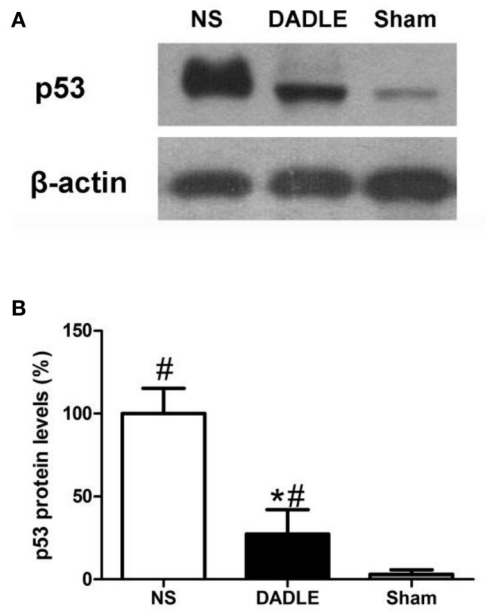

FIGURE 5 | Representative western blot of p53 **(A)** and quantitative analysis of p53 protein **(B)** in the ischemic spinal cords at 48 h after reperfusion. The samples of spinal cords were harvested from the spinal cord I/R and sham rabbits at 48 h after operation. The expression of p53 protein was up-regulated in the NS group, which was significantly decreased by DADLE treatment. Bars represent mean ± SD (n = 6-8). *$P < 0.05$ vs. the NS group. #$P < 0.05$ vs. the Sham group.

and NO, meanwhile increased the activities of SOD and GSH-Px, indicating that the neuroprotective benefits of DADLE are probably related to antioxidant capacity. Secondly, we found that DADLE administration inhibited the expressions of caspase-3 and p53 which are highly related to apoptosis, suggesting that one protective mechanism of DADLE on spinal cord I/R injury is related to its antiapoptotic property. So we suggest that the delta-opioid peptide DADLE increases the survival of spinal cord exposed to I/R injury via at least two mechanisms: antioxidative and antiapoptotic pathway.

Among the proposed mechanisms involved in spinal cord I/R damage, oxidative stress caused by reactive oxygen species (ROS) including superoxide, hydrogen peroxide, hydroxyl and reactive nitrogen species (RNS) including NO contribute to the pathological process of secondary neuron injury. Under physiological condition, superoxide accumulation was scavenged by the antioxidant enzymes such as GSH-Px and SOD. However, overproduction of oxidants following tissue hypoxia and/or ischemia overwhelm the antioxidant capacities of the cells, resulting the pathogenic outcome of the cell death (Lièvre et al., 2000). The spinal cords mostly consist of lipids and particularly susceptible to peroxidation induced by free radical. Peroxidation of lipids may leading to respiratory dysfunction, mitochondrial permeability transition and consequently cause cell death (Hall et al., 2016). MDA, the production of damaged polyunsaturated fatty acids, generates DNA-protein cross-links and is a highly toxic molecule. It serves as a marker for the extent of lipid peroxidation (Del Rio et al., 2005). Tsao et al. (1998) reported that DADLE functioned as a free radical scavenger in alleviating the generation of superoxide anions, hydroxyl free radicals and lipid peroxidation *in vitro*. In this study, DADLE has been found to share similar mechanism underlying neuroprotection in the ischemic spinal cords. Our current results show that ischemic spinal cords damage increased the serum concentration of MDA compared with the Sham group, which was mitigated by DADLE treatment. In addition, NO as a kind of oxidative stress biomarker has been shown to exert pivotal role in organ I/R injury. Higher NO concentration mediated inflammatory processes following I/R, depressed mitochondrial function and even triggered cellular death (Schulz et al., 2004). In our study, NO was upregulated in the spinal cords after I/R injury, while DADLE partially reversed it.

GSH-Px is the most important antioxidant enzyme by modulating the redox status of proteins in the cell membrane (Zhang et al., 2014). In the present study, we investigated the antioxidant capacity of DADLE by measuring the GSH-Px activity in the blood of rabbits exposed the spinal cord I/R. Consequently, spinal cord I/R injury tended to decrease the GSH-Px level, while DADLE intervention restored it. SOD is another critical antioxidant enzyme that scavenges free radicals generated by the electron transport chain to form H_2O_2 and oxygen (Wang et al., 2009). Consistent with GSH-Px, our data demonstrated that serum SOD activity in rabbits of spinal cords exposed to ischemic insult was reduced, which was reversed by DADLE treatment. Our results are consistent with the rat model of cerebral I/R injury, in which DADLE increased the activity of GSH-Px and SOD, and decreased the MDA and NO levels (Yang et al., 2009). Therefore, our results suggest that DADLE may protect spinal cord exposed to I/R insult through antioxidant functions.

Apoptosis plays a prominent role in the pathophysiology of secondary injury to ischemic spinal cord. This harmful process lasts for a long time and results motor neuron death, even if blood flow would be restored later (Hayashi et al., 1998). During this procedure, caspase-3 activation was essential for the execution step in programmed cell death and was a crucial mediator of ischemic injury (MacCorkle et al., 1998). It has been proven that inhibition of apoptosis may be a promising approach for therapeutic intervention in spinal cord injury (Ali-Khan and Hales, 2003). In addition, Hayashi et al. (2002) reported that DADLE showed potent antiapoptotic effects in serum-deprived pheochromocytoma cells. Zheng et al. (2012) suggested that DADLE could protect the brain from I/R injury most likely through the antiapoptotic pathway.

In our present study, we further tried to find that weather DADLE by regional reperfusion into abdominal aorta could inhibit the apoptosis of spinal cord motor neurons. Firstly, we examined if DADLE could affect caspase-3 expression in a rabbit model of spinal cord I/R injury. The results showed higher proportion positive cells exposed to I/R injury compared with the Sham group, indicating the importance of caspase-3 to activation of apoptotic process in spinal cord ischemia. Unsurprisingly, DADLE reduced the caspase-3 expression, suggesting that blocking caspase-3 activation may be a possible mechanism involved in DADLE neuroprotection.

Next, we further investigated the levels of p53 mRNA and protein to evaluate whether DADLE could alter the expression. Because p53 is an associated marker for apoptosis and the suppression of p53 was reported to protect neurons exposed to ischemia insults (Li et al., 1994). Consequently, we found that p53 expression increased in accordance with the result of caspase-3, meanwhile DADLE mitigated these changes, which suggested that DADLE limited neuron apoptosis possibly via inhibiting p53 signaling pathway.

