BRAIN HYPOXIA AND ISCHEMIA: NEW INSIGHTS INTO NEURODEGENERATION AND NEUROPROTECTION

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BRAIN HYPOXIA AND ISCHEMIA: NEW INSIGHTS INTO NEURODEGENERATION AND NEUROPROTECTION

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Image: Natalia Nalivaeva

Impaired oxygen supply (hypoxia) or reduced blood flow (ischemia) to the brain causes significant metabolic changes in neuronal and non-neural cells. It first leads to a rapid change in membrane lipid composition and enzyme activities and then to long-term changes in gene expression and levels of protein synthesis. They are often considered as major factors leading to cognitive impairment, seizures, and other neurological disabilities. The data accumulated to date suggest that vascular factors

and reduced levels of oxygen supply to the brain are linked with the pathogenesis of various neurodegenerative disorders, in particular of Alzheimer's disease (AD), and can affect their progression.

Importantly, the central nervous system can withstand cerebral hypoxia or ischemia for a limited amount of time, a phenomenon called primary hypoxic–ischemic tolerance. With an appropriate time interval and dosage, when a non-injurious hypoxic exposure (known as preconditioning) is performed, tolerance can be increased and cells protected against lethal hypoxia exposures. Furthermore, the hypoxic preconditioning-induced neuronal tolerance appears to be universal and represents increased resistance not only to hypoxic/ischemic insults but also to other injurious factors including various types of stress.

Chapters in this eBook discuss recent developments and progress in our understanding of the effects of hypoxia and ischemia on the brain at the molecular, morphological and physiological levels. It also focuses on therapeutic avenues that are currently being developed to protect the brain and reduce pathology under various types of external and internal hypoxic challenge.

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EDITORIAL
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Editorial: Brain Hypoxia and Ischemia: New Insights Into Neurodegeneration and Neuroprotection

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Keywords: Alzheimer's disease, ischemic tolerance, hypoxic preconditioning, mitochondria, fatty acids, miRNAs, nanoparticles, prenatal hypoxia

Editorial on the Research Topic

Brain Hypoxia and Ischemia: New Insights Into Neurodegeneration and Neuroprotection

Impaired oxygen supply (hypoxia) or reduced blood flow (ischemia) to the brain causes significant metabolic changes in neuronal and non-neural cells. It first leads to a rapid change in membrane lipid composition and enzyme activities and then to long-term changes in gene expression and levels of protein synthesis. They are often considered as major factors leading to cognitive impairment, seizures, and other neurological disabilities. The data accumulated to date suggest that vascular factors and reduced levels of oxygen supply to the brain are linked with the pathogenesis of various neurodegenerative disorders, in particular of Alzheimer's disease (AD), and can affect their progression.

Importantly, the central nervous system can withstand cerebral hypoxia or ischemia for a limited amount of time, a phenomenon called primary hypoxic–ischemic tolerance. With an appropriate time interval and dosage, when a non-injurious hypoxic exposure (known as preconditioning) is performed, tolerance can be increased and cells protected against lethal hypoxia exposures. Furthermore, the hypoxic preconditioning-induced neuronal tolerance appears to be universal and represents increased resistance not only to hypoxic/ischemic insults but also to other injurious factors including various types of stress.

The Research Topic "Brain hypoxia and ischemia: new insights into neurodegeneration and neuroprotection" evaluates recent progress in our understanding of the effects of hypoxia and ischemia on the brain at the molecular, morphological, and physiological levels. It also focuses on therapeutic avenues that are currently being developed to protect the brain and reduce pathology under various types of external and internal hypoxic challenge.

Taking into account the Developmental Origins of Health and Disease hypothesis (DOHaD) originally outlined for fetal programming of coronary heart diseases and later extended to mental health disorders, the review article by Nalivaeva et al. presents a detailed analysis of the effects of prenatal hypoxia during pregnancy on fetal brain development and increased risk of neurodegeneration in later life. It specifically outlines the importance of epigenetic reprogramming during embryonic development in response to various adverse environmental factors, which makes the organism vulnerable for development of neurological disorders, in particular AD. The authors evaluate various animal models for studying the consequences of prenatal hypoxia on brain physiology and biochemistry. A related research paper by Vasilev et al. gives an experimental example of how maternal hypoxia

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in certain periods of rat embryogenesis affects neuronal cell migration and formation of the brain structures involved in development of motor reactions and memory in the offspring in later life resulting in early cognitive deficit. In relation to AD pathogenesis, Kerridge et al. present cellular data on the effects of hypoxia on expression of the major amyloid-degrading enzyme neprilysin whose role in AD pathogenesis has recently been shown both in human and animal studies. These data explain how prenatal hypoxia can lead to reduced levels of the amyloid-precursor protein C-terminal fragment AICD which was shown to regulate neprilysin expression predisposing to AD. Furthermore, systemic hypoxia caused by carbon monoxide poisoning was also shown to promote Parkinson's disease as outlined in the article by Chang et al.

Discussing the response of organisms to reduced oxygen levels, Lukyanova and Kirova give a detailed analysis of the changes in energy metabolism in cells, especially in cortical neurons, which are the most vulnerable cells to reduced oxygen supply. They describe the role of mitochondria in development of immediate and delayed molecular mechanisms for adaptation to hypoxic challenge by reprogramming the respiratory chain function and switching from oxidation of NAD-related substrates (complex I) to succinate oxidation (complex II). They link succinate-related energy synthesis with stabilization of HIF-1α and initiation of its transcriptional activity which underlie long-term adaptation reactions.

In this regard the data presented in the research article by Brose et al. also reveal a novel neuronal specific pathway for adaptation to hypoxia through increased fatty acid biosynthesis. They have suggested that activation of fatty acid synthesis maintains reduction potential and reduces lactoacidosis in neuronal cells under hypoxia. Their work clearly demonstrates that fatty acids may serve as hydrogen acceptors under hypoxia supporting oxidation reactions including anaerobic glycolysis.

Biochemical changes which accompany brain ischemia often lead to accumulation of a toxic intermediate of methionine metabolism, homocysteine. Its detrimental effect on neuronal cells is underlined by accumulation of reactive oxygen species (ROS) and post-translational modifications of proteins via homocysteinylation and thiolation. In their review paper, Lehotský et al. summarize the effect of ischemia on intracellular signaling, especially in the mitogen-activated protein kinase (MAPK) protein pathways following ischemic injury. They provide evidence for the interplay and tight integration between ERK and p38 MAPK signaling mechanisms in response to homocysteine and also in association with ischemia and ischemic preconditioning challenge in the rat brain.

Summarizing the results of studying the molecular mechanisms and physiological responses of the organism to repeated mild hypoxia, Rybnikova and Samoilov discuss the effectiveness of specially designed hypoxic pre- and post-conditioning treatments. They demonstrate beneficial effects of these treatments not only on the outcome of the episodes of severe hypoxia but also on the reactions of individuals to various factors of a psycho-emotional nature. The mechanisms of hypoxic preconditioning which mobilize the defense processes and lead to development of brain tolerance are multileveled. They involve not only activation of intracellular cascades and changes in expression of multiple regulatory proteins in susceptible brain areas but also to modifications of the hypothalamic-pituitary-adrenal endocrine axis regulating various functions in the organism. A special role in these processes belongs to the epigenetic regulation of gene expression. In particular, changes in histone acetylation lead to chromatin remodeling which ensure access of pro-adaptive transcription factors activated by preconditioning to the promoters of target genes.

Another approach to increase brain tolerance as suggested by Horowitz et al. is activation of neuroprotective mechanisms following prolonged exposure to high ambient temperatures (heat acclimation). It alters molecular programs underlying cross-tolerance and enhances "on-demand" protective pathways evolved during acclimation. The protection achieved is long lasting and limits the need for de novo recruitment of cytoprotective pathways upon exposure to novel stressors. In particular using mouse and rat acclimated phenotypes, it was shown that the impact of heat acclimation is beneficial after traumatic brain injury as well as in global hypoxia models.

The emerging role of small non-coding RNA molecules (miRNAs) in regulation of brain and cellular functions also suggests their involvement in the response of organisms to hypoxia. Their neuroprotective potential under hypoxic conditions as discussed by Minhas et al. involves regulation of genes in oxygen and glucose deprived brain areas and is associated with the circadian rhythms. The authors suggest that alternate breathing or yogic intervention techniques can be considered as important non-invasive measures to protect the brain against hypoxia-associated pathology and discuss its efficacy for treatment of such neurodegenerative disease as AD.

Currently there are various pharmacological compounds which are neuroprotective against the damaging action of hypoxia and ischemia and they are widely discussed in various sections of the articles of this Research Topic. Special attention has been paid to the mechanisms of the protective action of an alkaloid sinomenine from a Chinese medicinal herb *Sinomeniumacutum* in a traumatic brain injury model. As demonstrated by Yang et al. this compound exerts its effect by activating the Nrf2–antioxidant response element pathway. Another neuroprotective approach against hypoxia-ischemic insult in newborns by cannabidiol treatment in association with hypothermia has been evaluated by Lafuente et al. They demonstrated the additive effects of both therapeutic factors resulting in reduced excitotoxicity, inflammation, oxidative stress, and overall cell damage in the brain if applied shortly after the insult.

Addressing an important problem of the delivery of neuroprotective or diagnostic compounds to the brain, Panagiotou and Saha discuss application of synthetic nanoparticles, which can cross the blood-brain barrier without compromising its integrity. They describe them as efficient carriers of therapeutic compounds designed for treatment of ischemic stroke.

This Research Topic clearly demonstrates the progress in our understanding of the molecular mechanisms underlying hypoxic-ischemic cell damage and the mechanisms of brain tolerance. However, it also makes clear that further studies of the complex interactions between multiple molecular pathways involved in epigenetic reprogramming of gene expression, energy metabolism, cell signaling, lipid- and protein homeostasis are needed to develop effective neuroprotective therapeutic measures against brain pathologies caused by insufficient oxygen supply to the brain.

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NN and ER reviewed the submissions and wrote the editorial.

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REVIEW
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Role of Prenatal Hypoxia in Brain Development, Cognitive Functions, and Neurodegeneration

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This review focuses on the role of prenatal hypoxia in the development of brain functions in the postnatal period and subsequent increased risk of neurodegenerative disorders in later life. Accumulating evidence suggests that prenatal hypoxia in critical periods of brain formation results in significant changes in development of cognitive functions at various stages of postnatal life which correlate with morphological changes in brain structures involved in learning and memory. Prenatal hypoxia also leads to a decrease in brain adaptive potential and plasticity due to the disturbance in the process of formation of new contacts between cells and propagation of neuronal stimuli, especially in the cortex and hippocampus. On the other hand, prenatal hypoxia has a significant impact on expression and processing of a variety of genes involved in normal brain function and their epigenetic regulation. This results in changes in the patterns of mRNA and protein expression and their post-translational modifications, including protein misfolding and clearance. Among proteins affected by prenatal hypoxia are a key enzyme of the cholinergic system-acetylcholinesterase, and the amyloid precursor protein (APP), both of which have important roles in brain function. Disruption of their expression and metabolism caused by prenatal hypoxia can also result, apart from early cognitive dysfunctions, in development of neurodegeneration in later life. Another group of enzymes affected by prenatal hypoxia are peptidases involved in catabolism of neuropeptides, including amyloid-β peptide (Aβ). The decrease in the activity of neprilysin and other amyloid-degrading enzymes observed after prenatal hypoxia could result over the years in an Aβ clearance deficit and accumulation of its toxic species which cause neuronal cell death and development of neurodegeneration. Applying various approaches to restore expression of neuronal genes disrupted by prenatal hypoxia during postnatal development opens an avenue for therapeutic compensation of cognitive dysfunctions and prevention of Aβ accumulation in the aging brain and the model of prenatal hypoxia in rodents can be used as a reliable tool for assessment of their efficacy.

Keywords: prenatal hypoxia, learning, memory, brain plasticity, amyloid-degrading enzymes, neprilysin, Alzheimer's disease

INTRODUCTION

In recent years, a growing body of clinical, epidemiological and experimental studies testify to a crucial role of gestational factors in brain development and functioning in postnatal life, which increases its vulnerability to later development of neurodegenerative disorders including Parkinson's and Alzheimer's diseases (Faa et al., 2014). It has clearly been demonstrated that factors such as diet, infectious disease, drug administration, smoking and alcohol consumption, as well as persisting maternal stress, significantly affect fetal brain development and its function after birth (for review see Charil et al., 2010; Li et al., 2012; Monk et al., 2013; Donald et al., 2015; Gawałek and Sliwowska, 2015; Kohlmeier, 2015; Labouesse et al., 2015). The periods of prenatal development and early postnatal life are extremely important for formation of brain structures which will be involved in cognitive functions, including learning and memory, and shape life experience and character of individuals (Babenko et al., 2015; Desplats, 2015). Any disruptions in these periods could result in compromised neuronal networking and manifest themselves at different stages of postnatal life predisposing individuals with aging to development of neurodegenerative diseases. This raises an important question about the importance of early intervention for preventing neurodegeneration caused by pre- and perinatal pathologies. However, to achieve this goal, clinicians need a deeper understanding of the pathological changes caused by prenatal stress and how to attenuate them using various pharmacological and behavioral approaches developed from basic science. For this, studies of the effects of various pathological conditions during pre- and early postnatal development using animal models which reproduce various stages of human embryogenesis, especially in fetal brain development, are of particular importance (for review see Maccari et al., 2016).

Despite intensive research and accumulation of significant amounts of experimental data, the mechanisms underlying developmental deficits caused by prenatal pathologies are still not well understood. In recent years the concept of epigenetic programming of neurological disorders attempts to explain how prenatal stress via epigenetic alterations of the developmental programme in the fetal brain affects mental health in later life (Babenko et al., 2015). This concept suggests latent early-life associated regulation mechanisms (LEARn) induced by environmental agents in the prenatal or early postnatal period which might underlie development of neurodegenerative disorders, including late onset Alzheimer's disease (Maloney et al., 2012).

In this review paper we will discuss various aspects of research accumulated to date, including our own multidisciplinary studies using the model of maternal hypoxia, with the aim to make a comprehensive analysis of the role of intrauterine hypoxia, in the development of neurodegeneration in later life as outlined in **Figure 1**. Since complications during pregnancy very often lead to insufficient oxygen supply to the fetus it is important to understand which changes they induce in the developing brain and how they can be prevented before, and compensated after, the birth. We have also summarized the main animal models of prenatal hypoxia developed to date. In some cases we also cite data obtained in the models of prenatal intrauterine ischaemia or neonatal hypoxia/ischaemia referring to the specific pathological conditions created in these models and their outcome for brain development. It is important to add that there are several animal models of neonatal hypoxia/ischaemia which have been extensively utilized and reviewed over the years (Vannucci et al., 1999; Vannucci and Hagberg, 2004; Patel et al., 2014; Rumajogee et al., 2016; Charriaut-Marlangue and Baud, 2018) and as such we have not specifically focused on this very important aspect of the research area.

RESPONSE OF THE DEVELOPING ORGANISM AND BRAIN TO DECREASED OXYGEN CONTENT

Brain hypoxia is one of the most common complications resulting from impaired circulation and brain metabolism, which can affect animals and humans at various stages of life. Hypoxia elicits a wide range of physiological responses of the organism at the systemic, tissue and cellular levels. Although brain ischaemia and hypoxia are often considered to be of similar nature, ischaemia is usually characterized by a reduction or arrest of blood flow to certain brain areas causing irreversible neuronal destruction while hypoxia, on the contrary, leads to an increase in cerebral blood flow which might result both in permanent or reversible changes of neuronal functions depending on its severity (Miyamoto and Auer, 2000).

It is generally accepted that neuronal cells are more vulnerable to the effects of altered oxygen supply and, in particular, hypoxia than other types of cells (Haddad and Jiang, 1993; Erecinska and Silver, 2001). Indeed, already 5 s after arresting oxygen supply to the brain a significant functional impairment of the CNS can be observed followed by total loss of CNS functions and unconsciousness after 8–12 s of anoxia with resuscitation time for the brain not exceeding 10 min (Oechmichen and Meissner, 2006). This explains why hypoxic insults to the brain have very significant consequences and lead to severe pathologies.

It is well-known that prenatal hypoxia caused by abnormal pregnancy and labor leads to dramatic changes in the developmental profile and behavioral characteristics of animals and is one of the most common reasons for mental retardation and cognitive deficit in children (Nyakas et al., 1996). Lack of oxygen supply to the fetal brain can be caused by various maternal pathologies including infection (Gabrielli et al., 2012), vascular diseases and pre-eclampsia (for review see Meister et al., 2016). Animal studies also provide evidence that preeclampsia-like conditions leading to increased blood pressure, proteinuria, growth restriction and, in the most severe cases, intrauterine mortality also resulted in increased apoptotic cell death in the fetal brain with the most sensitive areas being the subventricular and pallidum zones (Pellicer et al., 2011). Despite the existence of various protective mechanisms during embryogenesis and at birth, exposure of the embryonic brain to maternal hypoxia results in a number of changes in its structure and functional

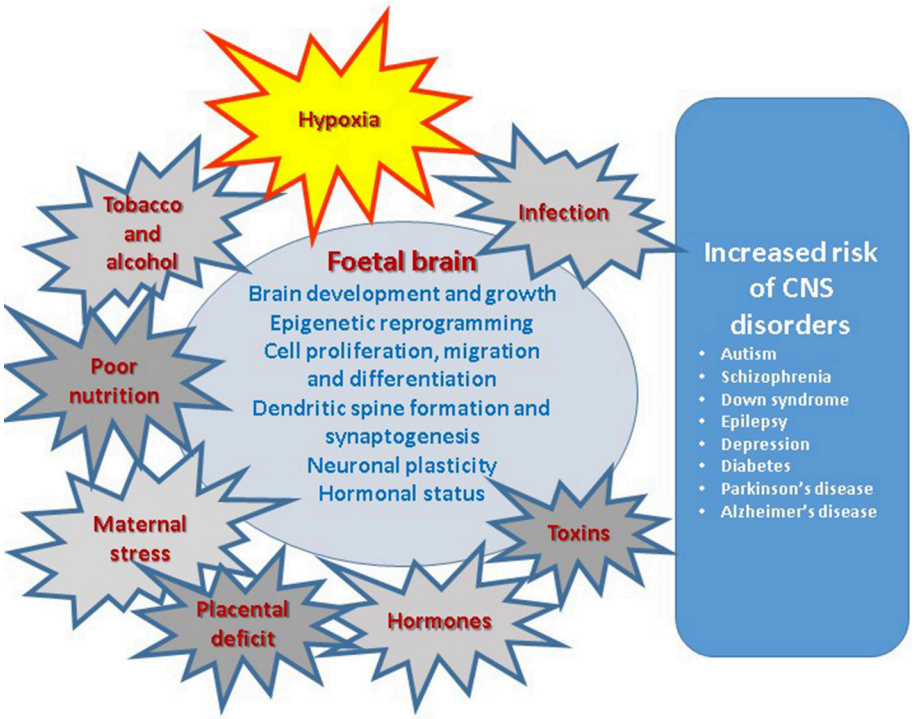

FIGURE 1 | Prenatal factors affecting various aspects of brain development result in increased risk of central nervous system disorders in postnatal life.

properties (Gross et al., 1981; Vasilev et al., 2016a,b; Zhuravin et al., 2018).

Oxygen Sensing Mechanisms

A decrease in O_2 content in the surrounding atmosphere is detected by oxygen sensors represented by central and arterial chemoreceptors (for review see Sharp and Bernaudin, 2004). Central chemoreceptors are located in the medulla of the brainstem, near the respiratory centers. The term "arterial chemoreceptors" is generic and describes aortic body chemoreceptors and carotid body chemoreceptors. Activation of carotid bodies and aortic arch chemoreceptors stimulates neurotransmitter release pathways (for review see Kemp et al., 2002).

One of the principle enzymes involved in the oxygen sensing response in the carotid body is a neutral endopeptidase, neprilysin (NEP), which modifies the cellular response to hypoxia by hydrolysing substance P (Kumar et al., 2000). Prenatal hypoxia was shown to impair the response of the developing organism to hypoxia in adulthood by alterations of the catecholaminergic components of the chemoafferent pathway contributing to impaired postnatal respiratory behavior (Peyronnet et al., 2007). Various studies in animals and cell models demonstrate that hypoxia down-regulates expression of NEP which might contribute to these alterations (Nalivaeva et al., 2004, 2012; Fisk et al., 2007; Kerridge et al., 2015). The adult rats exposed to intermittent hypoxia in the neonatal period demonstrate augmented carotid body and adrenal chromaffin cell response to hypoxia and irregular breathing, which are associated with increased oxidative stress (Nanduri and Prabhakar, 2015).

In addition to chemoafferent pathways, oxygen sensing is also controlled by pulmonary neuroendocrine cells, which are mainly located in the neuroepithelial bodies, and exert chemosensitivity, which is especially important in early life (Caravagna and Seaborn, 2016). Apart from oxygen sensing, chemosensory response to olfactory and gustatory stimuli in early life play a very important role in the development of new-born organisms. However, the effects of prenatal hypoxia on development of these chemosensory systems have barely been studied. However, there are data reporting deficits in motor responses to olfactory stimuli in rabbits submitted to prenatal hypoxia (Tan et al., 2005), which might be linked to a decrease in NO-dependent signal transduction (Drobyshevsky et al., 2012). Interestingly, in healthy adult humans, hypoxia caused by mimicking high altitude oxygen content at 4,000 m above sea level resulted in reduced olfactory sensitivity and intensity (Huppertz et al., 2018).

Tolerance to Hypoxia

Although the fetus during development in the womb grows under the conditions of reduced oxygenation, to withstand the reduced oxygen supply its cells and organs have developed several compensatory responses to hypoxia in the process of evolution (for review see Giussani, 2016). However, these defense and tolerance systems are sometimes not sufficient for protecting the developing brain against acute or chronic reduction in oxygen

supply caused by various pathological conditions (Gunn and Bennet, 2009).

The unifying theory of hypoxia tolerance at the molecular and metabolic levels was put forward by Hochachka and colleagues in the 1990s based on a comparative analysis of the reactions of cortical neurones and hepatocytes from high anoxia-tolerant and more hypoxia-sensitive systems (Hochachka et al., 1996). According to this hypothesis, the general mechanisms which are involved in the hypoxic response of cells include modulation of ATP-demands and ATP-supply pathways, suppression of protein synthesis, changes in metabolism and membrane function, which collectively affect ion transport and activity of cellular receptors and channels. In hypoxia-sensitive cells this translational arrest seems to be irreversible whereas, in the hypoxia-tolerant system, activation of the rescue mechanisms during an extended period of oxygen deficiency results in preferential expression of the same key proteins. Additionally, hypoxia-tolerant cells and tissues under hypoxic conditions use anaerobic metabolism, not for replenishing the energy deficits as in the sensitive cells, but for sustaining reduced energy turnover (Arthur et al., 1997).

The main difference between the reaction of liver and brain cortical cells of highly hypoxia-tolerant animals (e.g., turtle) to hypoxia is that in the hepatocytes the ATP demands for ion pumping are achieved by generalized "channel" arrest while the energy saving in the brain is mainly achieved by down-regulation of firing rates of synaptic transmission (so-called "spike arrest") (Pamenter et al., 2011). More recently, this hypothesis has been further specified and the term of "synaptic arrest" introduced (Buck and Pamenter, 2018). Synaptic arrest happens mostly due to the down-regulation of excitatory amino acid (especially glutamate) release accompanied by increased release of inhibitory amino acids. This results in less pronounced (by 50%) metabolic suppression in brain cells than in the liver cells where metabolic rate is decreased down to 10% of the normoxic levels. In terms of energy expense it means that the ATP turnover rates in anoxic turtle neurons are higher than in the liver cells. These specific changes in the neuronal cells dictated by reduced oxygen supply are accompanied by altered functional expression of ion channels in various brain cell types (Peers, 2002). Alteration of channel expression as an adaptive reaction to prenatal hypoxia may contribute to development of various neuropathologies in later life, including AD, whose pathogenesis involves impairment of various types of ion channels (Hynd et al., 2004).

Unlike chronic hypoxia, exposure of organisms to repetitive episodes of transient mild hypoxia leads to development of brain hypoxic/ischaemic tolerance, the phenomenon termed hypoxic preconditioning (for review see Rybnikova and Samoilov, 2015). This preconditioning by mild hypoxia induces adaptive changes in the organism and the brain, effectively preparing them to sustain more severe hypoxic or ischaemic conditions. Although there are no extensive data on the effects of hypoxic preconditioning during the prenatal period, the available literature testifies to its preventive action against further hypoxic or ischaemic insults (Nalivaeva et al., 2004; Zhao and Zuo, 2005; Giusti and Fiszer de Plazas, 2012). In particular, prenatal hypoxic preconditioning was shown to reduce neuronal loss and apoptosis of brain cells after cerebral ischaemia in new-born rats which was iNOS-dependent (Zhao and Zuo, 2005). Our own data also suggest that mild hypoxic preconditioning protects some enzymes of amyloid metabolism from their decrease caused by severe prenatal hypoxia and ischaemia, and can be considered neuroprotective (Nalivaeva et al., 2004).

Role of Placenta in Response to Hypoxia

The damaging effect of hypoxia on the fetal brain can be compensated by various defense mechanisms in the placenta regulating blood supply to the fetus (Schneider, 2009) and allowing the brain to develop at the expense of other organs (Browne et al., 2015), although fetal circulatory redistribution does not necessarily spare the brain from harmful effects of hypoxia (Roza et al., 2008). Adaptive mechanisms found in fetal as well as neonatal tissues show striking similarities to survival strategies seen in mammals with a high tolerance to severe hypoxia like hibernators or deep-sea divers (Singer, 1999). The special ability of the mammalian fetus/neonate to tolerate a considerable degree of hypoxia in the perinatal period has been regarded as protection against hypoxic threats inherent in the birth process (Mortola, 1999). Increased tolerance to hypoxia, as such, is a result of various metabolic responses including "hypoxic hypometabolism" aimed at economizing oxygen consumption (Rohlicek et al., 1998).

In response to hypoxia and ischaemia, the placental-fetal unit initiates several response reactions aimed to allocate oxygen and nutrients preferentially to the fetus and increase its chance for survival (for review see Murray, 2012; Smith et al., 2016). Exposure to ischaemia-hypoxia leads to changes in metabolic and energy demand (Wheaton and Chandel, 2011) and angiogenesis (Ishimura et al., 2009) both in the placenta and fetus. In the developing brain it leads to decreased expression of proteins involved in cortical angiogenesis and reduced capillary density in the fetal brain (Cohen et al., 2014).

Another important protein in the placenta, namely the thyroxine and retinol transporter transthyretin (TTR), which plays an important role in fetal brain development (Makover et al., 1989; Chan et al., 2009), was also shown to be significantly upregulated by hypoxia (Patel et al., 2012). Although increased levels of this protein might be considered as a compensatory mechanism for stabilizing transport of hormones to the fetuses under hypoxic conditions, the data on increased levels of misfolded TTR aggregates in human trophoblast cells under hypoxic conditions suggest that hypoxia might also lead to its misfolding and aggregation and contribute to placental pathogenesis (Cheng et al., 2016). Indeed, increased levels of misfolded and oxidized forms of TTR have been reported in the amniotic fluid of pregnant women with preeclampsia that can serve as a predictor of developing pathology (Vascotto et al., 2007). Increased levels of TTR protein expression have also been found in the choroid plexus of rat pups subjected to prenatal hypoxia (Vasilev et al., 2018) which suggest its role in providing pathologically developing fetal brain with an increased supply of thyroxine and retinol.

FETAL Response to Hypoxia

One of the important features of developing fetuses is that, under decreased oxygen supply, blood flow is drastically redistributed to the brain and heart from other organs increasing up to 90 and 240%, respectively, and this reaction is similar both in the pre-term and near-term fetuses (Richardson et al., 1996). In response to hypoxia the fetal brain also depresses its oxygen consumption via increased levels of adenosine acting on neuronal A1 receptors and vasodilatation through activation of A2 receptors on cerebral arteries (for review see Pearce, 2006). Apart from adenosine, hypoxia-induced release of nitric oxide also accounts for cerebral vasodilatation observed in the fetus (Cai et al., 1998). However, the reaction of fetal brain to acute and chronic hypoxia has different underlying mechanisms (Pearce, 2006).

Generally, at the cellular level, the initial response to hypoxia causes changes in expression of hypoxia-inducible factor (HIF), which regulates a number of genes, including practically all genes of the glycolytic pathway (for review see Semenza, 1998). It helps the organism to enhance its survival under hypoxic conditions by promoting erythropoiesis, angiogenesis and vasodilation. For example, hypoxia increases expression and secretion of the hormone erythropoietin, which improves systemic oxygen supply by enhancing the rate of erythrocyte formation, as well as transferrin, vascular endothelial growth factor (VEGF), leptin and other factors of angiogenesis and vascular tone (Chen et al., 2006). Other cell responses promote cellular survival by enhancing the expression of glycolytic enzymes, cell membrane glucose transporters including GLUT1, GLUT3, and other genes that tend to protect the cell from more severe oxygen deprivation (Vannucci et al., 1996). Other target genes for HIF-1 include enzymes of extracellular matrix metabolism (MMPs, plasminogen activator receptors and inhibitors), as well as factors of cell proliferation and apoptosis (Carmeliet et al., 1998). Deficiency of HIF-1 in the maternal organism leads to placental abnormalities which makes the fetus vulnerable to oxygen deprivation after mid-gestation (Kenchegowda et al., 2017). During embryonic and postnatal brain development, HIFs and specific HIF target genes are widely involved in early and highly active maturation processes via modulation of cell proliferation and differentiation (Trollmann and Gassmann, 2009). Although these genetic changes aim to protect neural tissue from ischaemia and hypoxia during development, their effects may persist and alter susceptibility for neurodegeneration later in life.

Prenatal hypoxia was shown to amend expression of GLUT4 and HIF-1α gene expression in fetal rat brain with different modality depending on the embryonic stage (Royer et al., 2000). At E14, exclusively, gestational hypoxia in rats was found to increase mRNA transcript levels of HIF-1α, GLUT3, GLUT, thyroid hormone receptors (TR) TRα2 and TRβ1 genes. However, hypoxia on E19 does not result in any response of HIF-1α and GLUT3 genes indicating differences in adaptation to hypoxia at these periods of development. This might explain the different impacts of prenatal hypoxia at different stages of rat embryogenesis on the development of cognitive functions in postnatal life as observed in our experimental model of prenatal hypoxia in the rat (Dubrovskaya and Zhuravin, 2010).

Microglia are now considered a very important safeguard of the healthy brain and their activation was shown to play an important role in the response of the neonatal brain to hypoxic-ischaemic injury (for review see Mallard et al., 2018). Prenatal hypoxia and ischaemia have also been shown to activate microglia in the developing brain. Thus, ischaemic insult in rats on E18 resulted in an increased number of microglial cells (Robinson et al., 2005). Moreover, it was shown that an insufficient oxygen supply leads to changes in the inflammatory/immune response in the fetal brain which can be detected in early postnatal life by an increased content of the pro-inflammatory cytokines IL-6, IL-10, and TNF-α in the CSF of new-born infants (Ellison et al., 2005). Furthermore, in a rat model of global fetal and perinatal asphyxia, changes in cytokine and ceramide metabolism genes in the prefrontal cortex, hippocampus and caudate-putamen were observed even at the age of 8 months after birth (Vlassaks et al., 2013). It was also shown that microglial activation in the brain of 1 day old rat pups subjected to hypoxia requires Notch signaling and activation of the NF-κB pathway (Yao et al., 2013).

EPIGENETIC CONSEQUENCES OF PRENATAL HYPOXIA

In the last decade a significant amount of studies have been focused on identification of the genetic and epigenetic factors that might link the effects of pre- and perinatal hypoxia and ischaemia with the risk of development of neurodegenerative disorders in later life. Since it has been shown that a number of specific genes in human cortex change their expression during fetal and early postnatal development, and that this pattern of gene expression is mirrored in aging and in neurodegeneration (Colantuoni et al., 2011), it is reasonable to expect that prenatal environment might, to various extents, affect these processes. One gene highly important in early brain development, which then changes in adult neurogenesis, is the RE1-silencing transcription factor, REST (Lunyak et al., 2002; Otto et al., 2007). Changes in expression of REST have been shown to correlate with mild cognitive disorders and Alzheimer's disease (Nho et al., 2015). Dysregulation of REST has also been implicated in the pathogenesis of Huntington disease and Down syndrome (Bahn et al., 2002; Buckley et al., 2010) and was shown to be involved in stress resistance in aging and Alzheimer's disease (Lu et al., 2014). This factor protects genomic integrity during embryonic development (Nechiporuk et al., 2016) and is induced in hypoxia leading to the changes in approximately 20% of hypoxia-repressed genes (Cavadas et al., 2016).

Although changes in the expression of hypoxia-responsive genes during prenatal development have an adaptive character, their epigenetic modifications may result in neurodevelopmental vulnerability and, in particular, underlie the attention deficit/hyperactivity disorders (ADHD) in children (Smith et al., 2016). Indeed, it was shown that SNP polymorphisms in angiogenic, neurotrophic and inflammatory genes are involved in response to an adverse prenatal environment and correlate with the severity of ADHD in children (Smith et al., 2014).

The epigenetic mechanisms which can be affected by prenatal hypoxia and ischaemia involve such processes as DNA methylation and histone modifications which regulate chromatin folding and gene activation or silencing (for review see Johnson and Barton, 2007) and brain development (Kato and Iwamoto, 2014; Tapias and Wang, 2017). Moreover, identification of a novel class of histone demethylases as true dioxygenases suggests that chromatin can act as an oxygen sensor coordinating cellular response to hypoxia (Melvin and Rocha, 2012).

Chromatin Modifications

Although not sufficiently studied existing data suggest direct involvement of chromatin modification at the levels of histone acetylation and DNA methylation in the fetal response to hypoxia. Some examples are listed below.

At the organ-specific level in a lamb model of high-altitude long-term prenatal hypoxia it was shown that fetal pulmonary arteries have reduced levels of global histone 4 acetylation and DNA methylation, accompanied by the loss of the cyclin-dependent kinase inhibitor p21 which were linked to development of pulmonary arterial remodeling and pulmonary hypertension of the new-born (Yang et al., 2012).

Analysis of expression of glucose 6-phosphatase (G6Pase), which is involved in gluconeogenesis, in a rat model of maternal hypoxia (11.5% atmospheric oxygen from E15 to E21) demonstrates that in male offspring decreased hepatic G6Pase mRNA and protein levels correlated with increased methylation of histone H3 surrounding the *G6Pase* promoter (Osumek et al., 2014).

Oxygen levels have also been shown to regulate epigenetically the fate of brain mid-gestational neural precursor cells via HIF1α-Notch signaling interaction and DNA demethylation of astrocytic genes (Mutoh et al., 2012). In normally developing brain, lower oxygen tension characteristic of the embryonic brain (below 5%) promotes differentiation of mid-gestational neuronal precursor cells into astrocytes via activation of the Notch-signaling pathway and up-regulation of transcription factor NFIA. This leads to DNA demethylation of such astrocyte specific genes as *gfap* and *S100β*. However, under normoxic conditions (21% O_2) these processes are inhibited. Considering these data, it is reasonable to suggest that decreased oxygen levels will have an even stronger promoting effect on astrocyte differentiation in developing brain resulting in a decreased neurone-astrocyte ratio. Indeed, in fetal guinea pigs subjected to maternal hypoxia (10.5% O_2 in the air, E52-62) the density of NeuN-immunoreactive neurons in the fetal CA1 hippocampal area was subsequently decreased (Blutstein et al., 2013). Additional treatment with nicotine in the same model significantly increased the number of astrocytes in the fetal hippocampus and resulted in reductions of both GFAP- and NeuN-positive cells in the CA1 in adulthood. It is important to note that comparing epigenetic landscapes of neuronal and glial cell genomes in normally and pathologically developing brains might in the future provide a powerful tool for creating genetic maps of normally developing and aging brain and provide clues for their changes in neurodegeneration (Mitchell et al., 2014).

Decreased expression of glucocorticoid receptors in developing rat brain caused by maternal hypoxia (10.5% O_2, E15-21) was also shown to involve increased DNA methylation in the area of exons 17 and 111. This decreased binding of the transcriptional factors Egr-1 and Sp1 to the promoters led to reduced levels of exon 17 and 111 mRNA variants (Gonzalez-Rodriguez et al., 2014).

Changes in DNA demethylation of the corticotropin-releasing hormone *Crhr1* gene was also observed in the hypothalamus of male offspring of Sprague–Dawley rats subjected to intermittent hypoxia during whole pregnancy (10.8% O_2, for 4 h per day, E1–E21). These data suggest the existence of hypoxia-triggered male-sex-dependent regulation of the *Crhr1* gene via demethylation at CpG sites in the promoter region leading to development of anxiety-like behavior in adulthood (Wang et al., 2013).

Demethylation of genomic DNA and a decreased level of DNA methyltransferase 3b expression *in vivo* was shown to result in elevated levels of APP, β- and γ-secretases in 3-month-old offspring of transgenic mice (APP^{SWE}/PS1ΔE9) exposed to an intermittent hypoxic environment (6 h/day) for 30 days. On the contrary, overexpression of DNA methyltransferase 3b reduced the levels of these proteins in *in vitro* cell models (Liu et al., 2016). Such alterations in expression of the Alzheimer's disease-related genes of hypoxic mice were accompanied by learning and memory deficits in later life.

Increased DNA methylation of a specific site in the BDNF gene harboring functional SNP rs6265 for the Val(66)Met allele predisposing the ValVal individuals to impaired working memory and increased risk of a schizophrenic phenotype, was reported in the peripheral blood mononuclear cells of children with a history of obstetric complications during labor-delivery (Ursini et al., 2016). Taking into account that brain DNA methylation in children cannot be studied *in vivo,* blood cell analysis might provide a non-invasive and useful approach for detecting epigenetic abnormalities caused by pathologies during pregnancy and labor.

Micro RNA

Accumulating data also support the involvement of a distinct class of small noncoding microRNAs (miRNAs) in post-transcriptional regulation of target genes in response to hypoxia (for review see Nguyen et al., 2013). Since the machinery of miRNA production and maturation depends on oxygen supply to the cells (Ho et al., 2012) and regulation of HIF expression, in turn, depends on miRNA species (Tanaka et al., 2013; Liu et al., 2015), in particular, on miR-17-92 (Taguchi et al., 2008), it is reasonable to suggest that miRNAs might also participate in the response of neuronal cells to hypoxia. Indeed, there are studies suggesting that hypoxia alters miRNA expression in rat cortical pericytes (Truettner et al., 2013) and hippocampus (Gao et al., 2017) which leads to cognitive dysfunction.

A reverse correlation between expression of miRNA species and REST in neuronal cells in response to hypoxia also suggests a regulatory role of reduced oxygen supply in maintaining neuronal miRNA profiles (Liang et al., 2014). Microarray analysis of neural progenitor cells has shown that 15 microRNAs were up-regulated at least 3-fold and 11 were down-regulated under

hypoxic conditions with specifically increased expression of miR-210 regulated by HIF-1α (Liu W. et al., 2011). In murine embryonic brain cortices during hypoxia-induced neuronal apoptosis, the miR-23b-27b cluster was also found to be downregulated depending on the transcription factor c-Myc (Chen et al., 2015). Taking into account that a number of recent studies have identified the role of miRNAs in neurodegenerative disorders, including AD, PD and Huntington's disease (Maciotta et al., 2013; da Silva et al., 2016; Salta and De Strooper, 2017), changes in the miRNA pattern in developing brain after prenatal hypoxia will certainly result in a neurodegeneration-prone phenotype in later life. Quantitative analysis of hypoxia-regulated miRNAs in the maternal blood may provide a tool for identifying risk of fetal hypoxia (Whitehead et al., 2013). Based on the recent data on aberrant hypoxia signaling due to pre-eclampsia, which involves deregulated expression of miR455, development of a non-invasive test based on miRNA analysis in the blood of pregnant women might be beneficial for early diagnostics of pre-eclampsia-related pathological changes in the placenta and fetal development (Lalevée et al., 2014).

Post-translational Modifications of Proteins

Post-translational modifications of proteins substantially affect their normal properties and hence metabolism (Nalivaeva and Turner, 2001). The accumulation of abnormally modified and folded proteins can lead to development of various neurodegenerative disorders including Alzheimer's, Parkinson's, and Huntington's diseases (Ren et al., 2014). Since proper protein folding and removal of misfolded proteins is an important part of proper brain development, analysis of the effects of prenatal hypoxia on the status of protein posttranslational modifications and aggregation in the brain is an important indicator of its healthy development.