The current study has several limitations. The specific site of action of DADLE remains unknown. However, the major goal of the current study was to investigate the effects of DADLE on oxidative stress and apoptosis. The future studies should include the investigation of whether DADLE provides neuroprotection in spinal cords at a delta-opioid receptor dependent-way or a delta-opioid receptor independent-way. Secondly, we did not assess the tolerance to DADLE upon long-term treatment in the spinal cord tissue. It is still an important area which we will investigate in the future studies.

In conclusion, our data demonstrated that DADLE can protect spinal cord neurons in the anterior horn exposed to I/R damage by decreasing MDA and NO levels and enhancing post-ischemic GSH-Px and SOD levels, and also by inhibiting apoptosis through a mechanism associated with caspase-3 and p53. These findings suggest that DADLE will be a promising therapeutic agent in the spinal cord I/R injury induced by aorta occlusion.

AUTHOR CONTRIBUTIONS

JY designed the study. DF and HL performed the experimental phase. DF and HL collected and analyzed data. DF drafted the manuscript. SL and LC contributed with reagents/materials/analysis tools. DF, HL, SL, LC, and JY have collaborated and approved the final manuscript version.

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Molecular Bases of Brain Preconditioning

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Preconditioning of the brain induces tolerance to the damaging effects of ischemia and prevents cell death in ischemic penumbra. The development of this phenomenon is mediated by mitochondrial adenosine triphosphate-sensitive potassium (K^+_{ATP}) channels and nitric oxide signaling (NO). The aim of this study was to investigate the dynamics of molecular changes in mitochondria after ischemic preconditioning (IP) and the effect of pharmacological preconditioning (PhP) with the K^+_{ATP}-channels opener diazoxide on NO levels after ischemic stroke in rats. Immunofluorescence-histochemistry and laser-confocal microscopy were applied to evaluate the cortical expression of electron transport chain enzymes, mitochondrial K^+_{ATP}-channels, neuronal and inducible NO-synthases, as well as the dynamics of nitrosylation and nitration of proteins in rats during the early and delayed phases of IP. NO cerebral content was studied with electron paramagnetic resonance (EPR) spectroscopy using spin trapping. We found that 24 h after IP in rats, there is a two-fold decrease in expression of mitochondrial K^+_{ATP}-channels ($p = 0.012$) in nervous tissue, a comparable increase in expression of cytochrome c oxidase ($p = 0.008$), and a decrease in intensity of protein S-nitrosylation and nitration ($p = 0.0004$ and $p = 0.001$, respectively). PhP led to a 56% reduction of free NO concentration 72 h after ischemic stroke simulation ($p = 0.002$). We attribute this result to the restructuring of tissue energy metabolism, namely the provision of increased catalytic sites to mitochondria and the increased elimination of NO, which prevents a decrease in cell sensitivity to oxygen during subsequent periods of severe ischemia.

Keywords: ischemic preconditioning, ATP-sensitive potassium channels, nitric oxide, mitochondria, neuroprotection

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INTRODUCTION

The resistance of the brain to the interruption of its blood supply and subsequent hypoxia can be increased by a pre-exposure to short episodes of ischemia/reperfusion or hypoxia (Bolanos and Almeida, 1999; Manukhina et al., 1999; Shmonin et al., 2012; Rybnikova and Samoilov, 2015), short periods of hypothermia (Maslov et al., 2012) and other moderate stress effects which are capable of activating endogenous protective mechanisms and increasing resistance to subsequent severe ischemia (Samoilenkova et al., 2008; Ding et al., 2012; Lim and Hausenloy, 2012). This

phenomenon is called "preconditioning." There is another phenomenon—post-conditioning, in which the protective impulse, e.g., brief interruptions of reperfusion, is applied after the onset of ischemia (Rybnikova and Samoilov, 2015; Wang et al., 2015).

Different cellular mechanisms are involved in realization of the protective effects of preconditioning and post-conditioning. Post-conditioning is mediated by functional responses of the Na^+/Ca^{2+} exchangers, the plasma membrane Ca^{2+}-ATPase, the Na^+/H^+ exchange, the $Na^+/K^+/2Cl^-$ co-transport and the acid-sensing cation channels, which main function is normalization of intracellular pH and calcium levels, and a number of signaling cascades (Cuomo et al., 2015).

The primary mechanism of the preconditioning-induced neuroprotection is K^+_{ATP}-channels opening (Samoilenkova et al., 2008). A decrease in ATP levels during ischemia promotes the activation of K^+_{ATP}-channels in the cell membrane, which restores the low concentrations of Na^+ and Ca^{2+} in the cytosol and restrains excessive depolarization. Kir6.2 is considered to be a predominant pore-forming subunit of neuronal plasmalemmal K^+_{ATP}-channel (Yamada et al., 2001). The opening of K^+_{ATP}-channels in the mitochondrial inner membrane is associated with the prevention of mitochondrial calcium overload (Murata et al., 2001) and the subsequent preservation of mitochondrial function (Mironova et al., 2007; Correia et al., 2010). Early immunohistochemical studies identified a mitochondrial subunit of this channel as Kir6.1 (Lacza et al., 2003b; Singh et al., 2003). However, further proteomic studies did not confirm these results (Brustovetsky et al., 2005), but identified the potential structural basis of mitochondrial K^+_{ATP}-channel as Kir1.1 (Foster et al., 2008, 2012; Foster and Coetzee, 2016), which has an N-terminal mitochondrial targeting signal and is encoded by *KCNJ1* (potassium voltage-gated channel, subfamily J, member 1) gene. In cardiomyocytes, Kir1.1 is localized only in the crista membranes, mostly in the center of mitochondria and the channel subunits are frequently grouped together (Talanov et al., 2016). Such clustering corresponds to the specific feature of a mitochondrial Kir allowing a 10-times higher sensitivity to ATP inhibition in mitochondria compared to liposomes and lipid bilayers (Mironova et al., 2004). Compared to liver or heart, brain mitochondria expresses six- to seven-times more K^+_{ATP}-channels, which opening is accompanied by a larger change in respiration (Bajgar et al., 2001). Although the expression of regulatory subunits SUR2A and SUR2B has been demonstrated in mitochondrial membranes (Zhou et al., 2007), Kir1.1 doesn't form a complex with SUR2B, but has the same sensitivity to glibenclamide as the native channel (Konstas et al., 2002). It was also hypothesized that mitochondrial K^+_{ATP}-channels may have a smaller molecular weight SUR variant (Lacza et al., 2003a).