Although this area of research is still not sufficiently developed, there are indications that hypoxia increases protein ubiquitination detected even 6 months after perinatal hypoxia (Capani et al., 2009; Grimaldi et al., 2012). Hypoxia also leads to activation of chaperone-mediated autophagy (Dohi et al., 2012) and it is reasonable to expect that autophagy mechanisms might be disrupted after prenatal hypoxic stress leading to accumulation of misfolded proteins, ER stress and metabolic syndrome in later life. As such, one of the neuroprotective strategies for treatment of prenatal hypoxia related brain disorders in postnatal life might be activation of autophagy-related cellular mechanisms (for review see Herrera et al., 2018).

RISK OF DEVELOPMENT OF NEURODEGENERATIVE DISORDERS

In this connection it is important to mention the Developmental Origins of Health and Disease hypothesis (DOHaD) based on the theory of fetal programming originally outlined for coronary heart diseases (Barker, 2007) but then extended to mental health disorders (Swanson and Wadhwa, 2008; O'Donnell and Meaney, 2017). The supporters of this hypothesis raise the question of the importance of epigenetic reprogramming during embryonic development in response to various adverse environmental factors, which makes the organism vulnerable for development of disease in later life. They also stress the importance of studying the fetal origin of mental health.

As already mentioned above and will be discussed further, accumulated research to date suggest a link between prenatal hypoxia and increased risk of development of Alzheimer's disease pathology in later life (Nalivaeva et al., 2012; Zhang et al., 2013; Wang et al., 2014; Liu et al., 2016). Although there have been experimental attempts to link prenatal hypoxia and other pathological factors in early life with the pathogenesis of Parkinson's disease, there is no direct evidence of such a correlation (Gardener et al., 2010). Nevertheless, there are reports that prenatal hypoxia results in selective and long-lasting impairments in the dopaminergic systems that can be detected even in adulthood (Burke et al., 1992; Chen et al., 1997). A search for other environmental factors which might affect development of the nigrostriatal dopaminergic system of the brain during embryogenesis revealed that exposure to some toxicant pesticides can predispose to Parkinson's disease in the male offspring (Barlow et al., 2004). Exposure to neurotoxins, e.g., bacterial lipopolysaccharides, during critical developmental periods in pregnancy might also be a risk factor since it leads to a decreased number of dopamine neurones in the brain of the offspring (Carvey et al., 2003).

Stronger evidence suggests a link between prenatal hypoxia and the development of schizophrenia (Van Erp et al., 2002; Howell and Pillai, 2014). Studies in rodent models indicate that chronic hypoxia leads to anatomical abnormalities often observed in schizophrenic patients (Asami et al., 2012; Andreasen et al., 2013). Moreover, rodent models have been found useful for investigating sex-related susceptibility to brain damage by neonatal hypoxia and development of schizophrenia (Mayoral et al., 2009).

MODELS OF PRENATAL HYPOXIA

For better understanding the role of prenatal hypoxia in fetal pre- and post-natal development, various animal models have been employed over the span of several decades (for review see Rees et al., 2008). Some have been developed based on restriction of uterine blood flow to the fetus using, for example, vascular occlusion in pregnant sheep which clearly demonstrated that prolonged hypoxia, induced by placental insufficiency of differing severity and duration, causes changes in fetal brain structure (Clark et al., 1982; Rees et al., 1998). Using this model it was demonstrated that transient hypoxia also affected morphology and functions of various types of neuronal cells and impaired development of neural processes and connections, involving the subplate neurons, which regulate brain development and play a critical role in establishing cortical connections to other brain regions (McClendon et al., 2017). Moreover, it was found that subplate neurons of the sheep fetus are surprisingly resistant to hypoxia and acquire some chronic structural and functional

changes that might be beneficial to neuronal survival and brain connectivity in postnatal life. The authors have clearly demonstrated that there are species-associated differences in the response to hypoxia between rodents and sheep that should be taken into account when interpreting and comparing the experimental data obtained in different animal models. The sheep models to date provide a platform for studying various physiological changes in the developing brain under restricted oxygen supply including electroencephalography (Abbasi et al., 2017).

Another model of prenatal hypoxia in sheep applied reduced oxygen content in a maternal ventilated gas mixture by partially replacing it with nitrogen (Tchirikov et al., 2011). Using this approach it was demonstrated that acute maternal hypoxia results in reduced placental blood perfusion in the hypoxemic fetuses and lower fetal pH and pO_2 compared to normoxic fetuses.

Among medium-size animals convenient species for modeling prenatal hypoxia are rabbits (Gingras and Long, 1988; Buser et al., 2010) and guinea-pigs (Mishra et al., 1988). For animals of this size an environmental chamber with reduced oxygen content can be used in which pregnant females are kept under lower oxygen content of different severity at various stages of pregnancy for the required duration (Oh et al., 2008). The experiments in rabbits have shown that prenatal hypoxia results in a wide spectrum of biochemical changes in brain tissue of the offspring (Gingras et al., 1995), including reorganization of the white matter, brain morphology and functions. These studies have significant implications for understanding the role of prenatal hypoxia for prematurity and cerebral palsy in children (Coq et al., 2016). The works of Mishra and colleagues utilizing the guinea pig model of prenatal hypoxia over a decade are summarized in a review article (Mishra and Delivoria-Papadopoulos, 1999) which underlines the enhanced susceptibility of the brain to hypoxia in the process of fetal development and increase in the demands of neuronal cells to oxygen supply. More recent studies in the guinea pig model provide detailed characterization of the changes in fetal brain energetics caused by chronic prenatal hypoxia (Wang et al., 2016). They also confirm that prenatal hypoxia affects various systems of the developing brain resulting in a decreased number of neurons in the cerebral cortex and dentate gyrus of the fetus correlated with reduced levels of BDNF (Chung et al., 2014, 2015).

Recently, development of transgenic mice modeling various neurodegenerative conditions has provided researchers with useful tools for studying the effects of prenatal hypoxia on acceleration of development of brain pathologies, including Alzheimer's disease (Zhang et al., 2013; Rueda-Clausen et al., 2014). These models allow investigation of detailed molecular mechanisms underlying development of brain pathology in the prenatal period and a search for possible therapies (Wang et al., 2014).

However, rats are still the most common experimental animals for modeling prenatal hypoxia and studying its short- and long-term effects on various aspects of brain development and characteristics (Golan and Huleihel, 2006). In rats, prenatal hypoxia can be achieved via reducing oxygen content in a chamber, where pregnant rats are housed and replacing it with an inert gas (commonly, nitrogen) (Gross et al., 1981; Zhuravin et al., 2004). Hypoxia can also be produced in a chamber with hypobaric conditions, which model high altitude hypoxia (Tyulkova et al., 2011) or by exposure of pregnant rats to chronic mild carbon monoxide concentrations (Beltran-Parrazal et al., 2010). Some chemical approaches e.g., administration of sodium nitrite (Nyakas et al., 1994) or a nitric oxide synthase inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME) can also be used (Pellicer et al., 2011) as well as surgical procedures restricting blood flow to the fetuses e.g., umbilical cord occlusions (Smotherman and Robinson, 1988) or unilateral uterine artery ligation (Magal et al., 1990; Tashima et al., 2001).

The list of animal models for studying the effects of hypoxia on the developing brain cannot be completed without mentioning an important area of research in peri- and neonatal hypoxia and ischaemia. These conditions mimic various pathologies to which the fetus might be subjected around the time of birth leading to neonatal hypoxic-ischaemic encephalopathy. Most of these models are based on the classic Rice-Vannucci model in which rat pups on P7 undergo unilateral ligation of the common carotid artery with subsequent exposure to 8% oxygen in the breathing air for several hours (Rice et al., 1981). Taking into account that the rat brain continues to mature during the first month after birth and at P7 is morphologically similar to the human fetal brain at 32–34 weeks of gestation, this model provides significant insights into how hypoxia and/or ischaemia affect immature brain cells. However, it is important to note that, after birth, brain metabolism in rat pups undergoes adaptation to extra-uterine oxygenation which might change the reaction of neurones to hypoxia-ischaemia. Nevertheless, utilization and modifications of this model over the decades have produced a significant amount of important information on the cellular mechanisms involved in reaction of the brain to hypoxia and have allowed development of therapeutic avenues for treatment of encephalopathy in children (Gancia and Pomero, 2012; Patel et al., 2014; Rumajogee et al., 2016; Edwards et al., 2017).

PRENATAL HYPOXIA IN RATS

In our studies for more than two decades we have developed and intensively utilized a model of normobaric hypoxia using laboratory Wistar rats at various days of pregnancy which is described in detail in our early work (Zhuravin, 2002; Lavreneva et al., 2003). For this we use a 100 L chamber supplied with gas analysis equipment, thermoregulation, and facility for removal of excess CO_2. The hypoxic conditions are achieved by replacing oxygen with nitrogen down to 7% O_2 concentration (or other desired level) during 10 min and then remaining at this level for 3 h. This paradigm provides a reliable and reproducible setting for maintaining hypoxic conditions and obtaining the material for further experiments either from the fetuses or rat pups during different stages of their postnatal development. The detailed analysis of the data obtained in these studies has recently been reviewed in Zhuravin et al. (2018). Below we shall discuss the main effects of prenatal hypoxia on rat brain anatomical,

biochemical and functional properties (**Figure 2**) comparing the results of our studies with the data of other research groups employing different hypoxia paradigms.

Structural Changes in Rat Brain After Prenatal Hypoxia

There is a significant amount of data demonstrating that prenatal hypoxia results in a set of physiological changes in rat embryos leading to functional and behavioral changes in the postnatal period including reduced body weight of newborn pups (Gross et al., 1981; Olivier et al., 2005; Dubrovskaya and Zhuravin, 2010). Although some authors have not observed significant changes in brain weight of rat pups after prenatal hypoxia (Gross et al., 1981; Liu Z. H. et al., 2011) they have reported changes in the brain to body weight ratio (Liu Z. H. et al., 2011) as well as DNA/protein ratio (Gross et al., 1981). In the experiments with chronic prenatal hypoxia (10.5% O_2, E4-E21) the decreased brain weight has been reported both in the fetuses and 6-week old offspring (Wei et al., 2016). However, other authors reported an increased brain weight in male offspring of Sprague-Dawley rats, subjected to maternal hypoxia (10.5% oxygen) on gestational day 21 (Zhang et al., 2016).

Obstructive sleep apnoea during pregnancy, and especially in late gestation, is a rather common complication in women. Intermittent hypoxia, to which the fetus is subjected during apnoea episodes, induces metabolic dysfunction which can be detected as increased body weight and higher adiposity index in adult male offspring. This suggests differential sex-dependent effects of the condition on expression of fetal genes (Khalyfa et al., 2017).

The major anatomical and structural alterations in rat brain after prenatal hypoxia are manifested at the level of the cellular composition of various brain structures (the cortex, hippocampus, striatum, cerebellum, etc.), including degeneration of neuronal cells, gliosis and apoptosis (Rees and Inder, 2005; Golan and Huleihel, 2006; Zhuravin et al., 2006; Liu Z. H. et al., 2011; Wang et al., 2017). Increased levels of apoptosis in rat brain after hypoxia correlated with upregulation of caspases, in particular of active caspase-3, which contributed to alteration in neuronal composition of different cortical layers (Vasilev et al., 2016a). Activation of apoptotic events caused by perinatal hypoxia modeling birth asphyxia was also shown in the cortex and CA1 area of the hippocampus in rat pups during the first 2 weeks after the insult resulting in reduced cell density and the accumulation of cells with nuclear fragmentation specific for apoptosis (Daval and Vert, 2004).

Importantly, it was also shown that prenatal hypoxia affects the cells in brain neurogenic zones and, in particular, the levels of expression of the protein paired box 6 (Pax6) which plays an important role in neurogenesis, cell proliferation, differentiation and survival during the development of the central nervous system (Simpson and Price, 2002). Although in the fetuses subjected to prenatal hypoxia the levels of Pax6 were increased in the subventricular zone and subgranular zone of the hippocampal dentate gyrus, they were significantly decreased in the cerebral cortex (So et al., 2017). This finding correlates with the reduced number of neuronal cells in rat cortex during the first month of postnatal life observed in our studies (Vasiliev et al., 2008).

Using light and electron microscopic techniques it was demonstrated that prenatal hypoxia caused a delay in differentiation of neurones and formation of synaptic contacts in rat neuropil as well as affecting myelination of nerve fibers at the ultra-structural levels both in the neocortex and basal ganglia (Zhuravin et al., 2006; Vasiliev et al., 2008; Vasil'ev et al., 2010). In particular, on postnatal days P10-30 in the brain cortex there was a significant decrease in the total number of pyramidal neurones (Vasiliev et al., 2008). However, this decrease was observed only during the first month of rat postnatal development and only in the group subjected to prenatal hypoxia on E14, but not on E18 (Dubrovskaya and Zhuravin, 2010; Vasilev et al., 2016b). Changes in cell composition have also been observed in the dorsal hippocampus of hypoxic rats, especially in the CA1 with increased number of neurones possessing retracted apical dendrites (Zhuravin et al., 2009a).

The effects of prenatal hypoxia were more profound when it was applied at mid rather than late gestation and became less apparent with development of rats in the postnatal period (Nyakas et al., 1996; Dubrovskaya and Zhuravin, 2010). Because formation of main brain anatomical architecture starts on embryonic day 12 (E12) and the precursors of cortical and striatal neurons actively proliferate on E14 and of the hippocampal neurons on E15 (Rice and Barone, 2000), the timing of hypoxia or other insults determines their impact on cellular composition and structure of specific brain regions and therefore affects formation of the physiological functions related to them. For example, prenatal hypoxia on E14 and E18 resulted in different outcomes of neuronal migration into the cortical layers of rat cortex and performance of behavioral tasks in postnatal life (Vasilev et al., 2016b).

Changes in Development of Brain Functions

There are many tests available to assess the development of brain integrity in rats at very early stages after birth including habituation, exploratory behavior, reactivity and motor coordination. The most commonly employed tests include the body-righting reflex, negative geotaxis, placing, homing, head elevation, ascending on a wire mesh, which are extensively reviewed in Rice and Barone (2000). In our model of prenatal hypoxia in rats we also observed a delay in pup maturation (reduced body weight during the first month of life, delayed eye opening time and the onset of separation of the external ear from the skin of the head) and development of various sensory-motor reactions including the body-righting reflex, negative geotaxis, forelimb placing reaction, maintenance of balance on a rotating grid etc (Dubrovskaya and Zhuravin, 2010). Some developmental features such as later separation of the external ear from the scull, the forepaw-placing reaction and the whisker placing reaction were found to be delayed only after prenatal hypoxia on E14 and not on E18 (Dubrovskaya and Zhuravin, 2010; Vasilev et al., 2016b).

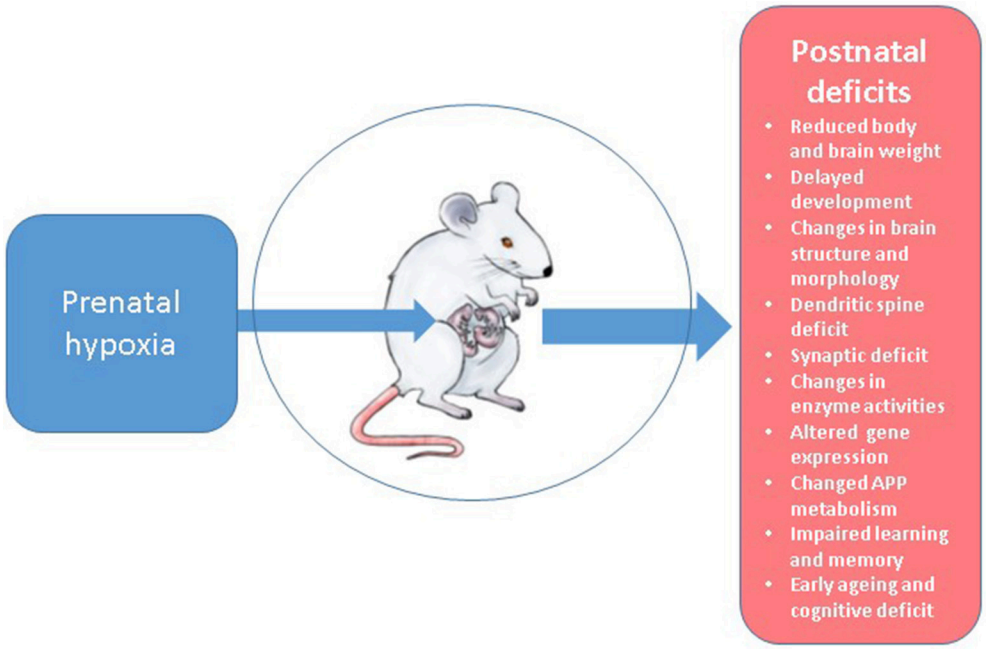

FIGURE 2 | Postnatal deficits observed in the rat model of prenatal hypoxia.

Although the deficit in innate motor reactions of new-born rats after prenatal hypoxia, observed in our studies, becomes less pronounced with pup development during the first month of postnatal life, execution of more skilful movements, e.g., reaching and pushing, and learning new motor reflexes were still compromised in adulthood (Zhuravin et al., 2002). This correlates with the observations that motor and coordination abilities remained partially impaired in the old rats subjected to prenatal hypoxia, especially under high oxygen demand (Jänicke and Coper, 1994). Some authors link the cause of motor deficit observed after prenatal hypoxia with a failure in the migration and maturation of oligodendroglial progenitor cells causing delay of myelination in the cerebellum (Barradas et al., 2016).

Unlike motor functions which are practically compensated and restored during the first month of pups' development, the cognitive deficits caused by prenatal hypoxia on E14 or E18 remain detectable at all tested stages of postnatal life. For example, various types of rat memory (working, short and long-term memory) assessed by 8-arm maze and novel object recognition were compromised and correlated with the structural changes observed in the hippocampus (Zhuravin et al., 2011; Nalivaeva et al., 2012; Cunha-Rodrigues et al., 2018). Some studies link working memory impairment observed both in juvenile and adult rats subjected to prenatal hypobaric hypoxia with increased levels of phosphatidylinositol 4,5-diphosphates and phosphatidylinositol 4-phosphates in the hippocampus and upregulated expression of the type 1 inositol 1,4,5-trisphosphate receptor (IP3R1) (Tyul'kova et al., 2015). Maternal hypoxia on days 15–21 was shown to result in increased activity of metalloproteinases and significant cell death in the hippocampus of rat pups on days 0–7 after birth, which correlated with worsened development of their neurobehavioral functions (Tong et al., 2010). On the other hand learning deficits in adult rats subjected to prenatal hypoxia was shown to correlate with a significant reduction in the number of neurones positive to the polysialylated markers in the dentate granular zone of the hippocampus (Foley et al., 2005).

Prenatal transient systemic hypoxia-ischaemia created in Sprague-Dawley rats by occlusion of uterine arteries for 60 min on E18 has recently been reported to cause a sustained motor deficit and poor social interaction in young adult rats, which were accompanied by impaired white matter microstructure and diffusion abnormalities in the hippocampus, striatum and thalamus (Robinson et al., 2018). In a similar model of prenatal hypoxia on E18 adult rat offspring also demonstrated increased anxiety behavior and reduced spatial exploration and deficit in habituation memory (Sab et al., 2013). Prenatal ischaemia induced by unilateral ligation of the uterine artery on E17 was also shown to induce motor hyperactivity and deficits in information encoding, and short- and long-term memory in adult offspring (P40 to P80) although no impairments in spatial learning or working memory were observed when animals were tested in the Morris water maze (Delcour et al., 2012).

According to our data, rats subjected to prenatal hypoxia demonstrate reduced ability to learn new instrumental reflexes. Thus, on postnatal days 20-30 the number of rats in the experimental groups capable of learning to push a piston inside a narrow tube was 30% lower compared to the control group and at the age of 3 months, the number of hypoxic rats capable of

learning this reflex for a certain duration was 40% lower than in the control group (Zhuravin et al., 2002). Analysing the ability of rats to remember the learnt task after a 5 week interval it was found that control rats were able to remember the learnt duration of the reinforced movements while hypoxic rats returned to the level before training, which implies a significant memory deficit caused by prenatal hypoxia.

Impaired learning abilities of rats were also reported in other paradigms of prenatal hypoxia. Thus, a 30-min hypoxic insult by complete clamping of the uterine vasculature on E17 was found to impair spatial memory in the Morris water maze and caused learning deficits in the passive avoidance test during the first month of development (Cai et al., 1999). These abnormalities the authors linked to the reduction in NOS expression and activity in the affected brain areas. On the contrary, gestational intermittent hypoxia induced by computer-controlled exposure of pregnant Sprague-Dawley rats either to room air or to 10% O_2 alternately every 90 seconds starting on E5 until delivery did not result in any changes in acquisition and retention of a spatial memory both at 1 and 4 months of age (Gozal et al., 2003). This outcome, however, might be attributed to the development of tolerance to hypoxia in the fetal brain caused by repeated short episodes of maternal hypoxia.

Synaptic Plasticity

Existing literature suggests that impaired brain functions caused by prenatal hypoxia are related to impaired neurotransmitter circuits and synaptic plasticity (Herlenius and Lagercrantz, 2004; Barradas et al., 2016; McClendon et al., 2017). In rats submitted to prenatal hypoxia on E14 we have also observed a significant reduction in the number of synaptopodin-positive dendritic spines (Zhuravin et al., 2011; Vasilev et al., 2016b) which are fundamental for the formation of synaptic contacts and memory (Martin et al., 2000; Zito et al., 2009; Segal, 2010). The decrease in the number of synaptopodin-positive dendritic spines was particularly evident in the molecular layer of the neocortex and in the CA1 area of the hippocampus which correlated with impaired working memory (Zhuravin et al., 2009b). This decrease in the number of labile dendritic spines in the CA1 area of the hippocampus might be related to the changes in the entorhinal cortex which, in humans, is considered to be the earliest event in the development of Alzheimer's disease (Killiany et al., 2002). Damage in the medial and lateral entorhinal cortices correlating with impaired memory have indeed been reported in adult rats subjected to prenatal hypoxia on E17 (Delcour et al., 2012). The reduction of the number of synaptopodin-positive spines along with decreased ability for learning is also observed in normally aging rats, which could be one of the reasons for cognitive decline related to advanced age, and in the sporadic form of Alzheimer's disease (Zhuravin et al., 2011; Arnold et al., 2013).

The mechanisms of impairment of neuronal interactions caused by prenatal hypoxia in rat brain are more complex and do not involve only the changes in the number of dendritic spines and neuronal contacts but also result in disruption in the development of various mediator systems in the postnatal period (Nyakas et al., 1994; Gerstein et al., 2005; Tyulkova et al., 2011). As we have also observed, prenatal hypoxia on E14 resulted in a decrease in the number of VAChT-positive cholinergic terminals which form synapses on the bodies of the pyramidal neurones in layers V-VI of the parietal cortex. On the other hand, the EAAT levels were found to be much higher in hypoxic animals resulting in spontaneous epileptogenic activity and increased kindling in response to pharmacological agents and other external stimuli (Zhuravin et al., 2018) and even a weak electric shock could induced seizure episodes in 1.5 years old rats subjected to prenatal hypoxia on E14 with more pronounced average duration than in control animals (Kalinina et al., 2015).

Changes at the Molecular Level

Structural and functional changes in rat brain after prenatal hypoxia are underlined by significant alterations in its biochemical characteristics including various classes of molecules (nucleic acids, proteins and lipids) and metabolic pathways (Gross et al., 1981; White and Lawson, 1997; Peyronnet et al., 2000; Beltran-Parrazal et al., 2010; Camm et al., 2011). For example, acute prenatal hypoxia on E14 affects activities of the different forms (cytosolic, membrane-bound, and soluble) of acetyl- and butyryl-cholinesterases (AChE and BChE) in the sensorimotor cortex detected at various stages of postnatal ontogenesis (Lavreneva et al., 2003; Kochkina et al., 2015). The increase in brain BChE activity might have a compensatory effect on the stress response of the brain due to the enzyme's ability for hydrolysing various toxic agents (for review see Lockridge, 2015). However, with aging it can lead to neurodegeneration and is considered as an indicator of Alzheimer's disease in humans (Greig et al., 2002). Changes in AChE and BChE activities after prenatal hypoxia are also observed in blood plasma of rats at various stages of postnatal development, which might affect their immune and stress responses (Kozlova et al., 2018).

Prenatal hypoxia on E14 also affected the levels of brain expression and activity of such peptidases as neprilysin and endothelin-converting enzymes (Nalivaeva et al., 2004, 2012) and altered the adenylate cyclase system (Zhuravin et al., 2002). In particular, the enzyme activity of adenylate cyclase in the striatum, which reversely correlates with the ability of rats to learn instrumental reflexes, was much higher in rats subjected to prenatal hypoxia and correlated with their learning deficits.

Hypoxia, and prenatal hypoxia in particular, are known to regulate expression of APP whose gene has a hypoxia-responsive element (Lahiri et al., 2003). This protein plays an important role in development of the nervous system (Young-Pearse et al., 2007) and the Aβ peptide produced from its precursor has a causative role in development of Alzheimer's disease (Hardy and Selkoe, 2002). Analysis of the content of APP in rats subjected to prenatal hypoxia also revealed significant changes in the levels of this protein in the sensorimotor cortex (Nalivaeva et al., 2003). Prenatal hypoxia not only led to an increase in the content of the membrane bound form of APP at different postnatal stages of rat development but also reduced production of its soluble forms (sAPP) which have protective neuritogenic properties (for review see Chasseigneaux and Allinquant, 2012). Moreover, the most significant changes after prenatal hypoxia on E14 were observed on P10-P30 when formation of rat brain neuronal networks is the most active and any deficit of neuritogenic factors might

underlie cognitive dysfunctions. These data also indirectly testify that prenatal hypoxia might modify the activity of α-secretase enzymes, which are important for releasing sAPPα and hence preventing formation of Aβ. The deficit of α-secretase after prenatal hypoxia might also explain the decreased production of soluble AChE since this activity can also be involved in AChE secretion (Nalivaeva and Turner, 1999). Moreover, maternal hypoxia in rats was shown to result in an increase in the activity of matrix metallopeptidases (MMPs) and decreases in the expression of tissue inhibitor of metalloproteinases (TIMPs) in the brain of neonatal rats, which can also underlie remodeling of neuronal circuits during brain development (Tong et al., 2010).

Although not studied in the models of prenatal hypoxia there is evidence that hypoxic conditions can alter expression of the γ-secretase complex (Liu et al., 2016) which not only regulates animal development via Notch signaling but also is a major enzyme involved in production of Aβ and Alzheimer's disease pathogenesis (Hartmann et al., 2001). Studies in transgenic mice modeling Alzheimer's disease have confirmed that prenatal hypoxia accelerates development of the pathology (Zhang et al., 2013).

One of the important factors which predisposes to formation of the sporadic form of Alzheimer's disease is the deficit of amyloid clearance (for review see Baranello et al., 2015). Our and other studies have shown that prenatal hypoxia leads to a significant deficit of the major amyloid-degrading enzyme neprilysin in rat brain at various stages of postnatal development (Nalivaeva et al., 2004, 2012; Wang et al., 2014). Together with the deficits of other amyloid-degrading enzymes e.g., endothelin-converting enzyme, angiotensin-converting enzyme and insulin-degrading enzyme, which are also affected by prenatal hypoxia or ischaemia (Nalivaeva et al., 2004), reduced NEP activity can lead to permanent insufficiency of amyloid clearance over the years and hence predispose to development of Alzheimer's disease pathology in later life (Nalivaeva et al., 2008; Wang et al., 2010).

On the other hand we observed an increased level of TTR expression in the choroid plexus of rat pups subjected to prenatal hypoxia (Vasilev et al., 2018). TTR is suggested to play a contributory role in regulation of the levels of brain Aβ (Li and Buxbaum, 2011; Du et al., 2012). Since APP expression in rat brain is also increased after prenatal hypoxia (Nalivaeva et al., 2004) it is possible that TTR increase might also function as a measure to protect the brain from potential accumulation of neurotoxic levels of Aβ and partially compensate for any reduction in NEP activity. However, TTR itself might undergo protein misfolding and aggregation leading to TTR amyloidosis (Coles and Young, 2012).

TREATMENT OF THE CONSEQUENCES OF PRENATAL HYPOXIA

There are several strategies to protect the developing brain against pathological effects of prenatal hypoxia which can be applied both during the pregnancy and in newborns. Some pharmacological approaches include maternal treatment with glutamate antagonists to prevent neuronal cell death caused by hypoxia-induced excitotoxicity. Epidemiological studies suggest that administration of magnesium sulfate ($MgSO_4$) to pregnant women with pre-eclampsia or during pre-term labor was protective against development of cerebral palsy in infants (Schendel et al., 1996). Further animal experiments revealed that injection of pregnant mice on gestational day 17 with $MgSO_4$ during 4 h prior to chamber hypoxia (9% O_2, 2 h) reduced motor disabilities and protected cerebellar cells in the postnatal development of the offspring (Golan et al., 2004). However, this treatment also had some side effects on the cells in the cerebral cortex and hippocampus which diminishes enthusiasm toward this rather common therapeutic approach. At the molecular level in the guinea pig model of prenatal hypoxia it was shown that post-hypoxic administration of $MgSO_4$ prevents increased nuclear Ca^{2+} influx and protects nuclear membrane function in neuronal cells (Maulik et al., 2005).

To prevent excessive calcium influx to neuronal cells caused by hypoxia several antagonists to voltage-sensitive calcium channels have been developed over the years. They subsequently demonstrated neuroprotective effects in the developing brain when given to pregnant females before or during hypoxic episodes (for review see Rees et al., 1998). In an experimental model of prenatal hypoxia it was shown that nimodipine was able to prevent hypoxia-induced inhibition of brain growth and long-term behavioral deficits in rat offspring (Nyakas et al., 1994) whereas flunarizine (an L-type VSCC blocker) reduced neuronal death in a model of cerebral ischaemia in neonatal rats (Gunn et al., 1989, 1994). Although flunarizine is more efficient that nimodipine in improvement of cerebral blood flow, it can cause fetal hypotension resulting in pre-term death, which diminishes its therapeutic value in pregnancy (Gunn et al., 1989). More recent clinical studies have revealed that $MgSO_4$ is more effective than nimodipine in women with severe pre-eclampsia in preventing seizures while demonstrating no significant difference to the neonatal outcome (Belfort et al., 2003).

Treatment of pregnant women with synthetic glucocorticoids is a widely accepted practice to reduce systemic morbidity and mortality in infants after premature birth caused by pathologic pregnancies including prenatal hypoxic and ischaemic insults (Barrett et al., 2007; Bennet et al., 2012). Clinical studies suggest that betamethasone and dexamethasone treatment reduce the rate of neonatal morbidity and mortality. Dexamethasone was also more effective in preventing brain hemorrhage in pre-term neonates (Elimian et al., 2007). However, the impact of glucocorticoid treatment on the developing brain during pregnancy is still not sufficiently investigated and their postnatal application often results in impaired development of brain function (French et al., 2004). Existing animal studies suggest that dexamethasone treatment during pregnancy reduces brain tolerance to hypoxia (Carlos et al., 1991). Moreover, maternal hypoxia can lead to a significant decrease in the levels of expression of glucocorticoid receptors and abolishes the neuroprotective effect of dexamethasone in rat pup brain (Gonzalez-Rodriguez et al., 2014).

During pregnancy the levels of neuroactive steroids increases both in the maternal circulation and developing brain. These

steroids contribute to a great extent to brain development and, under hypoxic conditions, their production is significantly increased (Hirst et al., 2009). Elevated levels of allopregnanolone, the most potent $GABA_A$ receptor modulator, plays an important role in protecting the brain against excitotoxicity caused by traumatic injuries and hypoxia (Djebaili et al., 2005). Treatment of pregnant rats with allopregnanolone was shown to protect the developing fetal hippocampus against hypoxic insults (Fleiss et al., 2012) although safe protocols for its clinical applications still require refinement.

A rather promising pharmacological strategy against the damaging effects of intrauterine hypoxia and prenatal asphyxia around birth is administration of selective nNOS inhibitors which was shown to reduce cerebral palsy outcome in a rabbit model (Yu et al., 2011). The underlying mechanism of this therapeutic approach is that nNOS (but no iNOS) inhibition preserves the integrity and functioning of brain mitochondria increasing neuronal cell survival (Rao et al., 2011). This promising approach is still being tested in larger animal models (Drury et al., 2013, 2014). Recently, studies have suggested that postnatal erythropoietin treatment might also be a beneficial therapeutic strategy since its administration results in recovery of structural integrity and myelination in developing rat brain and improves behavioral and memory deficits caused by perinatal brain injury (Robinson et al., 2018).

There are some other reports on beneficial approaches which include intranasal administration to rats on postnatal days 13–15 of a homeostasis protective peptide, Pro-Gly-Pro capable of preventing the negative effects of acute prenatal hypoxia (Graf et al., 2006). The effect of this peptide was similar to the effects of other neuroprotective peptides such as semax and β-casomorphin-7 developed by scientists from Moscow State University (Maslova et al., 2001). The authors explain the observed protective effect of Pro-Gly-Pro by its ability to normalize blood supply to organs and tissues (Badmaeva et al., 2006).

Since prenatal hypoxia induces a wide range of oxidative stress reactions in developing fetal brain (Sab et al., 2013) treatment with antioxidants during hypoxic pregnancies is also beneficial against fetal metabolic impairment (Okatani et al., 2000). Given prenatally, antioxidants were shown to protect rat brain neurones against high-altitude hypoxia even in postnatal life (Wu et al., 2011). Prenatal supplementation with docosahexaenoic acid has also been found protective against neuronal damage in a rat model of perinatal hypoxia-ischaemia (Berman et al., 2009). Administration of L-arginine was also found neuroprotective for the fetal brain against maternal stress during pregnancy (Mahmoudi et al., 2016).

With regard to the early postnatal interventions against neuroinflammation caused by perinatal hypoxia, application of hypothermia and N-acetylcysteine is currently considered to be effective although the underlying neuroprotective mechanisms are not yet fully investigated (Jenkins et al., 2009). Hypothermia combined with cannabidiol treatment were also shown to provide additive protecting effect against hypoxia on neuronal metabolism in the developing brain of new-born piglets when applied shortly after the insult (Lafuente et al., 2016).

For minimizing the risk factor of insufficient oxygen supply to the fetal brain during pathological pregnancy maternal voluntary exercise was shown to be beneficial (Akhavan et al., 2012) providing long-lasting protection against neurodegeneration and AD-related pathology in later life (Herring et al., 2012).

In recent years hypoxic pre- and post-conditioning has been suggested as a non- invasive method to improve brain function after severe hypoxic episodes (for review see Rybnikova and Samoilov, 2015). This approach was also shown to be protective against the damaging effects of prenatal hypoxia (Nalivaeva et al., 2004; Basovich, 2013). The neuroprotective effect of hypoxic preconditioning was also confirmed in a chick embryo model of prenatal hypoxia via induction of HIF-1 mechanisms (Giusti and Fiszer de Plazas, 2012).

There are reports suggesting that ozone therapy, which is beneficial for treating various medical conditions e.g., peritonitis, chronic skin ulcers, infected wounds, initial gangrene and burns (Valacchi et al., 2005; Safwat et al., 2017) might have application for treatment of neurological disorders (Re et al., 2008; Frosini et al., 2012). Although ozone, as a very reactive air pollutant, can cause various complications, its safe and well-defined clinical administration might reinforce the antioxidant system of the organism and protect cells and tissues against oxidative stress (Smith et al., 2017). This is of particular importance in new-born organisms with an undeveloped endogenous antioxidant system (Davis and Auten, 2010). In relation to perinatal injuries, it was shown that ozone therapy decreases neuronal apoptosis and improves memory in rat pups subjected to hypoxic/ischaemic brain injury on P7 (Resitoglu et al., 2018). However, protective application of this approach for treatment of neurological disorders caused by prenatal hypoxia is still rather controversial.

Another complementary approach to restore the impaired brain functions caused by hypoxia in pregnancy and at birth includes pre- and postnatal environmental enrichment (Durán-Carabali et al., 2017). Enriched environment was shown to increase enzyme activity of the important neuropeptidase neprilysin capable of degrading Aβ and reducing amyloid burden in Alzheimer's disease mice as well as to upregulate expression of genes associated with learning and memory (Lazarov et al., 2005).

Searching for strategies to increase expression and activity of the amyloid-degrading enzymes, in particular of neprilysin, in rat brain we have found that valproic acid not only upregulates expression of NEP levels in the cortex and hippocampus reduced by prenatal hypoxia but also improves cognitive functions of the adult animals (Nalivaeva et al., 2012). This process involves regulation of the *NEP* gene via binding of the C-terminal fragment of APP (termed AICD) to the *NEP* promoter (Belyaev et al., 2009). In a cell model of hypoxia the reduced NEP expression and activity were shown to be related to an increased cleavage of AICD due to upregulation of caspases and this process was reversed by caspase inhibition (Kerridge et al., 2015). Increased caspase expression and activity observed in the cortex of rats subjected to prenatal hypoxia also correlated with decreased AICD and NEP levels which were restored after intraventricular administration to rats of the caspase-3 inhibitors, Z-DEVD-FMK or Ac-DEVD-CHO (Kozlova et al., 2015; Vasilev et al., 2016a). Administration of the inhibitors also resulted in

prolonged improvement of learning and short-term memory in rats subjected to prenatal hypoxia up to the levels of control animals when tested in the two-level maze even one and half months after injections (Vasilev et al., 2016a).

Antioxidants, in particular epigallocatechin gallate (EGCG), which was shown to increase NEP expression in cell models (Melzig and Janka, 2003), were also found capable to improve neurological deficits in our model of rat prenatal hypoxia (Zhuravin et al., 2018) and in a mouse model of Alzheimer's disease (Chang et al., 2015). The increase in NEP activity correlated with an improvement of rat performance in the radial maze and with restoration of both short- and long-term memory in the novel object recognition test (Zhuravin et al., 2011, 2018; Nalivaeva et al., 2012).

Undoubtedly, there are other pharmacological and epigenetic approaches to restore expression of genes down-regulated by hypoxia. Using cell models we have tested several compounds (e.g., Gleevec and bexarotene) which are able to upregulate expression of neprilysin and other amyloid-clearing proteins, including a transport protein transthyretin and insulin-degrading enzyme (Kerridge et al., 2014; Nalivaeva et al., 2016). These compounds are currently being tested in our rat model of prenatal hypoxia.

CONCLUDING REMARKS

Despite intensive studies of the molecular mechanisms underlying impaired brain development and functioning caused by prenatal hypoxia we are still far from a complete appreciation of all the changes at the molecular and epigenetic levels which shape individual development in postnatal life. Further studies using models of prenatal hypoxia will allow us and others to gain a deeper insight into the mechanisms of dysregulation of neuronal functions during fetal development and design new preventive strategies to restore brain integrity and cognitive functions.

AUTHOR CONTRIBUTIONS

NN has outlined and written the manuscript, AT has discussed and edited the manuscript, IZ has selected literature and discussed the manuscript.

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Prenatal Hypoxia in Different Periods of Embryogenesis Differentially Affects Cell Migration, Neuronal Plasticity, and Rat Behavior in Postnatal Ontogenesis

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Long-term effects of prenatal hypoxia on embryonic days E14 or E18 on the number, type and localization of cortical neurons, density of labile synaptopodin-positive dendritic spines, and parietal cortex-dependent behavioral tasks were examined in the postnatal ontogenesis of rats. An injection of 5′ethynyl-2′deoxyuridine to pregnant rats was used to label neurons generated on E14 or E18 in the fetuses. In control rat pups a majority of cells labeled on E14 were localized in the lower cortical layers V-VI while the cells labeled on E18 were mainly found in the superficial cortical layers II-III. It was shown that hypoxia both on E14 and E18 results in disruption of neuroblast generation and migration but affects different cell populations. In rat pups subjected to hypoxia on E14, the total number of labeled cells in the parietal cortex was decreased while the number of labeled neurons scattered within the superficial cortical layers was increased. In rat pups subjected to hypoxia on E18, the total number of labeled cells in the parietal cortex was also decreased but the number of scattered labeled neurons was higher in the lower cortical layers. It can be suggested that prenatal hypoxia both on E14 and E18 causes a disruption in neuroblast migration but with a different outcome. Only in rats subjected to hypoxia on E14 did we observe a reduction in the total number of pyramidal cortical neurons and the density of labile synaptopodin-positive dendritic spines in the molecular cortical layer during the first month after birth which affected development of the cortical functions. As a result, rats subjected to hypoxia on E14, but not on E18, had impaired development of the whisker-placing reaction and reduced ability to learn reaching by a forepaw. The data obtained suggest that hypoxia on E14 in the period of generation of the cells, which later differentiate into the pyramidal cortical neurons of the V-VI layers and form cortical minicolumns, affects formation of cortical cytoarchitecture, neuronal plasticity and behavior in postnatal ontogenesis which testify to cortical dysfunction. Hypoxia on E18 does not significantly affect cortical structure and parietal cortex-dependent behavioral tasks.