Alternatively, it is suggested that a succinate dehydrogenase or resporatory complex II is the regulatory component of mitochondrial K^+_{ATP}-channels within a super-complex of proteins with an inverse relationship between complex II and mitochondrial K^+_{ATP}-channel activities (Wojtovich et al., 2013). Complex II has also been recognized as a modulator of a superoxide production by respiratory chain complexes I and III (Dröse, 2013). This fact is important for understanding of functional consequences of a reactive oxygen species (ROS) production, which might be cardioprotective when ROS formed in complex III, but deleterious in case of the ROS produced in complex I (Madungwe et al., 2016). Generation of ROS at low levels can mediate the protective effect of preconditioning (Kalogeris et al., 2014) apparently by the activation of K^+_{ATP}-channels. ROS oxidize thiol groups of mitochondrial protein kinase Cε (PKCε; Korichneva et al., 2002), which is co-localized with K^+_{ATP}-channels in the inner mitochondrial membrane (Jabůrek et al., 2006). Activated PKCε phosphorylates K^+_{ATP}-channels leading them to consistently open state (Garlid et al., 2013). An increase of PKCε interaction with cytochrome c oxidase subunit IV has also been observed under conditions of myocardial IP and is associated with enhanced respiratory complex IV activity (Guo et al., 2007). However, tissue oxygen consumption can be inhibited due to NO overproduction and binding to cytochrome c oxidase (Brown and Cooper, 1994). The properties of NO action depend on the intensity of its production and the physiological state of the surrounding tissue. NO overproduction in stroke causes damage to structural and regulatory components of cells (Bolanos and Almeida, 1999; Jung et al., 2006; Terpolilli et al., 2012). Moderate activation of NO during preconditioning may exert a neuroprotective effect (Schulz and Ferdinandy, 2013), activating antioxidant enzymes, triggering antiapoptotic mechanisms, and increasing cerebral blood flow (Jung et al., 2006; Terpolilli et al., 2012). The protective effect of moderate NO production may also be mediated by an interaction with K^+_{ATP}-channels opening (Sasaki et al., 2000). An inverse relation between these elements in the context of the neuroprotective effect of preconditioning has not been yet proven because of the absence of experimental approaches, precise methods of NO detection and verification of results obtained *in vivo*.

Different pre- and post-conditioning strategies has been tested in clinical settings since 1990s (Calabrese, 2016). However, the clinical studies have shown controversial results (Thuret et al., 2014). The protective effect of preconditioning is known to be lost in old age and in metabolic disorders (Rana et al., 2015; Calabrese, 2016), but it is possible to restore it by physical exercises and caloric restriction. There is a need to define the therapeutic targets for rapid pharmacological restoration of the responsiveness to preconditioning and it requires an understanding of key molecular events starting from the point of the protective changes induction, through the development of protective phenotype to the final realization of protective mechanisms under ischemia conditions. Thus, we conducted the present study to investigate the mitochondrial protein composition in brain cortex of rats after IP and to evaluate the changes of NO production in two models of ischemia—mild ischemia with reperfusion (IP) and the severe ischemia without reperfusion (ischemic stroke). Preliminary K^+_{ATP}-channel blockade and opening were used to study the relationship between K^+_{ATP}-channels and the NO system.

MATERIALS AND METHODS

Animals

Experiments were performed with 5 months old naïve male albino rats (Rattus norvegicus) weighing 300–500 g ($n = 102$) and kept under standard conditions (light regimen of 12/12 h, day/night). All manipulations were performed under general anesthesia with a long-acting aliphatic hypnotic drug chloral hydrate [ChlH, 400 mg/kg intraperitoneally (i/p)]. The study was conducted in accordance with and approved by the Bioethics Committee of Lomonosov Moscow State University and in accordance with the ARRIVE guidelines.

Study of the IP Molecular Basis

In the first experiment, we studied the effects of IP on the cortical expression of electron transport chain enzymes, mitochondrial K^+_{ATP}-channels, neuronal, and inducible NO-synthases, as well as the dynamics of nitrosylation and nitration of proteins during early and delayed phases of IP (**Figure 1A**). IP was performed by alternate ligation of the right and left common carotid arteries for 5 min followed by 5 min of reperfusion. Overall 6 cycles were repeated for each animal within 1 h. The early (3 h) and the delayed (24 h) phases of the protective IP effect were subsequently studied. An indirect immunohistochemical method was applied with secondary antibodies (**Table 1**) labeled with fluorochromes—fluorescein (FITC) and phycoerythrin (PE). Karnua solution was used as fixative. For the S-nitrosoCys study brain tissue samples were fixed in a solution of 4% paraformaldehyde and 1% of glutaraldehyde. Alcohol-chloroform processing and paraffin-embedding were carried out before slicing paraffin blocks into sections of 3 μm thickness. After deparaffinization, antigen retrieval was performed in microwave. Permeabilization was performed sequentially with 0.3, 0.2, and 0.1% solutions of TritonX-100 in sodium phosphate buffer (PBS-T). Slices were incubated with 5% goat serum in PBS-T 0.3% for 30 min at room temperature. Then slices were rinsed 3 times with PBS, permeabilization was repeated and they were incubated at 37°C for 1 h with a solution of primary antibodies in Antibody Diluent (ab64211—Abcam plc, Cambridge, UK). For double staining, repeated washing and incubation with the next primary antibody solution were performed. After washing of primary antibodies, slices were incubated at room temperature with secondary antibodies dissolved in deionized water. After secondary antibody washing, coverslips were mounted over the slides using UltraCruz Hard-set mounting medium (sc-359850—Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Scanning of slices was performed using a laser scanning confocal Carl Zeiss LSM780 microscope, with 5 images taken lengthwise the frontal section of the frontal lobe cortex for each slide and with one slide stained with primary antibodies per each animal. A fixed scanning mode was used for evaluation of all the slides in order to minimize the variability of fluorescence intensities (normalization was not possible due to unstable DAPI staining pattern): Plan-Apochromat 63x/1.40 Oil DIC M27 lens; 40 micron pinhole; fluorescent signal filtering in 499–560 nm range. The following laser intensity parameters were used for fluorescence excitation: 355 nm—5%; 561 nm—2%; 488 nm—10%. The expression of each marker was studied at the same day with the interchange of slides from different experimental groups to prevent the influence of natural fluorescence extinction. The subsequent quantitative analysis of results was done in Image-Pro v.4.5 program. Fluorescence intensity was calculated as the percentage of specific fluorescence points in the area of interest. The snapshots of pyramidal neurons were transferred into 16-bit images and processed through a macro that enables deduction of a non-specific background fluorescence from the analysis results, using the brain slices stained only with secondary antibodies as a control. This approach was chosen because of the background auto-fluorescence in paraffin blocks, the maximum values of which were observed in glutaraldehyde fixation. A qualitative evaluation of fluorescence intensity using the 5-point scale was also applied in order to confirm the quantitative analysis. Both methods showed comparable results, with a slightly lower significance of differences measured in the qualitative evaluation (data not shown). Co-localization of markers was defined in ImageJ program with subsequent analysis of 0.19–2.0 μm^2 areas.