Keywords: prenatal hypoxia, brain development, cortex, neurogenesis, cell migration, neuronal plasticity, dendritic spines, rat behavior

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INTRODUCTION

The consequences of action of pathological factors during brain embryogenesis on development of brain structure and animal or human behavior in later life depend to a great extent on the period of pregnancy when they have been applied. However, the mechanisms underlying different outcomes of pathologic conditions are not fully understood. Development of the cortical plate of rat fetuses is a good model to investigate the specific character of damage in different cell populations resulting from insults at various stages of embryogenesis. The period of radial migration of neuroblasts into the cortical plate is crucial for development of proper cortical architecture including stratification and formation of minicolumns which underlie behavioral reactions and cognitive functions in postnatal ontogenesis (Rakic, 2006; Hammond et al., 2010; Sekine et al., 2011). Earlier it was shown that neurons fail to acquire their proper position and remain scattered within inappropriate cortical layers in the fetuses when exposed to ionizing radiation (Rakic, 1988), ultrasound (Ang et al., 2006), or toxic agents (Miller, 1986; Aronne et al., 2011) during the period of radial cell migration into the cortical plate resulting in behavioral dysfunctions (Caviness and Rakic, 1978).

Hypoxia is a very common prenatal pathology, which often leads to brain malfunctioning in the postnatal period. Although the effects of oxidative stress caused by hypoxia are short-term (within a few days), hypoxia in the embryonic period leads to various structural and functional abnormalities in the postnatal period (Zhuravin, 2002; Golan and Huleihel, 2006; Vasilyev et al., 2008; Zhuravin et al., 2009). Although there are some studies of neuroblast migration under the effect of prenatal stressors the data on their long-term consequences (Rakic, 1988; Ang et al., 2006; Aronne et al., 2011) are insufficient. Prenatal hypoxia was shown to cause functional abnormalities in postnatal ontogenesis of animals (Zhuravin et al., 2004; Golan and Huleihel, 2006; Dubrovskaya and Zhuravin, 2010) but the underlying mechanisms were not sufficiently analyzed. In particular, there was no comparative evaluation of the effects of hypoxia at different stages of pregnancy on the character of neuronal migration in embryogenesis and cortical organization and functions in early postnatal life and adulthood. The aim of this work was to investigate the consequences of prenatal hypoxia on different days of gestation and cortical neurogenesis (E14 or E18) on cell generation and migration into the developing cortical plate by evaluating the number, type, and localization of cortical neurons, the density of labile synaptopodin-positive dendritic spines and behavior in the postnatal ontogenesis of rats.

METHODS

Animals

All animal experiments were performed in accordance with the Directive #86/609 of the Council of European Communities for the protection of animals used for experimental and other scientific purposes and in accordance with the guidelines of the Russian Academy of Sciences (RAS) approved by the Scientific Council of the Institute of Evolutionary Physiology and Biochemistry of RAS, St. Petersburg, Russia.

Cell Labeling and Prenatal Hypoxia

Pregnant female Wistar rats (total $n = 64$) on the 14th or 18th day of gestation were injected with a DNA-replication marker 5′ethynyl-2′deoxyuridine (EdU, one intraperitoneal injection in a dose of 25 mg in 0.5 ml of saline) to label cells generated in the proliferative zone (Rakic, 1988) destined to the cortical layers V and VI (14th embryonic day—E14) or to the superficial cortical layers II-III (18th embryonic day—E18). Within the same day (1 h after injection) some females were exposed to acute normobaric hypoxia in a 100 l chamber where oxygen content was decreased from 20.7 to 7.0% and maintained at this level for 3 h. Concentration of CO_2 in the chamber was no >0.2% and the temperature was +22°C. Another group of EdU-injected animals was kept under normal oxygen content. The offspring of all groups of animals was analyzed in further experiments on different days of postnatal ontogenesis. When calculating the age of rat pups, the day of birth was taken as P0.

Animal Behavior

To analyze the effects of prenatal hypoxia on the functional state of brain sensorimotor cortex and development of animal reactions the following specific tests have been applied.

Whisker Placing Test

This test allows quantification of the functional state of the sensorimotor cortex and is widely used for assessing the severity of the lesions of this brain region after experimental stroke or other brain pathologies (Schallert, 2006). On days P0 to P20 the rats from the control group ($n = 13$) and rats subjected to prenatal hypoxia on E14 ($n = 14$) or E18 ($n = 16$) were tested for whisker placing reaction. For this an animal was lifted by the tail and the whisker area was touched by a sharp end of a horizontally oriented pencil (Thullier et al., 1997). The forelimb placing reaction was assessed for 1 min by a four-point scale: 0–absence of reaction; 1–weak, chaotic elevation of the limb without making contact with the stick; 2–rotatory movements of the head and elevation of the limb to the support; and 3–extension of the snout in the direction of the stick with accurate and precise lifting of both forelimbs onto the support. The mean ranking was then calculated for each animal and compared in the control and both hypoxic (E14 and E18) groups.

Training to Perform an Instrumental Reflex

Young (P20) or adult (P90) male rats were trained to touch or push a piston attached horizontally to the front wall of the experimental chamber with an automatic feeder and instruments for recording movements and delivery of reinforcement (Zhuravin and Bures, 1986, 1989) with some modifications (Dubrovskaya and Zhuravin, 1995; Zhuravin et al., 2002). Young rats of the control ($n = 20$), E14 ($n = 12$), and E18 ($n = 15$) groups were trained only to touch the piston while adult rats of the control ($n = 13$), E14 ($n = 14$), and E18 ($n = 16$) groups were trained to push the piston for longer than a specified period of time (usually 50 ms). Every day during two cycles of

training, consisting of 64 movements each, the rats were trained to use the forelimb to touch (young rats) or to press (adult rats) the piston. Young rats were considered being able to learn the reflex well when they touched the piston during all 64 movements in the cycle. Adult rats were considered to learn the reflex well when the average time of pushing the piston in each session was ≥50 ms for 6 or 7 successive sessions ($p < 0.05$; *t*-tests). The rats whose average pushing time in a session did not significantly change during the training were considered being unable to learn the test.

Analysis of Migration and Positioning OF Cortical Neurons Generated on E14 or E18

To evaluate the position of neuronal cells generated on E14 or E18, the pups of female rats injected with EdU were decapitated on P5 when the number of labeled cells is high enough to be analyzed. The number of pups in each group is given in **Table 1**. The brains were extracted, fixed in 10% formalin in phosphate-buffered saline (PBS, pH 7.4), cryoprotected in 20% sucrose in PBS, frozen, and sectioned at the coronal plane. Brain slices (20 μm) were stained with Hoechst 33342 to reveal cortical stratification and to count the total number of cells. To expose the final position of the cells generated on E14 or E18 and labeled with EdU the sections were stained using the Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Invitrogen, USA) following the manufacturer's protocol. In selected sections, the cells were also immunolabeled with a neuronal marker Fox3 (ab104224; Abcam, Bristol, UK; dilution 1:1000) to prove that the EdU-positive cells are neurons. To analyze the pattern of migration and positioning of E14- or E18-generated cortical neurons, the number of EdU-labeled cells within a 500 μm—wide area of the parietal cortical tissue from the layer I to VI from Bregma +0.20 mm (Paxinos and Watson, 2006) (see **Figure 2B**) was calculated. A ratio of the EdU-positive cells to total Hoechst-labeled cells was calculated in each investigated cortical slice. The mean ratio for different animal groups was compared using unpaired two-tailed Mann–Whitney *U*-tests ($p < 0.05$). The ratio of the number of EdU-labeled cells within the superficial cortical layers (the layers II-III) and lower layers (V-IV) layers was also calculated and compared in control and hypoxia-exposed pups using the unpaired two-tailed Mann–Whitney *U*-test ($p < 0.05$).

Morphological Analysis of Cortical Neurons in Postnatal Ontogenesis

Light Microscopy

The rats ($n = 8$ for each group) were decapitated on P10, P20, P30, P60, and P90 and their brains were fixed in 10% formalin in PBS (pH 7.4), cryoprotected in 20% sucrose in PBS, frozen and sectioned at the coronal plane. Brain slices (20 μm) were Nissl stained and analyzed by light microscopy using an ImagerA microscope (Zeiss, Germany). Ten cortical slices (the same area of the parietal cortex as in the EdU visualization experiment) were analyzed for each animal. The area of the cell body, as well as the longest and the shortest axes with the nucleus being oriented in the section plane were measured for each cell using the "Videotest: Master Morphology 4.2" computer program (VideoTest, Russia). The cells were classified by two numerical parameters: the size (area) and shape (ratio of the longest and shortest axes passed through the cell nucleus) of the cell body using cluster analysis (Ward's method), as described in (Vasilyev et al., 2008). The size and shape of the cell body are essential parameters to differentiate the projection cells (pyramidal cells with a high axis ratio) and interneurons (with axis ratio about 1) in different cortical layers. It allows the analysis of the number of cells belonging not only to different morphotypes but also to different functional groups. The mean number of cells belonging to the different morphotype classes was counted for each cortical slice in rats exposed to hypoxia on E14 or E18 and compared to control group. The two-tailed t-student test for independent samples ($p < 0.05$) was used to compare the values obtained in control and hypoxia-exposed animals.

Immunohistochemistry

We have also performed an analysis of the number of cells labeled by neuronal and glial marker proteins in the same area of the parietal cortex as in the EdU visualization experiment. For this a sequence of sections (10 sections per animal with 80 μm between them) were randomly selected and used for immunolabeling. Sections were incubated for 3 h at 37°C in

TABLE 1 | Pattern of migration and positioning of cortical neurons generated on E14 or E18.

	Number of pups	Number of cortical slices analyzed	Number of counted cells	Total number of EdU-labeled cells per 500 μm wide cortical column (mean ± SEM)	Number of EdU-labeled cells scattered in the layers II-III (for the labeling on E14), or in V-VI (for E18) cortical layers, presented as % to the total number of EdU-labeled cells (%)
Control rats (EdU labeling on E14)	14	140	586460	17.5 ± 0.9	19.1 ± 5.2
Hypoxia on E14 (EdU labeling on E14)	12	120	479400	9.7 ± 0.8	42.0 ± 9.4
Control rats (EdU labeling on E18)	9	90	377460	62.8 ± 0.6	17.3 ± 7.2
Hypoxia on E18 (EdU labeling on E18)	10	100	407800	23.1 ± 0.8	28.1 ± 9.8

The mean number of EdU-labeled cells in the parietal cortex of ras subjected to hypoxia on E14 or E18 is lower than in control animals. The number of EdU-positive cells per 500 μm wide cortical column (500 μm -wide area of the cortical tissue from the layers I to VI) is presented as mean ± SEM. The mean number of EdU-labeled cells scattered in the II-III cortical layers for cell labeled on E14 or in the layers V-VI for cell labeled on E18 is given as % of total EdU-labeled cells. These cells failed to migrate to the cortical layers to which they are destined.

PBS containing 2% bovine serum albumin and 0.3% Triton X-100 (Merck, Darmstadt, Germany), and then overnight with a primary antibody at 4°C. Visualization of the glial cells with glial fibrillary acidic protein (GFAP-positive glia) was performed using a monoclonal Cy3-conjugated anti-GFAP antibody (C9205, Sigma, 1:100) and analyzing the emission of Cy3 at 550–650 nm wavelength. The number of neurons was analyzed using mouse monoclonal anti-Fox3 (=NeuN) antibody (ab104224; Abcam, Bristol, UK; dilution 1:500). After thorough rinsing, all sections were incubated for 1 h at 37°C with a FITC-conjugated secondary antibody against mouse IgG (ab7007, Abcam, 1:200) diluted in the blocking serum. The rat hepatic tissue was used as a negative control. For excitation of the signal a 488 nm wavelength He/Ar laser was used with emission being observed in the 496–537 nm wavelength range. The number of immunopositive cells with the optical density of cell bodies 300% higher than the background was compared in the parietal cortex of control and hypoxia-exposed animals using the unpaired two-tailed Mann–Whitney U-test ($p < 0.05$).

Distribution of Synaptopodin-Positive Dendritic Spines in the Parietal Cortex

Immunohistochemistry

Synaptopodin is a specific marker of labile mushroom spines, which play an important role in neuronal network plasticity (Deller et al., 2003). The number of labile axon-spine inter-neuronal contacts in the molecular (Ist) layer of the parietal cortex in adult (P90) control rats as well as in animals exposed to hypoxia on E14 or E18 was analyzed using a random sampling method described in the previous section. Selected sections were incubated with a rabbit anti-synaptopodin antibody (S9567, Sigma 1:200) overnight at 4°C and then with a phycoerythrine PE-conjugated secondary antibody against rabbit IgG (ab7007, Abcam, 1:200). Some slices were double stained for synaptopodin and a postsynaptic marker protein PSD95 (mouse monoclonal ab2723 antibodies, Abcam, 1:1000, overnight) or synaptopodin and a presynaptic terminal marker protein synaptophysin (S5768, Sigma, 1:1000, overnight) and visualized by a FITC-conjugated secondary antibody against mouse IgG (ab7007, Abcam, 1:200). Rat hepatic tissue was used as a negative control. For excitation of PE and FITC a 488 nm wavelength He/Ar laser was used with emission of PE being detected at 652–690 nm and FITC at 496–537 nm wavelength. The number of synaptopodin-positive spines was calculated in a 100 × 100 μm area of the molecular layer of the parietal cortex just above the area where we performed counting of the number of neurons. The data obtained in hypoxia-exposed animals were compared with controls using the unpaired two-tailed Mann–Whitney U-test ($p < 0.05$).

Western Blotting

The parietal cortical tissue sections (the same areas of the parietal cortex as described in the previous experiments) of adult (P90) control rats and of animals subjected to hypoxia on E14 or E18 were homogenized on ice in 0.5 M Tris-HCl buffer (pH 7.4) with 1% (of volume) of Triton X-100 and centrifuged at 2500 g (5 min, 4°C.) The amount of synaptopodin protein in the supernatant was analyzed by electrophoresis in 8% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS), followed by immunoblotting on polyvinylidene difluoride (PVDF) membranes. The membranes were incubated overnight at 4°C with a rabbit anti-synaptopodin antibody S9567 (Sigma 1:1000). Immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, ab6721, Abcam, 1:5000, 1 h) and visualized using Optiblot ECL Ultra Detect Kit (1.2 pg-2 ng) (ab133409, Abcam,). The amount of actin in the same samples was analyzed using a rabbit anti-actin antibody (A5060, Sigma, 1:5000). The amount of a postsynaptic marker protein PSD95 was analyzed using a mouse monoclonal antibody (ab2723, Abcam, 1:1000, overnight) followed by a secondary horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (ab6808, Abcam, 1:5000, 1 h). The amount of an active presynaptic terminal marker protein synaptophysin was analyzed using a mouse primary antibody S5768 (Sigma, 1:1000, overnight) and visualized with the ab6808 secondary antibody. The relative intensity of immunoreactive bands on the membranes was quantified by densitometry using the "Videotest: Master Morphology 4.2" software program (VideoTest, Russia). Rat hepatic tissue was used as a negative control. A ratio of the intensity of the bands corresponding to the protein of interest (synaptopodin, PSD95 or synaptophysin) to actin was calculated for each sample and the data of hypoxia-exposed animals were compared with controls using unpaired two-tailed Mann–Whitney U-test ($p < 0.05$).

Statistics

The data were analyzed using the PAST statistical software package (Hammer et al., 2001; http://palaeo-electronica.org/2001_1/past/issue1_01.htm). All data are presented as mean ± SEM. The data for hypoxia-exposed pups were compared with a control using the unpaired two-tailed t-student tests or Mann–Whitney U-test ($p < 0.05$). The Fisher's exact significance test (Mehta et al., 1984) was used in the analysis of the instrumental reflex data obtained in two animal groups.

RESULTS

Animal Behavior

Whisker Placing Test

Analyzing animal motor reactions during the first postnatal month (from P0 to P20) we have observed that the pups exposed to prenatal hypoxia on E14 had a significant delay in the development of the whisker placing reaction compared to controls and pups subjected to prenatal hypoxia on E18. The animals subjected to prenatal hypoxia on E14 compared to control rats or rats exposed to hypoxia on E18 starting from P0 had a significantly lower score of forelimb placing after stimulation of their whiskers and this difference practically disappeared at P20 (**Figure 1A**). It indicates a disruption in development of the sensorimotor coordination caused by prenatal hypoxia on E14 but not on E18.

Training to Perform an Instrumental Reflex

Using an instrumental learning paradigm, we have revealed that prenatal hypoxia on E14 resulted in reduced ability to learn a

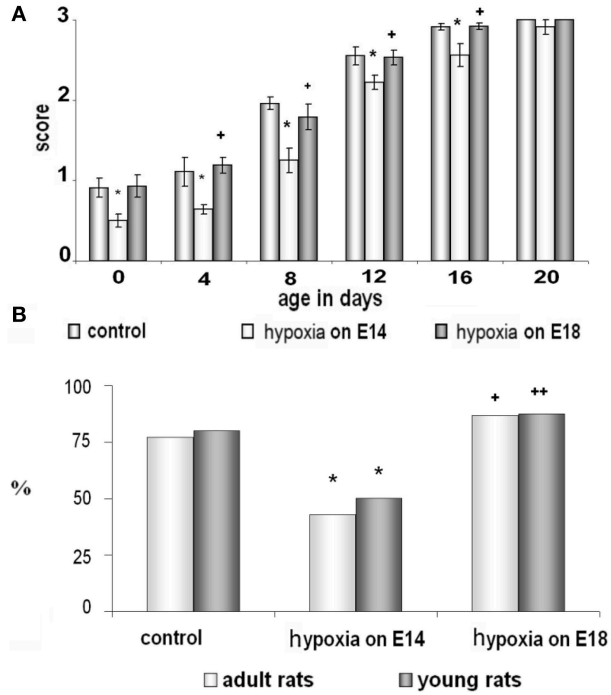

FIGURE 1 | Behavioral testing of control rats and rats subjected to prenatal hypoxia on E14 and E18. (A) Development of the whisker placing reaction during the first month of postnatal ontogenesis on P0-P20. Data presented as the mean of whisker placing score ± SEM. Small asterisks and large asterisks—significant ($p \leq 0.05$ and $p \leq 0.001$, correspondingly) difference compared to control rats. Small cross and large cross—significant ($p \leq 0.01$ and $p \leq 0.001$, correspondingly) difference between the groups of rats exposed to hypoxia on E14 and E18. **(B)** Ability of young (dark columns) and adult (white columns) rats to learn an instrumental reflex presented as percent of animals which learn the task well from the total amount of animals taken as 100%. *Significant ($p \leq 0.05$) difference between the control rats and rats subjected to hypoxia on E14. + and ++-significant ($p \leq 0.05$ and $p \leq 0.01$, correspondingly) difference between two groups of rats exposed to hypoxia on E18 and on E14.

complex operant reflex (reaching or reaching with pushing by a forepaw) when tested both in young (P20) and adult (P90) animals (**Figure 1B**). The ability of rats to learn the task was expressed as a ratio of animals able to learn the task well to the total number of trained animals. In the group of rats, exposed to hypoxia on E18, the number of young and adult rats able to learn the reflex was not statistically different from the age-matched controls (**Figure 1B**). Thus, prenatal hypoxia on E14 caused significant disruption of motor behavior and learning ability of rats connected with their cortical functions, while prenatal hypoxia on E18 did not cause such changes compared to controls.

Analysis of EdU-Labeled Cortical Neurons Generated on E14 or E18, in Rat Postnatal Ontogenesis

Control Group

Double-labeling cells on P5 with EdU and a neuron-specific marker Fox3 (EdU+Fox3) showed that the majority of the cells generated on E14 or E18 in control, as well as in hypoxia-exposed animals, were neurons (**Figures 2A,C**). A majority of the cells EdU-labeled on E14 were localized in the lower cortical layers V-VI (**Figure 2E**, **Table 1**) while the cells labeled on E18 were mainly found in the superficial cortical layers II-III (**Figure 2G** and **Table 1**). In the course of rat development (P10-P20) the number of labeled cells decreased to 54.0 ± 8.7% compared to P5 (**Figures 2D,I,J** and **Table 1**).