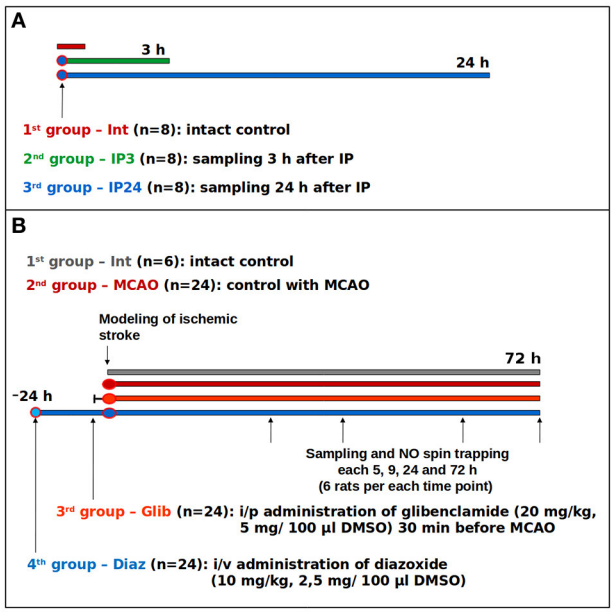

FIGURE 1 | Study protocols: **(A)** study on the effects of early and delayed IP phases on the expression of immunohistochemical markers; **(B)** study of glibenclamide and diazoxide effects on NO levels in the brain of the rats with ischemic stroke.

Study of the PhP Effect on Brain NO Content

In the second experiment, we examined the effects of preliminary K^+_{ATP}-channels blockade and opening on free cerebral NO following middle cerebral artery (MCA) occlusion (MCAO; **Figure 1B**). The objective was to evaluate an integrated contribution of K^+_{ATP}-channels in regulation of NO levels. PhP

TABLE 1 | Panel of antibodies.

Marker	Primary antibody	Secondary antibody
COX1	Mouse monoclonal [1D6E1A8] anti-MTCO1 antibody, mitochondrial marker (ab14705*), 1:200	Goat anti-mouse IgG_{2a}-FITC (sc-2079**), 1:100
SDHA	Rabbit polyclonal anti-SDHA antibody (sc-98253), 1:50	Goat anti-rabbit IgG-PE (sc-3739), 1:100
KCNJ1	Rabbit polyclonal anti-KCNJ1 antibody, cytoplasmic domain (ab80967), 1:20	Goat anti-rabbit IgG-PE (sc-3739), 1:100
nNOS	Rabbit polyclonal anti-nNOS antibody (ab106417), 1:200	Goat anti-rabbit IgG-PE (sc-3739), 1:100
iNOS	Mouse monoclonal [C-11] anti-NOS2 antibody (sc-7271), 1:50	Goat anti-mouse IgG_1-FITC (sc-2078), 1:100
S-nitrosoCys	Mouse monoclonal [HY8E12] anti-S-nitrosocysteine (conjugated) antibody (ab94930), 1:1000	Goat anti-mouse IgG_1-FITC (sc-2078), 1:100
3-nitroTyr	Mouse monoclonal [39B6] anti-3-Nitrotyrosine antibody (ab61392), 1:200	Goat anti-mouse IgG_{2a}-FITC (sc-2079), 1:100

*≪ab≫ *product IDs refer to Abcam plc, Cambridge, UK.*
**≪SC-≫ *product IDs refer to Santa Cruz Biotechnology, Santa Cruz, CA, USA.*

was performed by injecting a solution of a non-selective opener of K^+_{ATP}-channels, diazoxide (Sigma-Aldrich Rus LLC, Moscow, Russia), at a dose of 10 mg/kg [2.5 mg/100 mcl of dimethyl sulfoxide (DMSO)] in the sublingual vein (i/v) 24 h prior to MCAO. For the purposes of non-selective blockade of K^+_{ATP}-channels, i/p administration of glibenclamide (Sigma-Aldrich Rus LLC, Moscow, Russia, 20 mg/kg, 5 mg/100 mcl DMSO) was applied 30 min before MCAO. The selection of dosage and injection time was based on literature data (Marshall et al., 1993; Liu et al., 2002; Shimizu et al., 2002).

Ischemic infarction in the fronto-parietal region of cerebral cortex was induced by electrocoagulation of the distal branch of left MCA and the adjacent vein, with simultaneous ligation of the ipsilateral carotid artery. This model of ischemic stroke was chosen because of its good reproducibility in experiments which demonstrated a 30–45% decrease of necrotic area after IP or PhP (Samoilenkova et al., 2007; Deryagin et al., 2016). The MCA coagulation altitude was chosen so that necrosis developed mainly in the frontoparietal cortex, without affecting subcortical structures, thereby avoiding complications of visceral functions and animal death. Simultaneous coagulation of the adjacent vein and ligation of the ipsilateral carotid artery stabilizes the size of the ischemic area, which also reduces the number of animals required for the experiment.

EPR spectroscopy with spin trapping (Mikoyan et al., 1997; Vanin et al., 2003) was used to measure NO cerebral content. For this purpose, animals received components of a trap 30 min before sampling: 500 mg/kg of sodium diethyldithiocarbamate (DETC) (Sigma-Aldrich Rus LLC, Moscow, Russia) in the amount of 8.4 ml/kg i/p, and a mixture of solutions of 37.5 mg/kg $FeSO_4$, 187.5 mg/kg of sodium citrate in a total volume of 6.6 ml/kg subcutaneously. As, a result of their interaction within the body, $DETC_2$-Fe^{2+} water-insoluble complex is formed, capable of capturing NO with the formation of $(DETC)_2$-Fe^{2+}-NO stable radical, which is detected by EPR [Bruker ER 200E SRC in X-band (9.50 GHz) spectrometer at 77 oC]. The method was described by us earlier (Gainutdinov et al., 2011, 2013). The trapping of NO was introduced 30 min before decapitation. For each time point (0, 5, 9, 24, and 72 h) in each group, six independent measurements were done. These time points were chosen, taking into account the activity of constitutive and inducible NO-synthases in the development of ischemic stroke: 5 and 9 h cover the period of constitutive NO-synthase activity, while the 24- and 72-h points make it possible to estimate the activity of inducible NO-synthase (Kitamura et al., 1998). The brain fragments were immediately frozen in separate plastic containers, overall 4 probes from each animal: the ischemic region of cortex in the left cerebral hemisphere; the remainder cortex of the left cerebral hemisphere; cortex of the right cerebral hemisphere; and cerebellum. The samples were weighted before the experiments and their average mass was 100 mg. Amplitude of EPR spectra was normalized on the mass of sample and on the EPR signal amplitude of standard sample. As a standard sample, we use the TEMPO radical solution (8.89 mM/l, $58*10^{17}$ spins/cm^3, $S = 1/2$, $g \sim 2.0023$; Barr et al., 2000).