Hypoxia on E14

Examination of brain tissue by Nissl staining on P5 has not revealed any mass cell death, changes in cell morphology, or any difference in cytoarchitecture of the cortical plate between the control and hypoxia-exposed (E14 or E18) pups. However, there was a decrease in the number of EdU-positive neurons in the parietal cortex of 5-day-old pups subjected to hypoxia on E14 (1.64 ± 0.01% of total cell number, see **Table 1**) compared to controls (2.93 ± 0.02% of total cell number) which suggests that prenatal hypoxia on E14 causes a decrease in neuroblast division rate. In animals subjected to hypoxia, as well as in controls, the majority of cells labeled on E14 were localized in the lower cortical layers V-VI. However, in 5-day-old pups subjected to hypoxia on E14, the number of labeled neurons scattered within the superficial layers of the parietal cortex was increased compared to controls (**Figure 2F**, **Table 1**) suggesting that prenatal hypoxia on E14 caused a failure of some neuroblasts to migrate into their proper position in the V-VI cortical layers.

Hypoxia on E18

On P5 in rats subjected to hypoxia on E18 we have also observed a decrease in the number of EdU-positive neurons in the parietal cortex (1.84 ± 0.02% of total cell number compared to 3.86 ± 0.01% in controls, see **Table 1**) suggesting that hypoxia at this stage of embryogenesis also caused a decrease in neuroblast division rate. The majority of cells labeled on E18 were localized in the superficial (II-III) cortical layers of rat parietal cortex (**Figure 2H** and **Table 1**). However, the number of EdU-positive cells scattered in the lower (V-VI) layers of the parietal cortex was higher than in controls (**Table 1**) suggesting that some neuroblasts failed to migrate into their proper position in the II-III cortical layers.

Morphological Analysis of Cortical Neurons in Rat Postnatal Ontogenesis

Control Rats

Analysis of cell morphology in rat parietal cortical tissue by Nissl staining on P10–P90 has been performed using such parameters as the size (area) and shape (ratio of the longest to the shortest axes) of the cell body. According to such parameters we have divided the cortical cells into four morphotypes according to DeFelipe and Fariñas (1992): 1–small non-pyramidal cells (glial cells and small non-pyramidal neurons) scattered in all cortical layers; 2–big non-pyramidal neurons (cortical interneurons) mostly localized in the layers II-III; 3–small pyramidal neurons (the ratio of the longest to the shortest axes greater than 2.5) localized in the layers II-III and V-VI of the cortex; and 4–big

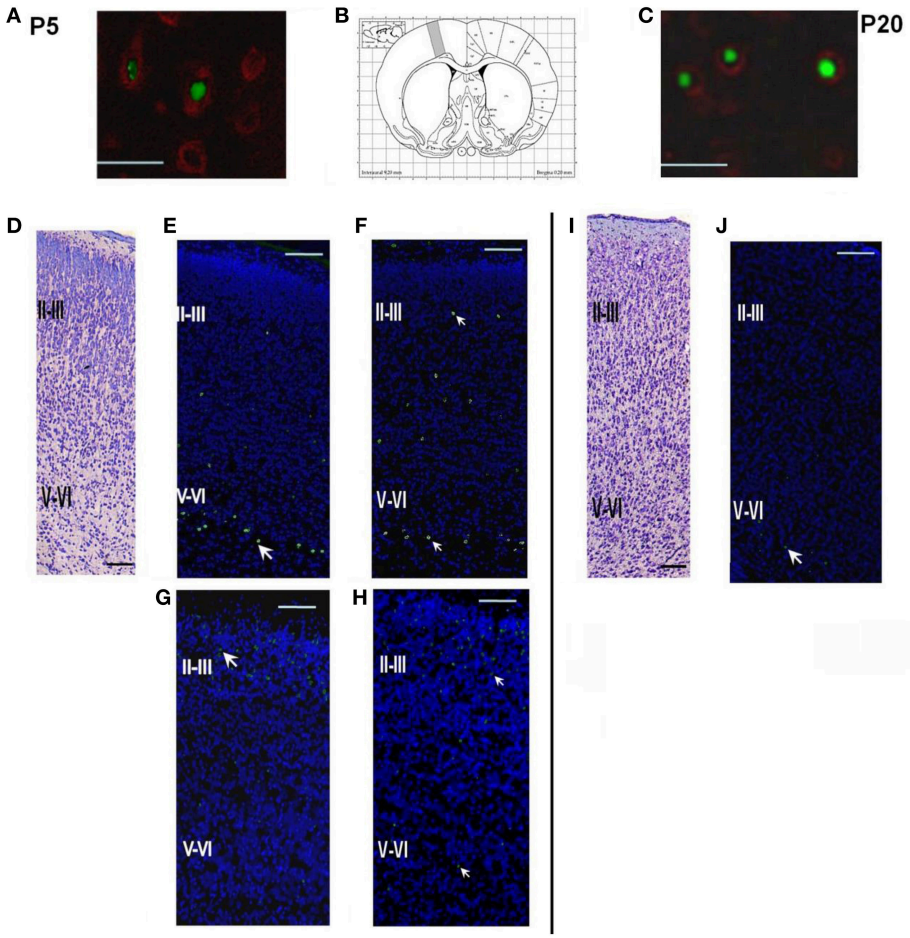

FIGURE 2 | Structure of the parietal cortex on P5 and P20 in control rats and rats subjected to prenatal hypoxia on E14 or E18. (A) Double labeling of cortical tissue in a control rat on P5 with EdU labeling performed on E14. EdU-positive cell nuclei were stained by AlexaFluor488, Fox3-positive neurons stained by PE-conjugated secondary antibodies. Scale bar 50 μm. **(B)** Position of the analyzed areas in the parietal cortex (Bregma +0.20 mm; Paxinos and Watson, 2006). **(C)** Double labeling of cortical tissue in a control rat on P20 with EdU labeling performed on E14. EdU-positive cell nuclei were stained by AlexaFluor488, Fox3-positive neurons stained by PE-conjugated secondary antibodies. Scale bar 50 μm. **(D)** A coronal section of the cortical tissue of a control rat on P5, Nissl staining. Scale bar 200 μm. **(E)** A coronal section of the cortical tissue of a control rat on P5. EdU labeling (indicated by an arrow) on E14. The majority of the cells labeled on E14 are placed in the lower (V-VI) cortical layers. **(F)** A coronal section of the cortical tissue of a rat, exposed to hypoxia on E14, on P5. EdU labeling was performed on E14. **(G)** A coronal section of the cortical tissue of a control rat on P5 with EdU labeling performed on E18. The majority of the cells labeled on E18 are placed in the superficial cortical layers II-III. **(H)** A coronal section of the cortical tissue of a rat, exposed to hypoxia on E18, on P5. EdU labeling was performed on E18. **(I)** A coronal section of the cortical tissue of a control rat on P20, Nissl staining. Scale bar 200 μm. **(J)** A coronal section of the cortical tissue of a control rat on P20. EdU labeling performed on E14.

pyramidal cells (the ratio of the longest to the shortest axes greater than 2.5, the longest axis is more than 15 μm) mostly localized in the V-VI layer.

Hypoxia on E14

In pups exposed to hypoxia on E14, the number of big pyramidal neurons in the layers V-VI of the parietal cortex on P10-P20 was lower compared to age-matched controls. At later stages of postnatal development (P20-P30) we have also observed a decrease in the number of small pyramidal and non-pyramidal cells in the layers II-III of the parietal cortex of hypoxic rats (**Table 2**). Thus, the total number of pyramidal neurons in the layers II-III and V-VI of the cortex after prenatal hypoxia on E14 was reduced on P10-P30 by 20–30% compared to control. The number of Fox3-positive neurons was also decreased on P10-P30 (**Table 2**) although in older rats (P60-90) there were no differences in the number of these neurons compared to an age-matched control. At all stages analyzed we have not seen any changes in the number of GFAP-positive cells after prenatal hypoxia (**Table 2**).

Hypoxia on E18

Unlike hypoxia on E14, prenatal hypoxia on E18 caused no changes in the ratio of pyramidal to non-pyramidal cells in developing cortex and there was no reduction in the total number of pyramidal neurons on P10-P30 (**Table 3**).

TABLE 2 | Number of cortical neurons belonging to different morphological types in rats exposed to hypoxia on E14.

Type of cells	Days after birth				
	P10	P20	P30	P60	P90
	The number of cortical cells in rats exposed to hypoxia on E14 in % to control				
Small non-pyramidal	101.7 ± 15.9	101.4 ± 18.7	100.8 ± 14.0	103.1 ± 15.5	110.7 ± 11.4
Big non-pyramidal	100.5 ± 9.8	81.6 ± 6.5*	84.3 ± 7.2*	102.7 ± 9.7	93.8 ± 16.3
Small pyramidal	92.8 ± 10.4	84.1 ± 7.2*	85.6 ± 9.8*	94.1 ± 15.2	105.8 ± 10.5
Big pyramidal	67.1 ± 11.3**	72.4 ± 8.3**	90.7 ± 12.9	100.6 ± 7.4	97.9 ± 15.5
Total cell number	78.6 ± 7.5**	59.2 ± 10.2**	92.9 ± 14.6	94.7 ± 8.4	97.9 ± 9.1
GFAP-positive cells	98.3 ± 5.1	105.8 ± 10.2	117.4 ± 12.5	104 ± 6.4	93.3 ± 6.5
Fox3 (NeuN)-positive	79.2 ± 6.3*	69.4 ± 7.1**	74.1 ± 5.4*	93.5 ± 7.7	105 ± 5.8

*Neural cells were classified by their size (area) and shape (ratio of the longest to the shortest axes) of the cell body. Data are presented as the ratio (mean number of cells in rats exposed to hypoxia/mean number of cells in control rats) in % ± Standard error of the difference between the means of two samples. 100%—cell number in control rats. In each group of animals (n = 8) 10 cortical tissue areas of each animal were analyzed by Nissl staining or immunohistochemistry. *$p \leq 0.05$; **$p \leq 0.001$ using two-tailed t-student tests for independent samples.*

TABLE 3 | Number of cortical neurons belonging to different morphological types in rats exposed to hypoxia on E18.

Type of cells	Days after birth				
	P10	P20	P30	P60	P90
	The number of cortical cells in rats exposed to hypoxia on E18 in % to control				
Small non-pyramidal	97.1 ± 12.6	110.4 ± 11.2	100.0 ± 11.5	92.0 ± 10.1	115.8 ± 21.1
Big non-pyramidal	95.0 ± 15.3	95.7 ± 10.5	115.6 ± 8.8	94.5 ± 7.8	99.7 ± 17.4
Small pyramidal	104.3 ± 5.8	101.4 ± 8.2	92.4 ± 15.2	115.1 ± 19.9	92.8 ± 15.2
Big pyramidal	102.8 ± 3.7	110.6 ± 11.6	108.4 ± 7.2	100.0 ± 4.4	97.9 ± 15.5
Total cell number	100.5 ± 4.4	99.1 ± 3.8	90.7 ± 9.8	105.2 ± 9.4	105.0 ± 9.2
GFAP-positive cells	101.7 ± 3.2	104.6 ± 4.8	105.2 ± 6.7	104 ± 6.4	94.7 ± 8.9
Fox3 (NeuN)-positive	96.8 ± 8.4	108.5 ± 9.6	91.4 ± 11.5	93.5 ± 7.7	94.1 ± 10.5

Neural cells were classified by their size (area) and shape (ratio of the longest to the shortest axes) of the cell body. Data are presented as the ratio (mean number of cells in rats exposed to hypoxia/mean number of cells in control rats) in % ± Standard error of the difference between the means of two samples. 100%—cell number in control rats. In each group of animals (n = 8) 10 cortical tissue areas of each animal were analyzed by Nissl staining or immunohistochemistry. No changes (compared to control) in rats, exposed to hypoxia on E18, were observed.

Synaptopodin Distribution in the Cortical Tissue of Adult Rats (P90)

Control Rats

The pattern of distribution of the actin-associated protein synaptopodin in control adult rats on P90 is given in **Figure 3A** demonstrating that the majority of this protein is located in dendritic spines (immunopositive dots, 1 μm in diameter) in the molecular layer of brain cortex. On the other hand, the immunolabeled postsynaptic protein PSD95 was mostly placed near the synaptophysin-positive presynaptic terminals in different (but mostly in the molecular) cortical layers.

Hypoxia on E14

Prenatal hypoxia on E14 resulted in a decreased number of synaptopodin-positive spines in the molecular layer of the cortex of adult rats (**Figure 3B**). Moreover, hypoxia also resulted in decreased overall expression of synaptopodin protein levels compared to controls (**Figures 3C,D**). However, there were no changes in the amounts of the postsynaptic marker protein PSD95 and presynaptic marker protein synaptophysin (**Figures 3C,D**). As such we can conclude that the hypoxia on E14 resulted in a specific decrease in the number of labile synaptopodin-positive dendritic spines in the first cortical layer belonging to the apical dendrites of pyramidal neurons but did not cause significant changes in the number of PSD95-positive postsynaptic terminals and total synaptic activity. It is remarkable that in the cortical tissue of young (P30) rats exposed to hypoxia on E14 there were decreased amounts of both postsynaptic proteins synaptopodin and PSD95 (**Figures 3E,F**) which might lead to some abnormalities in dendritic spines formation.

Hypoxia on E18

In rats exposed to hypoxia on E18 we have not observed any changes either in the number of synaptopodin-positive dendritic spines in the molecular layer of the cortex (**Figure 3B**) or in the total synaptopodin, synaptophysin, or PSD95 content (**Figure 3D**).

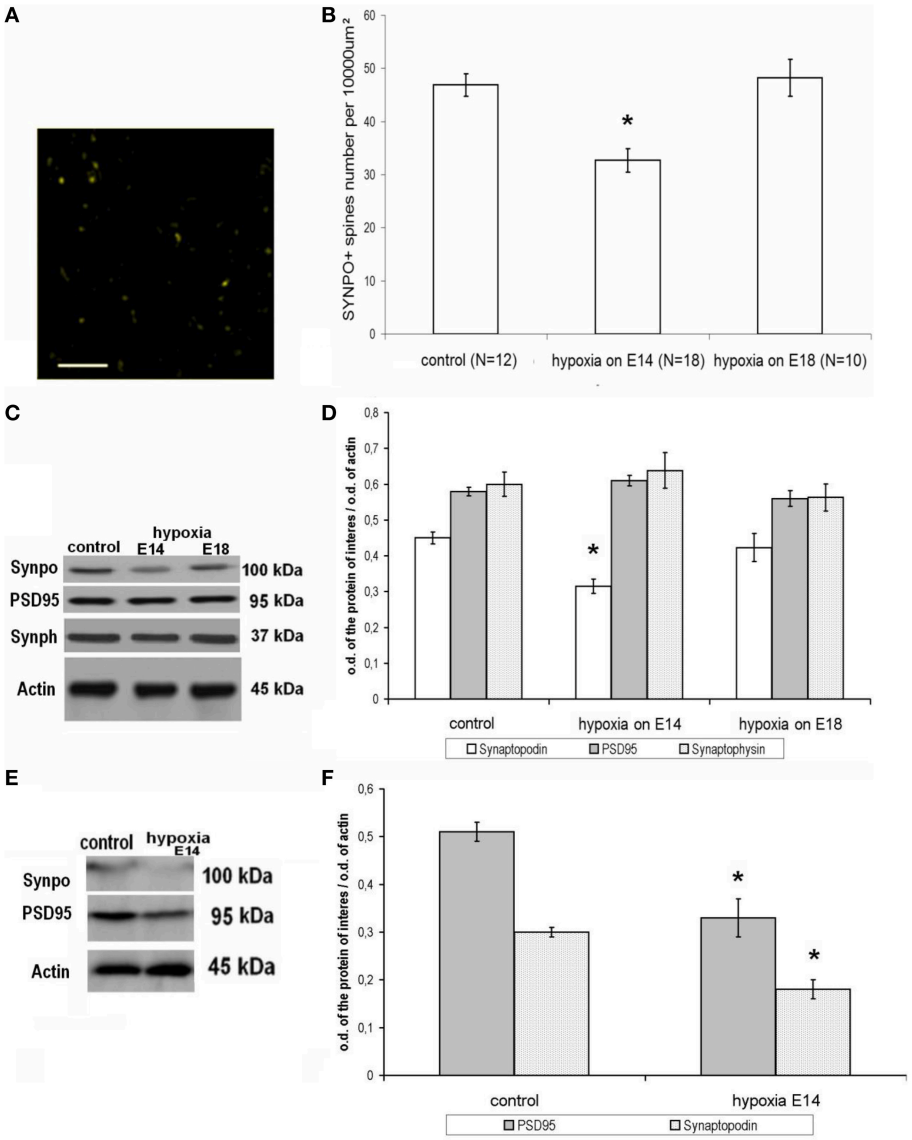

FIGURE 3 | Expression and distribution of synapse-associated proteins in rat cortical tissue on P90. (A) Distribution of an actin-associated protein synaptopodin in the molecular layer of the parietal cortex, adult control rat. Scale: 10 μm. Indirect immunofluorescence, excitation—488 nm wavelength, emission—552–690 nm wavelength. **(B)** Number of labile synaptopodin-labeled dendritic spines in the molecular layer of the parietal cortex in adult rats (P90). Ordinate: number of synaptopodin-positive spines per 100 × 100 μm area. *Statistically significant changes compared to the control ($p \leq 0.001$). **(C)** Immunoblotting of proteins from adult rat cortical tissue with antibodies against synaptopodin (100 kDa), PSD95 (95 kDa), synaptophysin (37 kDa), and actin (45 kDa). **(D)** Ratio of the intensity of the bands corresponding to the proteins of interest (white columns—spine-associated protein synaptopodin, gray columns—postsynaptic marker protein PSD95, dotted columns—presynaptic marker synaptophysin) to the intensity of the band of actin. Data presented as the mean ratio of optical density ± SEM. *Statistically significant decrease in synaptopodin expression in adult rats subjected to hypoxia on E14 compared to controls, $p \leq 0.05$. No significant changes in PSD95 and synaptophysin can be seen $n = 8$ in each group of animals. **(E)** Electrophoretic separation of young (P30) rat cortical tissue proteins and subsequent immunoblotting against synaptopodin (100 kDa), PSD95 (95 kDa), and actin (45 kDa). **(F)** Ratio of the band intensity of the protein of interest (white, dotted columns—spine-associated protein synaptopodin, gray columns—the postsynaptic marker protein PSD95) to the actin band intensity in young (P30) rat cortical tissue. Data presented as mean optical density ratio ± SEM. Asterisk—significant difference in synaptopodin or PSD95 protein expression between control rats and rats exposed to hypoxia on E14 (two-tailed Mann–Whitney *U*-test with independent samples, $p \leq 0.05$). No significant changes were shown in rats exposed to hypoxia on E18. $N = 4$ in each group of animals.

DISCUSSION

Analysis of the consequences of prenatal hypoxia at different stages of embryogenesis on brain development, as well as motor and cognitive functions in animals and humans is important for understanding the underlying mechanisms of neonatal pathologies and prognosis of their outcome in later life. Investigation of behavior in rats, subjected to hypoxia on E14

or E18 performed in our study showed that prenatal hypoxia in earlier embryogenesis results in much more pronounced effect on animal motor activity and ability to learn an instrumental reflex. Using a test, which allows the analysis of the effects of various pathological factors on development of fronto-parietal neocortical regulation of motor reactions by estimating how a rat can perform forelimb placing after whisker stimulation (De Ryck et al., 1992; Fan et al., 2008), we have demonstrated that rats exposed to prenatal hypoxia on E14, but not on E18, have delayed development of this motor function in early ontogenesis. The data obtained in the operant reflex paradigm clearly demonstrated that prenatal hypoxia on E14, but not on E18, also causes a reduced ability to learn reaching movements in young and adult rats. To investigate the underlying mechanisms of the impairment caused by prenatal hypoxia on E14 compared to hypoxia on E18 we have performed detailed analysis of development of the parietal cortex in both groups of animals compared to controls.