Statistical Analysis

Statistical processing was performed using Excel 2010 and SPSS 17.0 software packages for Windows. Data distribution was evaluated with Shapiro–Wilk test and it didn't meet the assumption of normality. Thus, non-parametric statistical methods were applied. When comparing more than two independent samples, Kruskal–Wallis test (H-criterion) was used. When comparing two independent samples, Mann–Whitney test (U-criterion) was used. Data of graphs is presented as Median (within IQR for the box plot graph) with Min and Max whiskers. Differences were considered significant with the permissible error probability (two-tailed p-value) of <0.05.

RESULTS

Effect of Delayed Phase of IP on Expression of COX1, SDHA, KCNJ1, nNOS, and iNOS

Moderately expressed cytoplasmic and predominantly perinuclear cytochrome c oxidase subunit 1 (COX1) expression of granular pattern was observed in cerebral cortical regions of intact animals (**Figures 2a,b**). A similar kind of a weak granular COX1 staining was detected in the neuropil. Twenty-four hours after performing IP, the median intensity of fluorescence was 70% higher than in the control group (Mann–Whitney test, $p = 0.008$, **Figure 3A**). However, a staining of succinate dehydrogenase flavoprotein subunit (SDHA) revealed no changes in intensity of SDHA expression in all the animal groups ($p > 0.05$, data not shown). An expression of mitochondrial K^+_{ATP}-channels (KCNJ1) was moderate and varied between animals (**Figures 2d,e**). KCNJ1

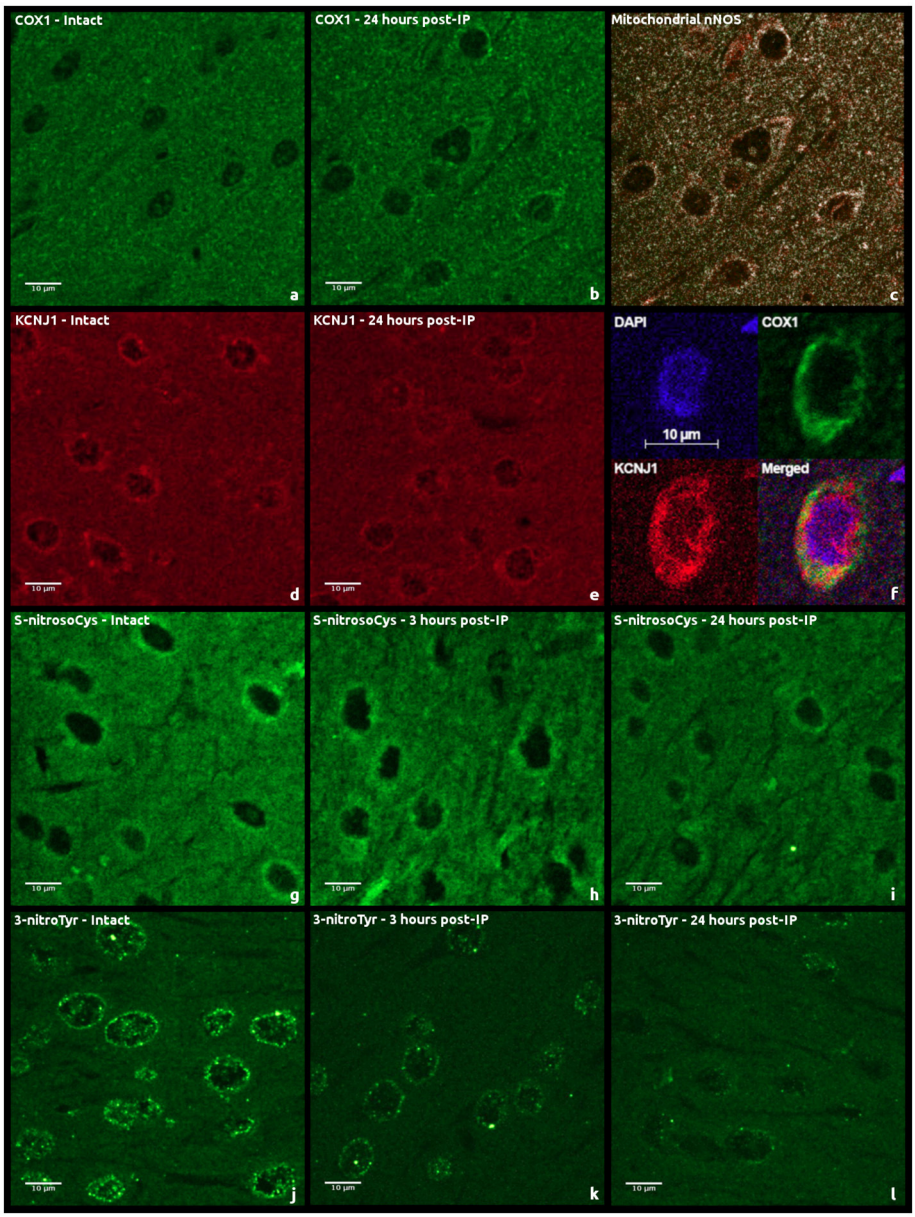

FIGURE 2 | Examples of immunohistochemical staining of rat cerebral cortex sections using second antibodies labeled with fluorochromes: **(a,b)** anti-COX1 antibody staining; **(c)** COX1 and nNOS co-localization; **(d,e)** anti-KCNJ1 antibody staining; **(f)** KCNJ1 and COX1 co-localization; **(g–i)** anti-S-nitrosylation (S-nitrosoCys) antibody staining; **(j–l)**, anti-tyrosine nitrosylation (3-nitroTyr) antibody staining.

staining was co-localized with COX1 (**Figure 2f**). IP led to a decrease in median KCNJ1 fluorescence intensity by 44% in cerebral cortex in its delayed phase, as compared to the intact group (Mann–Whitney test, $p = 0.012$, **Figure 3B**).

Expression of neuronal NO-synthase (nNOS) had cytoplasmic staining pattern, mostly fine-grained. The intensity of fluorescence varied from moderate to strong, more intense in the cells located closer to arterioles. Expression of nNOS was comparable in intact and IP24 groups (Mann–Whitney test, $p = 0.376$). COX1/nNOS co-localization analysis made it possible to evaluate the expression level of mitochondrial nNOS fraction (**Figure 2c**). There was no statistically significant difference found between intact rats and IP24 group (Mann–Whitney test, $p = 0.261$). An evaluation of the area and the perimeter of double stained zones in mitochondria revealed no differences between the two groups. Both in intact rats and after IP, only minimal expression of inducible NO-synthase (iNOS) was detected in cerebral cortex, which does not allow estimating the staining results reliably.

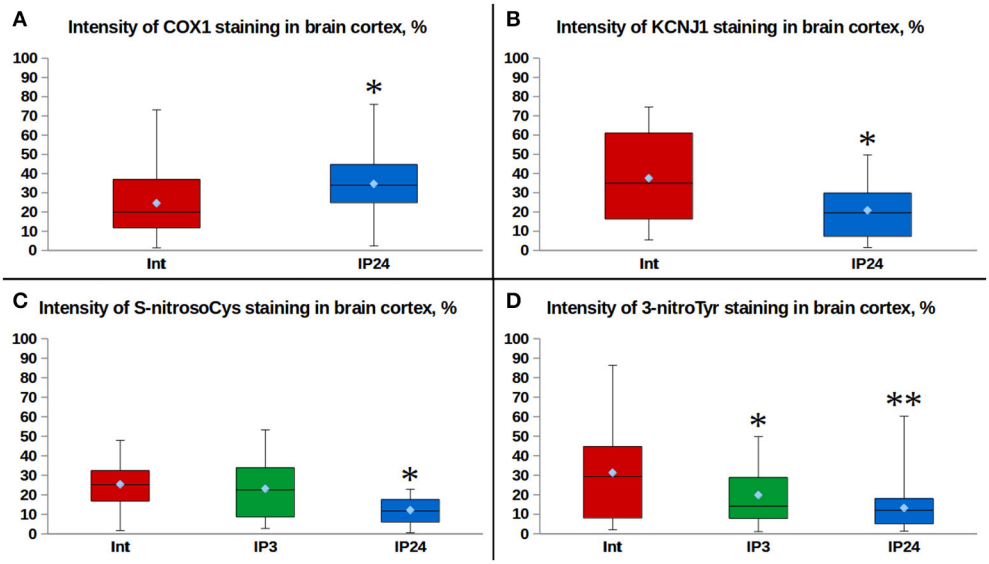

FIGURE 3 | Comparison of fluorescence intensities: **(A)** effects of delayed IP phase on the expression of COX1 in rat cerebral cortex cells. *$p = 0.008$, Mann–Whitney test (Int. vs. IP24); **(B)** effects of delayed IP phase on the expression of KCNJ1 in rat cerebral cortex cells. *$p = 0.012$, Mann–Whitney test (Int. vs. IP24); **(C)** effects of delayed IP phase on cysteine S-nitrosylation levels in rat cerebral cortex cells. *$p = 0.0004$, Mann–Whitney test (Int. vs. IP24); $p = 0.001$, Mann–Whitney test (IP3. vs. IP24); **(D)** effects of delayed IP phase on tyrosine nitration levels in rat cerebral cortex cells. *$p = 0.053$, Mann–Whitney test (Int. vs. IP3); **$p = 0.001$, Mann–Whitney test (Int. vs. IP24); $p = 0.019$, Mann–Whitney test (IP3. vs. IP24).

Effect of Early and Delayed Phases of IP on Processes of Nitrosylation and Nitration

A variable pattern of cysteine S-nitrosylation marker (S-nitrosoCys) fluorescence was observed in the cerebral cortex specimens. Its cytoplasmic staining was mostly diffuse, with the greatest intensity in intact animals and in rats 3 h after IP (**Figures 2g–i**). The quantitative analysis of data revealed a 2-fold (**Figure 3C**) statistically significant decrease in the median S-nitrosoCys fluorescence intensity in delayed phase of IP, in comparison with both the intact and the IP3 groups (Mann–Whitney test, $p = 0.0004$ and $p = 0.001$, respectively). When specimens were stained with tyrosine nitration marker (3-nitroTyr), a weak diffuse perinuclear fluorescence was observed, as well as the pronounced dot-like staining in both the perinuclear zone and in the nucleus (**Figures 2j–l**). The median intensity was higher in intact animals, 2- and 2.5-fold more prominent than at 3 and 24 h after IP, respectively (Mann–Whitney test, $p = 0.053$ and $p = 0.001$, **Figure 3D**).

Impact of K^+_{ATP}-Channels on NO Cerebral Content in Rats with Ischemic Stroke

The cortical levels of $(DETC)_2$-Fe^{2+}-NO complex in control groups of rats with MCAO were two times less than in intact animals at all the time points (Kruskal–Wallis test, $p < 0.0001$, **Figure 4**). The median level of NO in the cerebral cortex of intact animals amounted to 1.16 nM/g (IQR = 0.95–1.52). Five hours after MCAO, the minimal median NO concentration of 0.23 nM/g (IQR = 0.17–0.27) was observed in the core of ischemia increasing with distance from it in penumbra (0.42 nM/g, IQR= 0.20–0.71) to 0.70 nM/g (IQR = 0.64–0.74) in the contralateral hemisphere and to 0.81 nM/g (IQR = 0.71–0.91) in the cerebellum. The median NO concentrations at 9, 24, and 72 h after the MCAO were 0.58 nM/g (IQR = 0.47–0.70), 0.58 nM/g (IQR = 0.45–0.88), and 0.73 nM/g (IQR = 0.52–0.95), respectively.

In the group of animals treated with glibenclamide, a 64% (1.19 nM/g, IQR = 0.84–1.41) increase in NO levels vs. the control was observed on the third day post-operation (Mann–Whitney test, $p = 0.0005$). Diazoxide administration 1 day before MCAO resulted in a decrease of NO levels at all time points by 21–56%, while statistically significant difference from the control group was observed for samples taken 72 h after MCAO [0.32 nM/g (IQR = 0.25–0.58); Mann–Whitney test, $p = 0.002$].