In rat pups exposed to various harmful stimuli during embryogenesis migration of neuroblasts is reported to be compromised in different cortical areas (prefrontal, temporal, parietal, entorhinal etc.) because the mechanisms of radial migration of neuroblasts are rather common (Rakic, 1988). There are data demonstrating that hypoxia on E17 also disturbs neuroblast migration into the hippocampus (Golan et al., 2009). Continuous hypoxic conditions during brain development were reported to affect both radial (Zechel et al., 2005) and tangential (Stevens et al., 2013) migration of cortical neuroblasts causing changes in the cortical plate structure in newborn rats. However, the consequences of the failure of cell migration after prenatal hypoxia at different stages of embryogenesis on the neuronal network formation in later ontogenesis were not previously investigated. Our data suggest that prenatal hypoxia both on E14 and E18 results in a failure of some neuroblasts to migrate into their proper position in the layers V-VI or II-III of the parietal cortex, respectively. We have also found that, independently of the period when rats were subjected to prenatal hypoxia (E14 or E18), neuroblast generation rates in developing neocortex were decreased. It is worth noting that prenatal hypoxia on E14 or E18 disturbed cell generation and radial migration in a similar way and caused similar changes in final positioning of generated neurons as observed in different non-hypoxic models of prenatal pathology (Ang et al., 2006).

However, as shown in our study, hypoxia on E14 and E18 causes a selective impact on different cell populations in rat neocortex. As we noted earlier (Vasilyev et al., 2008), and confirmed in this investigation, the number of pyramidal neurons in the parietal cortex was reduced compared to the controls only in rats subjected to prenatal hypoxia on E14 but not on E18. This investigation also showed that after prenatal hypoxia on E14 the number of pyramidal neurons decreases earlier (P10-P20) in postnatal development than of non-pyramidal neurons (P20-P30). Although in adult animals subjected to prenatal hypoxia the total number of neuronal cells is normalized with aging and is not different from the controls, they still demonstrate memory deficit (Zhuravin et al., 2009). This might be a result of impairment not only in the cortex but also in the hippocampus and their neuronal networks causing long-term memory impairment (Dubrovskaya and Zhuravin, 2010; Nalivaeva et al., 2012).

Although a selective impact of prenatal hypoxia on different cell populations was suggested in the literature (Ang et al., 2006) the quantitative analysis of neurons belonging to different morphotypes in early postnatal ontogenesis after prenatal hypoxia has not been reported. The analysis of the number of cortical neurons as well as immunohistochemical evaluation of glial and neuronal marker proteins performed in this study confirmed a selective impact of prenatal hypoxia on different cell populations. Our data imply that hypoxia on E14 disturbs generation of pyramidal neurons in the lower cortical layers (V-VI) which are the first generation of cortical neurons known to be crucial for formation and function of the cortical minicolumn (DeFelipe and Fariñas, 1992). These changes in neuronal cell number and composition correlate with the deficit of motor reactions in animals observed during the first month of postnatal ontogenesis but not detected in adult animals. Since pyramidal neurons of the parietal cortex generated on E14 are known to be a part of the motor control system (DeFelipe and Fariñas, 1992) hypoxia at this period of embryogenesis leads to some dysfunctions observed as delayed development of the forepaw placing reaction. On the other hand, the decreased ability to learn reaching movements might be provoked by the structural changes underlying cortical network formation and synaptic plasticity later in postnatal life.

Development of neuronal plasticity to a great extent depends on formation of labile mushroom-shaped dendritic spines which are well-known to be capable of fast changes of their form and size resulting in modulation of synaptic transmission. One of the proteins of the spine apparatus, synaptopodin, which is regarded as a specific marker of labile mushroom spines, is important for remodeling of the spine cytoskeleton and neuronal network plasticity (Asanuma et al., 2005). There is a correlation between memory and the density and plasticity of the labile spines in the cortical areas of the brain (Deller et al., 2003). In addition to the data reported by us previously (Zhuravin et al., 2009) the results obtained in this study demonstrate that in rats subjected to prenatal hypoxia on E14 (but not on E18) in the molecular layer of the neocortex there is a decrease in the number of labile synaptopodin-positive spines, which belong to the dendrites of the pyramidal neurons, not affecting the total number of PSD95-positive postsynaptic terminals. The changes in the number of the labile spines in adult rats, exposed to prenatal hypoxia, could be provoked not only by cell death during brain development or by degeneration of the dendrite system of the cortical pyramidal neurons, but also by some specific molecular mechanism regulating spine formation. The decrease in the number of synatopodin-positive labile spines might underlie changes of neuronal network plasticity (Okubo-Suzuki et al., 2008) and testify to the impairments provoked by prenatal hypoxia.

Taking into account our data we suggest that prenatal hypoxia in the period of generation of the pyramidal neurons in the layers V-VI (E14) might affect formation of the cortical minicolumns in the postnatal ontogenesis affecting neuronal plasticity and motor functions of rats. Hypoxia in the later period of cortical

neurogenesis (E18) appears not to be as crucial for neocortex formation and causes no significant changes in its structure and related specific motor functions. Further investigation of various aspects of abnormalities in brain formation caused by harmful factors acting on the organism during prenatal development can provide us with better understanding of the mechanisms of their long-term consequences and insights into pathogenesis of certain neurodegenerative disorders characteristic for human aging including dementia and Alzheimer's disease.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the experimental design and writing the paper. Dr. IZ, DV, and ND performed animals studies (hypoxia and labelleing), Dr. DV and Dr. NT have done morphology experiments, Dr. DV performed biochemical studies, Dr. ND and Dr. IZ have done behavioral experiments.

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Hypoxia Affects Neprilysin Expression Through Caspase Activation and an APP Intracellular Domain-dependent Mechanism

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While gene mutations in the amyloid precursor protein (APP) and the presenilins lead to an accumulation of the amyloid β-peptide (Aβ) in the brain causing neurodegeneration and familial Alzheimer's disease (AD), over 95% of all AD cases are sporadic. Despite the pathologies being indistinguishable, relatively little is known about the mechanisms affecting generation of Aβ in the sporadic cases. Vascular disorders such as ischaemia and stroke are well established risk factors for the development of neurodegenerative diseases and systemic hypoxic episodes have been shown to increase Aβ production and accumulation. We have previously shown that hypoxia causes a significant decrease in the expression of the major Aβ-degrading enzyme neprilysin (NEP) which might deregulate Aβ clearance. Aβ itself is derived from the transmembrane APP along with several other biologically active metabolites including the C-terminal fragment (CTF) termed the APP intracellular domain (AICD), which regulates the expression of NEP and some other genes in neuronal cells. Here we show that in hypoxia there is a significantly increased expression of caspase-3, 8, and 9 in human neuroblastoma NB7 cells, which can degrade AICD. Using chromatin immunoprecipitation we have revealed that there was also a reduction of AICD bound to the NEP promoter region which underlies the decreased expression and activity of the enzyme under hypoxic conditions. Incubation of the cells with a caspase-3 inhibitor Z-DEVD-FMK could rescue the effect of hypoxia on NEP activity protecting the levels of AICD capable of binding the NEP promoter. These data suggest that activation of caspases might play an important role in regulation of NEP levels in the brain under pathological conditions such as hypoxia and ischaemia leading to a deficit of Aβ clearance and increasing the risk of development of AD.

Keywords: neprilysin, hypoxia, caspases, AICD, APP

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INTRODUCTION

Cerebral hypoxia is a condition in which the brain is deprived of oxygen and, as a result, cells elicit a wide range of adaptive responses and major metabolic alterations. Currently, vascular disorders such as ischaemia and stroke are considered as established risk factors for the development of neurodegenerative diseases, in particular of Alzheimer's disease (AD) (O'Brien and Markus, 2014).

The pathology in familial AD is caused by overproduction and accumulation in the brain of abnormally high concentrations of the amyloid-β (Aβ) peptide and its oligomers causing synaptic loss and neuronal cell death (Hardy and Higgins, 1992; Walsh et al., 2002; Hardy, 2009). It is now becoming evident, however, that Aβ levels in the brain represent a dynamic equilibrium between its production from the amyloid precursor protein (APP) and removal by a cohort of amyloid clearance proteins which can be either enzymes (proteases) or binding/transport proteins. The group of enzymes capable of proteolytic degradation of Aβ currently embraces more than 20 members (for review, see Nalivaeva et al., 2012a, 2014). Several of the main amyloid-degrading enzymes (ADEs) are members of the neprilysin peptidase family: NEP, NEP2 and endothelin converting enzymes (ECE-1 and ECE-2) (Turner, 2003; Marr and Spencer, 2010; Nalivaeva et al., 2012a; Pacheco-Quinto and Eckman, 2013). Another metallopeptidase which plays an important role in Aβ metabolism is insulin-degrading enzyme (IDE) (Qiu and Folstein, 2006; Leissring and Turner, 2013). With aging and under pathological conditions expression levels and activity of these enzymes decline (Nalivaeva et al., 2004; Caccamo et al., 2005; Kochkina et al., 2015) leading to an amyloid clearance deficit, which is now considered to be one of the major factors of the sporadic form of AD (Selkoe, 2012; Pluta et al., 2013).

As we have shown earlier, hypoxia leads to reduced NEP levels and activity both in cellular models and *in vivo* (Nalivaeva et al., 2004, 2012b; Fisk et al., 2007). A prolonged exposure to a hypoxic environment has also been reported to increase Aβ levels significantly accelerating the hyperphosphorylation of tau and contributing to neuronal cell death (Jendroska et al., 1995; Li et al., 2009; Fang et al., 2010). Regulation of NEP expression is complex as the enzyme appears to have a constitutive regulatory pathway (D'Adamio et al., 1989; Li et al., 1995) as well as an epigenetically-regulated component (Pardossi-Piquard et al., 2005; Belyaev et al., 2009). The latter involves competitive binding of a transcription factor, namely the APP intracellular domain (AICD) produced in the β-secretase amyloidogenic pathway (Belyaev et al., 2010), to the NEP gene promoter leading to activation of mRNA synthesis while histone deacetylases inhibit this process. As the effects of hypoxia on NEP expression may represent an important pathological trigger in AD, the factors affecting NEP dysregulation under these conditions need to be better understood. AICD is an extremely labile peptide being a substrate of various intracellular peptidases including caspases (Bertrand et al., 2001) which might result in dysregulation of AICD-dependent NEP expression under various pathological conditions related to caspase activation. In particular, hypoxia was shown to be accompanied by increased levels of caspase expression and activity in the brain (Khurana et al., 2002). The aim of this study was to assess whether activation of caspases might be a factor leading to dysregulation of NEP gene expression and activity under hypoxic conditions. For this we have employed human neuroblastoma NB7 cells which possess high endogenous levels of NEP and, as has been demonstrated, are responsive to hypoxia (Fisk et al., 2007; Belyaev et al., 2009).

METHODS

Cell Culture and Hypoxia Treatment

The NB7 (SJ-N-CG) neuroblastoma cell line, which expresses high endogenous levels of NEP, was obtained from St Jude Children's Research Hospital (Memphis, USA, kind gift of Dr. Vincent J. Kidd). The NB7 cells were cultured in RPMI-1640 media supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 2 mM glutamine (all from Cambrex Bio Science Ltd., Wokingham, Berkshire, UK) at 37°C in 5% (v/v) CO_2 and sub-cultured every 7 days. After reaching the confluent stage, cells were incubated in an O_2/CO_2 incubator (MC0-175M, Sanyo) for 24 h under 1% O_2. The cells were collected 24 (or 48 h) later, washed twice with 10 ml PBS, scraped into 10 ml of PBS (pH 7.2), pelleted at 3000 g for 5 min and used for mRNA and protein content analysis as well as for the activity assays.

Cell Viability Determination by Trypan Blue Exclusion

Cells were washed twice in PBS, incubated in trypsin/EDTA for 5 min at 37°C and knocked from the surface of the flask prior to adding 5 ml of media then pelleting at 400 g for 5 min. Pellets were resuspended in 1 ml of media and a 1:1 dilution of cell suspension in 4% trypan blue was prepared. Twenty microlitres of cell suspension was loaded under a cover slip on a haemocytometer and allowed to fill the chambers by capillary action. The number of total and viable cells was determined by viewing under a light microscope at 40X magnification. Non-viable cells take up the trypan blue and appear dark (Strober, 2001).

Gene Expression Analysis

Cell RNA was prepared using the RNeasy extraction kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. RNA was treated with DNase I (Invitrogen, Paisley, UK) and cDNA was prepared using the iScript cDNA kit (BioRad, UK). cDNA was amplified using conventional PCR or real-time PCR as in Zuccato et al. (2007). DNA amplified by conventional PCR was analyzed in 2% agarose gels containing ethidium bromide (1 μg/ml) and visualized on a Molecular Imager Gel Doc XR System with Quantity One 4.6.1 programme (BioRad). Image densitometry was performed using Aida Array Analyzer 4.15 software. Real-time PCR was performed in an iCycler Thermal Cycler with Multicolour PCR detection system, (Biorad, Hercules, CA) using SYBR Green (BioRad) incorporation and expression reported relative to actin mRNA. Primer sequences used were as follows:

NEP F- CCTGGAGATTCATAATGGATCTTGT
R- AAAGGGCCTTGCGGAAAG
Caspase-1 F- TGCTTTCTGCTCTTCAACACC
R-CACAAGACCAGGCATATTCTTTC
Caspase-3 F-GAGGCCGACTTCTTGTATGC
R-AATTCTGTTGCCACCTTTCG
Caspase-8 F-AGAGCCTGAGGGAAAGATGTC
R-TCACATCATAGTTCACGCCAGT
Caspase-9 F-CGTGGTGGTCATCCTCTCTC
R-GAGCATCCATCTGTGCCATA

U6 F- CTCGCTTCGGCAGCACA
R- AACGCTTCACGAATTTGCGT
Actin F- CGCAGCAGTCAGGGACATTT
R- TTCACATACAGCTTGGGAAGC

Preparation of Cell Lysates

Cells were washed twice with phosphate-buffered saline (PBS: 1.5 mM KH_2PO4, 2.7 mM Na_2HPO4, 150 mM NaCl, pH 7.4), harvested and pelleted by centrifugation. For detection of proteins from cell lysates, cells were lysed in RIPA buffer [10 mM Tris/HCl pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 5 mM EDTA, 1X complete inhibitor mix (Roche diagnostics)] on ice for 20 min. Lysates were homogenized through 22 G needles 10 times then clarified by centrifugation at 2800 g for 10 min. Protein concentration of lysates was determined using the bicinchoninic acid (BCA) assay (Sigma Aldrich).

Electrophoresis and Western Blotting

Samples (30 μg or, for AICD detection, 50 μg, protein) were resolved on 10% polyacrylamide gels or, for AICD detection, 10–20% tricine gels (Invitrogen, Paisley, UK), and transferred onto Hybond-P poly(vinylidene) difluoride (PVDF) membranes (Amersham Life Sciences, Buckinghamshire, UK). The membranes were blocked overnight at 4°C in Tris-buffered saline (TBS: 50 mM Tris, 150 mM NaCl) containing 0.1% (v/v) Tween-20 (TBST) and 5% (w/v) skimmed milk powder. Membranes were incubated with the primary antibodies for 3 h. Primary antibodies used for blotting included: NEP (1:100, mouse monoclonal, NCL-CD10-270, Novocastra, Newcastle, UK or rabbit anti-rat polyclonal antibody, US Biological); GLUT-1 (1:500, rabbit polyclonal, from Prof. S. Baldwin, University of Leeds); anti- β-actin (1:500, rabbit polyclonal, Sigma-Aldrich Co., Poole, Dorset, UK); anti-APP C-Terminal fragment A8717 and anti-actin (both Sigma-Aldrich) used at 1:1000 and 1:10,000, respectively. Membranes were washed in TBST for 1 h before incubation with peroxidase-conjugated rabbit anti-mouse or donkey anti-rabbit secondary antibodies (GE Healthcare, Bucks, UK) at a dilution of 1:4000. Membranes were washed further before detection of proteins using the enhanced chemiluminescence method. Western blots were quantified using AIDA image analyzer v.4.22 (Straubenhardt, Germany).

Neprilysin Activity Assay

NEP activity was measured in a two-step coupled fluorescence assay. NEP cleaves between Ala-Phe of the fluorogenic substrate succinoyl-Ala-Ala-Phe-7-amido-4-methylcoumarin (Mumford et al., 1980) and, in the presence of leucine aminopeptidase, releases the fluorophore (AMC) from Phe and prevents quenching allowing fluorescence to be detected. Protein samples (1 μg) were loaded into opaque 96-well plates in 100 mM Tris-HCl (pH 7.4) with or without 20 μM of the NEP inhibitor thiorphan and incubated at 37°C for 20 min. The substrate Suc-AAF-AMC (50 μM) and Leu-aminopeptidase (40 munits/ml) were added to each well (100 μl total volume/well) and fluorescence was observed on a FLUOstar Omega multidetection microplate reader (excitation = 355 nm, emission = 460 nm) at suitably frequent time points. NEP activity was determined as the difference between fluorescence measurements in the absence and presence of 20 μM thiorphan.

Caspase 3/7 Activity Assay

Caspase-3 and caspase-7 activities were determined using a luminescent Caspase-Glo® 3/7 activity assay (Promega, Southampton, UK) as per manufacturers' instruction. Briefly, approximately 20,000 cells were added to each well of a 96 well, white-walled plate. At room temperature, the Caspase-Glo reagent was made up by mixing the Caspase-Glo substrate with the Caspase-Glo buffer and 100 μl was added to each well. Contents were mixed by shaking for 1 min then incubated at 37°C for 1 h. Luminescence was measured on a MicroLumat Plus LB 96V (Berthold Technologies).

Chromatin Immunoprecipitation Analysis

ChIP was performed as described previously (Zuccato et al., 2007; Belyaev et al., 2009). Cells were fixed, extracts sonicated and, following incubation with protein-G-Sepharose, primary antibodies were applied overnight. After de-crosslinking and DNA extraction, analysis was performed by real time PCR. Data are represented as the fold enrichment of DNA pulled down with the specific antibody over that immunoprecipitated with IgG. Antibodies used were anti-AICD (Covance, Harrogate) and anti-IgG (mouse) (negative control) (Abcam). Promoter primer sequences used were as follows:

NEP F- GGTGCGGGTCGGAGGGATGC
R- CTCCCAGCGCCCTGGGCGCTCG

Statistics

All results are given as mean ± SEM from at least three experiments. Results were compared using unpaired two-tailed Student's *t*-test with a threshold of $p < 0.05$.

RESULTS

Cell Viability and GLUT1 Expression in NB7 Cells after Hypoxia

Hypoxia elicits a wide range of cellular adaptations and the severity and duration of a hypoxic episode can significantly affect hypoxia-induced apoptosis (Banasiak and Haddad, 1998). In order to show that the NB7 cells were responding to hypoxic conditions and were still viable, a cell viability assay was carried out as well as a Western blot analysis of the established hypoxia marker, GLUT1. Acting through the hypoxia- inducible factor-1, GLUT-1 expression is enhanced in hypoxia, stimulating glucose transport and maintaining cell homeostasis (Ouiddir et al., 1999; Zhang et al., 1999). After incubation at 1% O_2 for 24 h, the viability of NB7 cells was not significantly different from those incubated under normal conditions (**Figure 1A**). In both conditions cell viability was above 90%. Although GLUT1 protein content was detected at high levels in both normoxia and hypoxia incubated cells, 24 h of hypoxia increased GLUT-1 protein levels significantly suggesting that the cells were responding to hypoxic insult by changing the level of expression of hypoxia-related genes (**Figure 1B**).

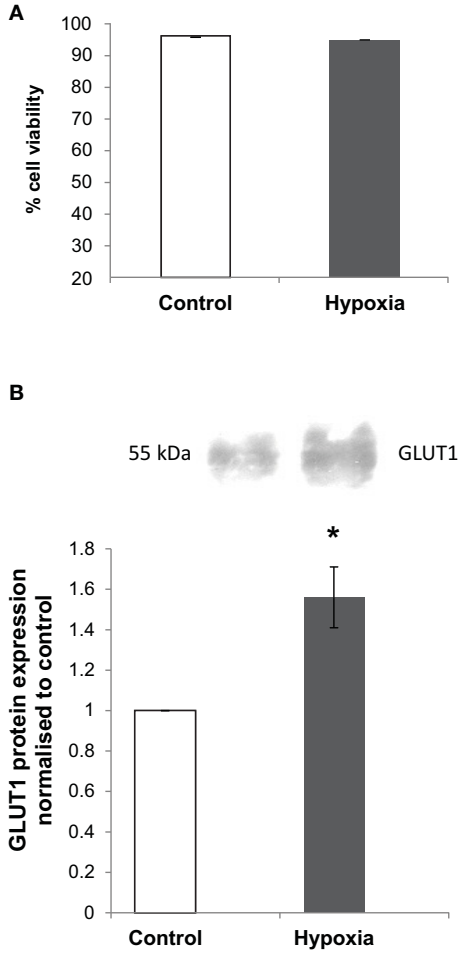

FIGURE 1 | The effects of hypoxia on cell viability and GLUT1 protein levels in NB7 cells. (A) A cell viability assay carried out in NB7 cells subjected to 24 h normoxia or hypoxia (1% O_2). **(B)** Representative Western blot and calculation of GLUT1 levels in whole cell lysates of NB7 cells subjected to 24 h normoxic or hypoxic (1% O_2) conditions. Bars represent mean ± SEM, $^*p < 0.05$ ($n = 3$).