DISCUSSION

In preconditioning, there are two phases in the development of the protective effect: the acute phase and the delayed phase, which may differ by the molecular basis of this effect (Nandagopal et al., 2001). Contradictory opinions exist regarding which of the phases plays the major role in the development of cerebral "ischemic tolerance." Some authors attribute the predominant role to the delayed phase, unlike the heart preconditioning, where defense mechanisms are triggered rather rapidly (Barone et al., 1998). Other researchers observed the emergence of cerebral tolerance to subsequent global ischemia as early as in half an hour after preconditioning (Perez-Pinzon et al., 1997). However, the protective effect of the acute preconditioning phase is believed to be short termed and only capable of delaying cell death (Kirino, 2002).

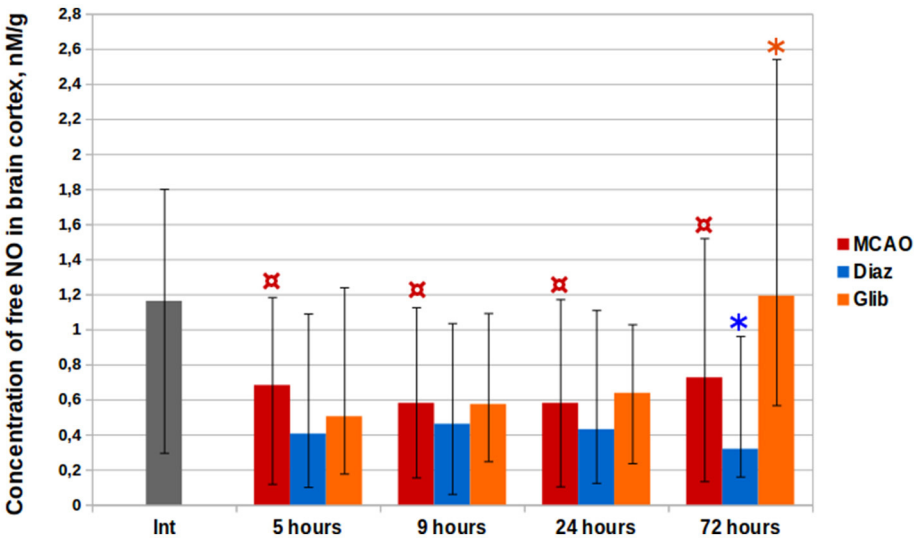

FIGURE 4 | Complex $(DETC)_2$-Fe^{2+}-NO time history in cerebral cortical structures in the rats with ischemic stroke. $^{*}p < 0.05$, Mann-Whitney test (MCAO vs. Glib, 72 h; MCAO vs. Diaz, 72 h); $^{¤}p < 0.0001$, Kruskal–Wallis test (MCAO, int. vs. 5, 9, 24, and 72 h).

We found that the density of mitochondrial K^+_{ATP}-channel subunit Kir1.1 (KCNJ1 staining) is reduced considerably after ischemic brain preconditioning (**Figure 3B**). This data explains why there is no reduction in the inner mitochondrial membrane potential in preconditioned cells in ischemia (Kim et al., 2006; Katakam et al., 2007). Another recent study reports that a 2-fold decrease in SUR2 regulatory subunit mRNA expression is observed 48 h after remote ischemic brain post-conditioning (Ezzati et al., 2016). Interestingly, an isoflurane anesthesia was applied for surgical procedures, which itself results in K^+_{ATP}-channels opening (Jiang et al., 2007; Swyers et al., 2014) and therefore might be a trigger of the subsequent SUR2 downregulation. Thus, a decrease in mitochondrial K^+_{ATP}-channels expression is observed after the mild ischemia/reperfusion or after pharmacological opening of these channels. Can it be the same mechanism that both downregulates mitochondrial K^+_{ATP}-channels expression after preconditioning and causes K^+_{ATP}-channels dysfunction with cardioprotective deficit in diabetic myocardium (Hassouna et al., 2006)? In opposite to mitochondrial K^+_{ATP}-channels, it was recently found that after hypoxic preconditioning in hippocampal neurons there is an increase in activity and expression of the plasmalemmal Kir6.2 subunit (Sun et al., 2015), which prevents membrane excitability and seems to be differentially regulated by the preconditioning impulse. Under severe ischemia myocardial Kir6.2 channels are removed by endocytosis due to phosphorylation by calcium/calmodulin-dependent protein kinase II (CaMKII; Gao et al., 2016). However, the way of how the mitochondrial K^+_{ATP}-channel becomes inactive in response to damaging factors remains to be evaluated.

The increase in cytochrome c oxidase density (COX1 staining) was detected in the delayed phase of IP (**Figure 3A**). Cytochrome c oxidase is known to be a negative regulator of free NO concentration (Torres et al., 2000; Antunes et al., 2007). The neuroprotective effect of the delayed preconditioning phase may persist for days or even weeks (Perez-Pinzon et al., 1997; Nandagopal et al., 2001), and this is consistent with the extraordinary stability of this enzyme (Saikumar and Kurup, 1985). This upregulation is consistent with previously published proteomic data (Cabrera et al., 2012), northern blot and reverse transcription–polymerase chain reaction results (McLeod et al., 2004) that demonstrated increased cytochrome c oxidase and succinate dehydrogenase mRNA expression in the context of delayed ischemic preconditioning. We found no difference in SDHA protein expression between the preconditioned animals and the control group.

Neither the difference in mitochondrial and overall nNOS fluorescence intensity between the groups nor positive staining of iNOS were observed in the preconditioned brain tissue. These results indicate that enzymatic NO synthesis in the delayed IP phase is not regulated at the level of gene expression. In IP models NO synthesis can be suppressed by CaMKII-dependent negative regulatory phosphorylation of nNOS (Wang et al., 2010, 2016). However, K^+_{ATP}-channels openers diazoxide and BMS-191095 may initiate NO production by increasing nNOS and eNOS activity due to the activation of positive regulatory phosphorylation through the phosphoinositide-3-kinase/AKT serine/threonine kinase 1 (PI3K-Akt) pathway (Katakam et al., 2013, 2016). Mitochondrial NO-synthase is unlikely to be involved in the preconditioning-induced signaling production of NO as K^+_{ATP}-channels opening decreases the mitochondrial capacity for Ca^{2+} ions (Ishida et al., 2004) and it is a Ca^{2+}-dependent enzyme (Elfering et al., 2002; Dedkova and Blatter, 2009).

Although an insignificant increase in protein S-nitrosylation (Shen and English, 2005) was noticed at 3 h time point, there was the decrease in S-nitrosoCys fluorescence intensity 24-h post-IP (**Figure 3C**). This data suggests that the protective effect of the delayed phase of IP do no depend anymore on the protein nitrosylation, which in the acute phase of IP leads to

preservation of mitochondrial energetics, reduction of cytosolic Ca^{2+} (Sun et al., 2007) and inhibition of the harmful ROS production (Chouchani et al., 2013; de Lima Portella et al., 2015). As early as 3 h post-IP, we observed a decrease in levels of 3-nitroTyr (**Figure 3D**)—the marker of NO-dependent oxidative stress (Mohiuddin et al., 2006). The above indirectly points to the activation of the antioxidant systems as a result of tissue conditioning and is confirmed by the studies of neuronal survival, in which the pretreatment with diazoxide prevented cell death via antioxidative pathway activation (Virgili et al., 2013; Shukry et al., 2015). Indeed, diazoxide-induced mitochondrial membrane depolarization (Xi et al., 2005; Vadziuk et al., 2010) can lead to uncoupling of mitochondrial respiration and phosphorylation of adenosine diphosphate molecules (Holmuhamedov et al., 2004) and to the moderate production of ROS (Andrukhiv et al., 2006; Katakam et al., 2016), which may stimulate the antioxidant defense. It is worth to mention that the accumulation of 3-nitroTyr marker was mainly tied to nuclear proteins (**Figure 2j**). Considering the focus of recent publications toward epigenetic regulation of ischemic tolerance (Aune et al., 2015) and the facts that NO activity is involved in inhibition of histone deacethylases (Illi et al., 2009) and activation of DNA damage repair (Bartz et al., 2015), we hypothesize that delayed neuroprotection caused by IP can be triggered by such ROS as peroxynitrite and nitric oxide (IV), which seems to have important roles in epigenetic regulation of gene expression.

EPR study detected a 2-fold decrease of free NO level (**Figure 4**) in cerebral cortex post-MCAO, which can be attributed to cerebral hypoperfusion. According to literature reports (Tominaga et al., 1994; Chen et al., 2002), a significant increase in the cerebral EPR signal is observed in MCAO models with spin trap 15 min after vessel occlusion. However, this effect is reversible: NO levels decrease gradually and reach the background values over time (Yuan et al., 2010). Under the IP impulse the production of NO in low-oxygen conditions can be mediated by non-enzymatic nitrite (NO_2^-) reduction. K^+_{ATP}-channels opening and an increase in K^+ flow is followed by intense accumulation of osmotically obligate water which results in mitochondrial swelling (Lim et al., 2002). In turn, an increase in matrix volume activates fatty acid β-oxidation (Halestrap, 1987), nicotinamide adenine dinucleotide (NADH) being one of the products of the process (Houten and Wanders, 2010). NADH is an electron donor for the electron transfer network and for molybdenum-containing xanthine oxidoreductase metalloenzyme capable of disoxidating NO_2^- to NO (Li et al., 2001). Apart from xanthine oxidoreductase, there are several other metalloproteins demonstrating NO_2^- reductase properties: mitochondrial aldehyde dehydrogenase (Golwala et al., 2009; Perlman et al., 2009), cytochrome c (Basu et al., 2008), neuroglobin (Tiso et al., 2011; Tejero et al., 2015), and cytoglobin (Li et al., 2012). It is possible that their combined effect leads to the appearance of signaling NO production.

We demonstrated that PhP with K^+_{ATP}-channels opener diazoxide causes a 2-fold reduction of free NO concentration 72 h post-MCAO. This relationship can be mediated by the enhancement of cytochrome c oxidase function after the preconditioning. Although it is reported that ischemia-associated signaling production of NO (Tominaga et al., 1994; Chen et al., 2002) results in inhibition of oxygen consumption by cytochrome c oxidase (Brunori et al., 2004; Palacios-Callender et al., 2007; Sarti et al., 2012), there are two possible ways of NO interaction. NO binds to metal ions in the active center of cytochrome c oxidase to produce either nitrosyl- or nitrite derivatives (Gibson and Greenwood, 1963; Brudvig et al., 1980). These reactions dominate over each other depending on the oxygen concentration and the electron flow in the respiratory chain (Sarti et al., 2000). In the first period after the preconditioning impulse a proportion of cytochrome c oxidase subunits is likely be found in a reduced state. Association with a reduced form of the enzyme under ischemia conditions is rapid and followed by a longterm inhibition of cell respiration until the dissociation of NO, while its interaction with an oxidized form of the enzyme initiates 1,000 times slower, has 10–20 times shorter duration and results in a production of NO_2^- (Giuffrè et al., 2000). In the delayed preconditioning phase, against the background of the increase in cytochrome c oxidase density, most of the enzyme molecules will be oxidized which creates favorable conditions for the second scenario of NO oxidation to NO_2^-, therefore reducing NO bioavailability and diminishing its damaging effect.

There was a sharp increase in cerebral tissue NO on the third day after stroke in the group of animals treated with glibenclamide immediately prior to MCAO (**Figure 4**). We associate the obtained result with the activation of iNOS, the level of which increases in the tissues in response to the inflammation. According to literature, the expression of microglial cell membrane K^+_{ATP}-channels is increased in response to pro-inflammatory signals, and exposure to glibenclamide enhances the extent of microglial cell activation considerably (Ortega et al., 2012) which results in increased cytokine, NO, and ROS production (Wang et al., 2004).

In conclusion, the opening of K^+_{ATP}-channels, which initiates a change in the mitochondrial matrix ionic composition and triggers the protective mechanisms, plays a key role in the development of preconditioning phenomenon. Mild ischemia/reperfusion leads to the reduction in density of mitochondrial K^+_{ATP}-channels that prevents a drop in the inner mitochondrial membrane potential in post-ischemic preconditioning period. The amount of protein nitrosylation and nitration is also lower in preconditioned tissue. We observed the decrease in NO cerebral content in the MCAO model after PhP with K^+_{ATP}-channels opener diazoxide and attribute it to the enhanced ability of the overexpressed cytochrome c oxidase to consume free NO, thereby preventing the reduction of mitochondrial sensitivity to oxygen in the period of severe ischemia. In addition to their involvement in the preconditioning process, plasmalemmal K^+_{ATP}-channels appear to play a special role in the regulation of the intensity of NO production and microglial inflammation in ischemic cerebral tissue.

Further research is required to study the feedback mechanisms of the changes in expression of key mitochondrial energy metabolism molecules in response to signaling production of ROS and NO—the unconditional mediators of preconditioning phenomenon.

AUTHOR CONTRIBUTIONS

OD, SG, and AG performed the experimental manipulations with the laboratory animals. OD and SB contributed to the acquisition and analysis of IHC data. KG, VA, and GY contributed to the acquisition and analysis of EPR data. SG and OD designed the experiments, interpreted the data and wrote the manuscript. VK, SB, and KG provided supervision, critical revision of the article, and final approval.

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