The Effects of Hypoxia on NEP mRNA Expression, Protein Level, and Activity

Assessing the effect of hypoxia on NEP expression in NB7 cells we have confirmed that both NEP mRNA and protein levels in the cells subjected to hypoxia for 24 h were reduced by approximately 30% compared to the control cells (**Figures 2A,B**). Similarly, a fluorogenic NEP enzyme activity assay has demonstrated that NEP activity was reduced in hypoxia by approximately 40% (**Figure 2C**).

The Effects of Hypoxia on mRNA Expression and Activity of Members of the Caspase Family

Caspases are a family of cysteine proteases that are crucial mediators of apoptosis. Hypoxic conditions are known to induce cellular apoptosis and the family of caspases are participants in the hypoxia-induced apoptotic cascade (Khurana et al., 2002; Li et al., 2005). In order to investigate the effect of hypoxia on the expression of caspases in our cellular model, mRNA levels for caspases-1, -8, and -9 were determined by real-time PCR and a caspase-3/-7 activity assay was also carried out. A three to five-fold increase in mRNA was detected for caspase-1 and -9 (**Figures 3A,C**). In accordance with other studies (Finlay et al., 2010), caspase-8 expression was extremely low in the NB7 cells. However, despite endogenously low levels, hypoxia also increased its mRNA expression (**Figure 3B**). Since caspase-3 and caspase-7 exhibit similar substrate specificity, the analysis of their activity was combined. Total caspase -3/-7 activity was increased by approximately four-fold in the NB7 cells incubated in hypoxic conditions compared with control (**Figure 3D**).

The Effect of Hypoxia on AICD Enrichment of the NEP Promoter

The APP C-terminal fragments (CTFs) have been identified as a target for caspase cleavage due to the presence of a caspase-specific cleavage site at aspartate residue 664 (for APP_{695} isoform) in the APP molecule (Weidemann et al., 1999). As caspases appear to be significantly elevated in hypoxia, ChIP analysis was used to determine whether the reduction in NEP may be due to reduced AICD binding to the *NEP* promoter region. After 24 h hypoxic incubation, AICD enrichment on the *NEP* promoter region was found to be significantly reduced by approximately five-fold (**Figure 4B**).

The Effects of Caspase Inhibition in Hypoxia on NEP Activity and AICD Enrichment on the NEP Promoter

Caspase-3 has been identified as the predominant caspase involved in the cleavage of the APP-CTF, including AICD (Gervais et al., 1999), therefore in order to investigate whether caspase-3 is involved in the reduction of AICD-induced NEP expression, cells were subjected to hypoxia with or without the caspase-3 inhibitor, Z-DEVD-FMK. As previously shown, NEP activity was significantly reduced in hypoxia compared to control, but cell treatment with the caspase-3 inhibitor restored NEP activity to approximately 90% of the control level (**Figure 4A**). Similarly, ChIP analysis has revealed that caspase inhibitor treatment to a significant extent protected the level of AICD binding to the NEP gene promoter (**Figure 4B**). This testifies to the role of caspases, and especially of caspase-3, in reduction of NEP expression in neuronal cells subjected to hypoxic conditions.

DISCUSSION

Hypoxia is an established factor for AD and brain deprivation of oxygen has been associated with an increase in Aβ levels and plaque deposition (Jendroska et al., 1995; Zhang and Le, 2010). The leading factor in Aβ accumulation might be deficit of its major degrading peptidase, NEP. Indeed, dysregulation of NEP

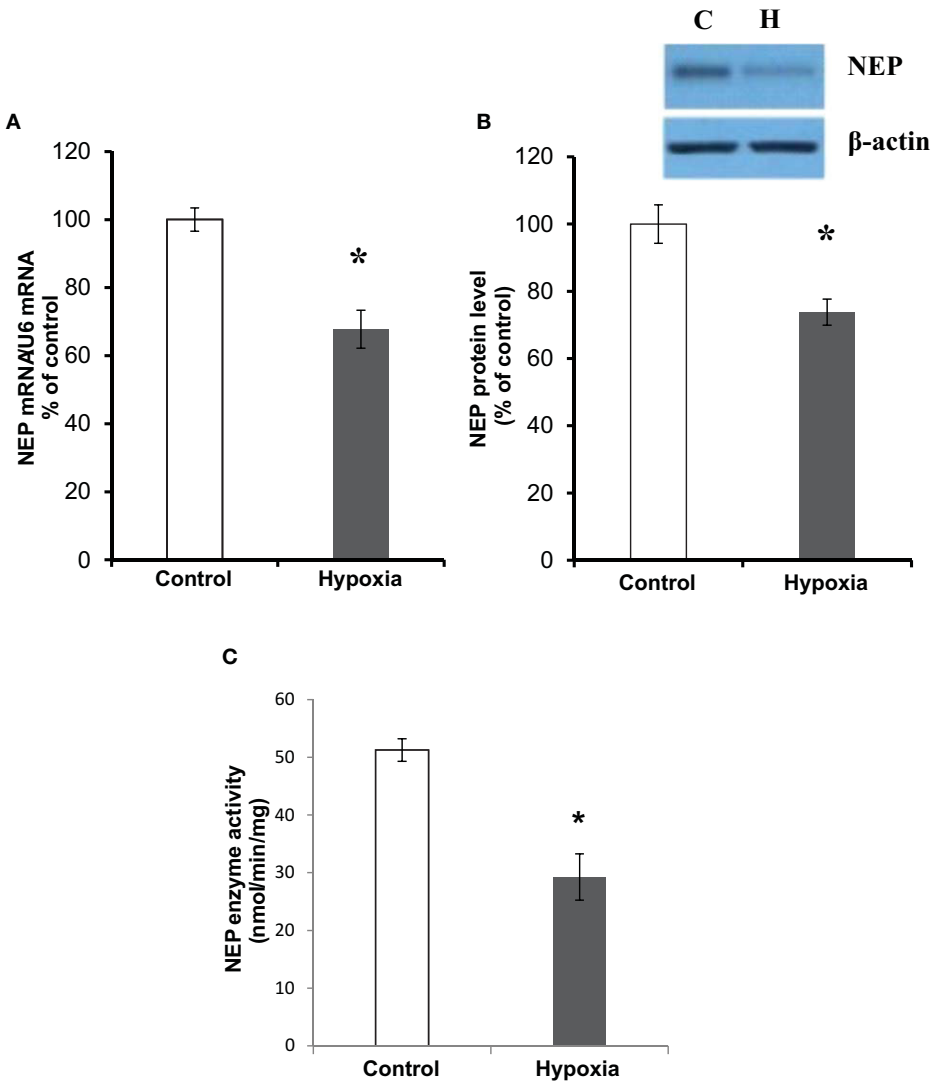

FIGURE 2 | The effects of hypoxia on NEP expression and activity. (A) NEP mRNA expression levels normalized to U6; **(B)** NEP protein levels showing a representative blot with CD10 antibody against human NEP and β-actin as loading control; **(C)** NEP enzyme activity (nmol/min/mg) in NB7 cells subjected to 24 h normoxia or 24 h hypoxia (1% O_2). Bars represent mean ± SEM, $^*p < 0.05$ ($n = 5$).

after exposure to hypoxia has been reported in many areas of the body including the lungs (Carpenter and Stenmark, 2001; Wick et al., 2011), carotid body (Kumar et al., 1990), eye (Hara et al., 2006), and brain (Nalivaeva et al., 2004, 2012b; Oh-hashi et al., 2005). The data of the present study report significantly reduced NEP expression and activity in the human neuroblastoma NB7 cell line subjected to hypoxia, which are consistent with other reports.

Oxygen homeostasis is regulated by the hypoxia inducible factor (HIF)-1α (for review see Semenza, 2006). HIF-1α binds specifically to the hypoxia-responsive element (HRE) in gene promoter regions and in hypoxic conditions which can significantly alter cellular gene expression. The effects of hypoxia on APP processing have been widely studied and it is now well established that hypoxia increases the amyloidogenic pathway by facilitating the up-regulation of expression of β-secretase (BACE1), as well as components of the γ-secretase complex resulting in increased production both of $A\beta_{40}$ and $A\beta_{42}$ (Sun et al., 2006; Wang et al., 2006; Zhang et al., 2007; Li et al., 2009). Sequence analysis, mutagenesis and gel shift studies have revealed the binding of HIF-1α to both the BACE1 and APH-1A promoter regions and Notch processing has also been increased after hypoxic incubation (Wang et al., 2006; Zhang et al., 2007). Hypoxia has also been shown to decrease levels of metalloproteinases ADAM10 and ADAM17 expression and consequently reduce APP processing through the non-amyloidogenic, α-secretase pathway (Webster et al., 2004; Rybnikova et al., 2012). A reduction in mature ADAM10 and a concomitant increase in immature ADAM10 has also been reported to alter the regulation

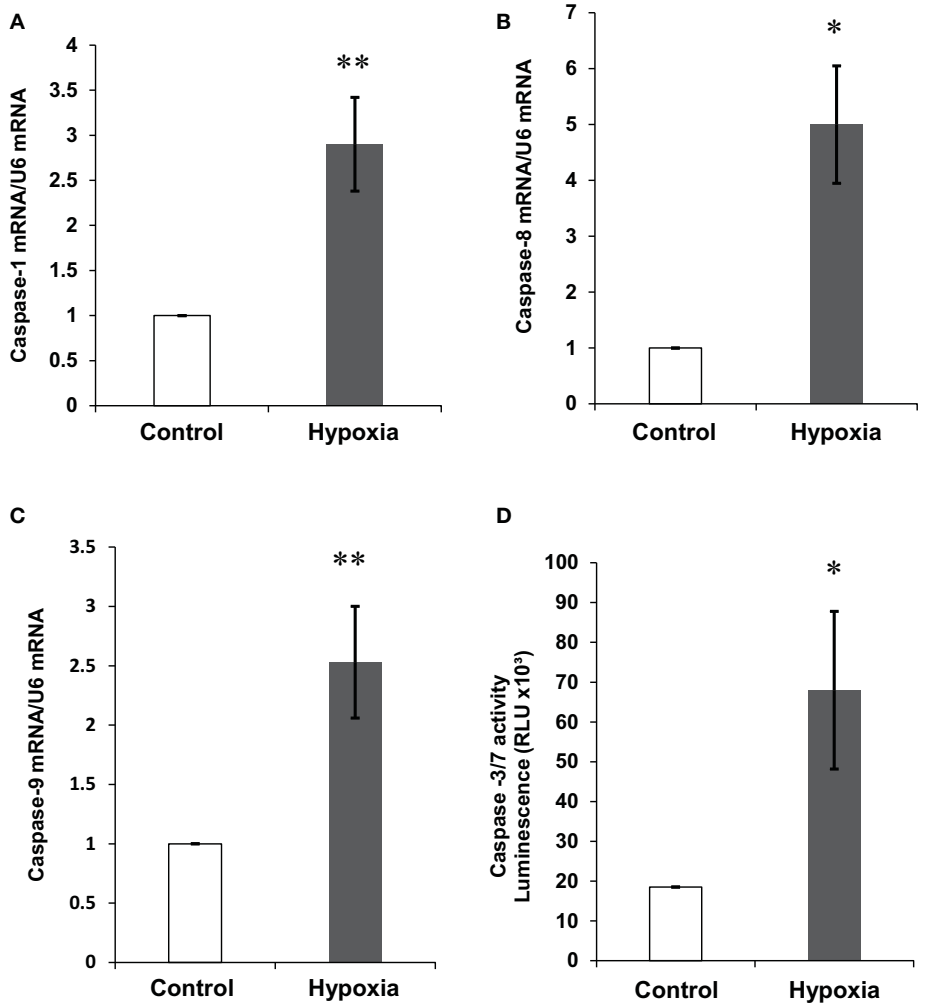

FIGURE 3 | The effect of hypoxia on caspase-1,-8, and -9 mRNA expression and caspase -3/-7 activity levels. Expression levels of **(A)** caspase-1, **(B)** caspase-8, **(C)** caspase-9 normalized to U6 in NB7 cells subjected to 24 h normoxia or 24 h hypoxia (1% O_2). **(D)** Caspase-3/-7 activity levels in NB7 cells subjected to 24 h normoxia or 24 h hypoxia (1% O_2). Bars represent mean ± SEM, $^*p < 0.05$, $^{**}p < 0.01$.

of α-secretase APP processing in hypoxia (Auerbach and Vinters, 2006).

The data in this paper provide evidence for an alternative mechanism by which hypoxia may increase Aβ levels. The hypoxia-induced activation of caspases may cause an increased cleavage of the APP-CTF and thus reduce AICD levels. This then results in the down-regulation of the AICD-mediated gene NEP and reduction of enzyme expression and activity which inevitably will lead to increase of Aβ content. It has been proven both in cell and animal models that NEP deficit is one of the factors leading to increased Aβ production and load in the brain (Iwata et al., 2000; Hanson et al., 2011) and up-regulation of NEP, on the contrary, leads to reduction of Aβ levels (Mohajeri et al., 2004; Kerridge et al., 2014). We have also shown that caspase-3 inhibition using the potent DEVD caspase inhibitor can protect NEP activity in the hypoxic conditions. Similar findings have been reported in dying motor-neurons deprived of trophic support. This type of cellular stress was shown to cause an increase in the expression of APP and the cell death protease caspase-3, as well as a concomitant increase in neurotoxic Aβ (Barnes et al., 1998). Although AICD and NEP levels were not investigated, treatment with a caspase-3 inhibitor significantly reduced the Aβ peptide content in these types of cells. Apoptotic stimuli activating the caspase-3 protease family have also been reported to cleave both PS1 and PS2 (Kim et al., 1997). As such, an alternative mechanism could involve an altered γ-secretase cleavage of APP thus generating APP-CTF incapable of translocating to the nucleus and/or regulating the transcription of NEP.

It was demonstrated that hypoxia affects NEP gene expression via histone modifications on its promoter region (Wang et al., 2011) which can affect accessibility of AICD to its binding site. As we have shown here, AICD binding to the NEP gene promoter is indeed reduced under hypoxic conditions but this decrease

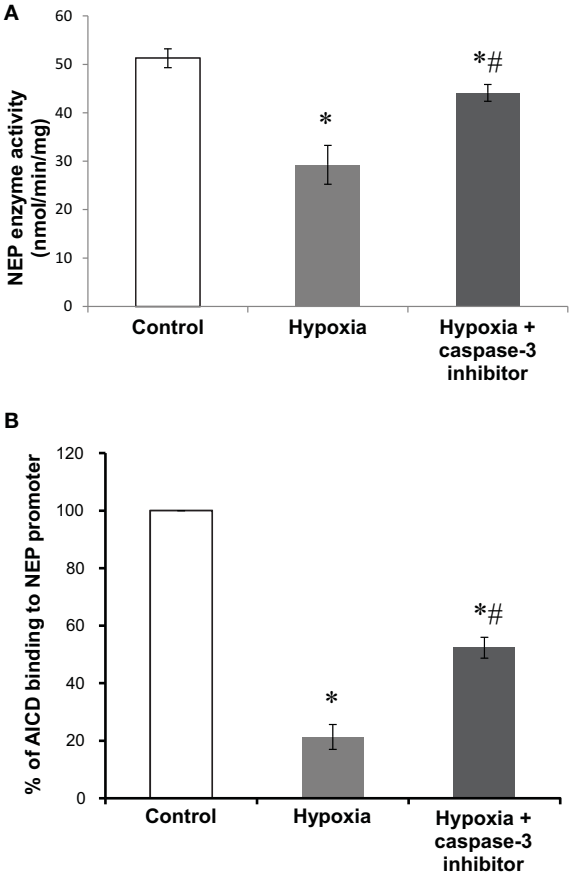

FIGURE 4 | The effect of a caspase-3 inhibitor on NEP activity and AICD binding to the NEP promoter in hypoxia. (A) NEP enzyme activity (nmol/min/mg) and **(B)** enrichment of AICD on the NEP promoter analyzed by ChIP in NB7 cells subjected to 24 h normoxia or 24 h hypoxia (1% O_2) with or without the caspase-3 inhibitor, Z-DEVD-FMK (100 μM, 24 h). Bars represent *n* of 3 ± SEM, $^{*}p < 0.05$ compared to control, $^{\#}p < 0.05$ compared to hypoxia.

can be prevented by a caspase inhibitor. Because inhibition of caspase activity does not restore completely the level of AICD binding to the NEP promoter observed in the control cells, it is reasonable to suggest that either there are other peptidases active under hypoxic conditions that degrade AICD, or changes in the NEP promoter structure due to histone deacetylation are still interfering with its activation by AICD. As we have shown previously (Belyaev et al., 2009; Nalivaeva et al., 2012b), inhibition of histone deacetylases by valproic acid leads to significant but not complete restoration of NEP gene expression and NEP enzyme activity after hypoxia which confirms the role of histone modifications in NEP regulation under reduced oxygen supply.

AICD was also shown to regulate HIF-1α expression (Kaufmann et al., 2013) and, therefore, decreased levels of this transcription factor under hypoxic conditions might result in a modified response of cells to hypoxic insults. As we have established, the Aβ-clearing protein TTR is also AICD-regulated (Kerridge et al., 2014) and is shown to be responsive to hypoxia (Ahmad et al., 2014). Alterations of the levels of this protein under hypoxic conditions may also affect amyloid metabolism leading to dysbalance of its production and clearance.

There is an increasing body of evidence that AICD levels can be regulated by various pharmacological agents (Eisele et al., 2007; Bauer et al., 2011; Kerridge et al., 2014) which opens an avenue for designing a therapeutic strategy for regulating important AICD-dependent genes. Because AICD-regulated genes have different roles in amyloid metabolism and are cell- and tissue-specific (for review see Beckett et al., 2012; Pardossi-Piquard and Checler, 2012) understanding the effect of various physiological conditions on its ability to regulate expression of the major amyloid-degrading enzyme is of great importance. This work extends further our knowledge about the role of hypoxia in NEP regulation via AICD and shows that caspase inhibitors can be potentially beneficial for protecting the brain against harmful effects of low oxygen supply.

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Metabolic Covariant Network in Relation to Nigrostriatal Degeneration in Carbon Monoxide Intoxication-Related Parkinsonism

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Presence of parkinsonian features after carbon monoxide (CO) intoxication is well known and the severity was found to relate to the pre-synaptic dopaminergic deficits. There is no systemic study to analyse the functional network involved in CO-related Parkinsonism. Forty-five CO-related parkinsonism patients and 25 aged-matched controls completed the 3D T1-weighted imaging and ^{18}F-fluoro-2-deoxyglucose positron emission tomography (FDG-PET). Voxel-based morphometry (VBM) was performed to assess the structural and functional brain differences between the patients and controls. Spatial covariant networks responsible for distinguishing patients and controls were constructed using independent component analysis. For validation, the pre-synaptic dopaminergic functional network was established by regression model using striatal TRODAT-1 SPECT as the independent variable. The clinical significance of both networks was determined by correlation with the Unified Parkinson's Disease Rating Scale (UPDRS). Compared with controls, the spatial covariant signals of FDG-PET were significantly lower in the medial and lateral frontal, caudate nucleus, dorsomedial prefrontal areas, and temporal-parietal regions while the spatial intensities correlated significantly with UPDRS total scores. The functional network that correlated with striatum pre-synaptic dopaminergic uptakes included the midbrain, thalamus, caudate, lateral frontal cortex, ventral striatum, ventral, or dorsal anterior cingulate cortex. Both networks overlapped considerably and the topographies reflected structural damage pattern. Our study provides evidence that glucose metabolism in CO-parkinsonism patients pertains to an organized covariant pattern in the cortical regions that is spatially coherent with the cortical map of pre-synaptic dopamine deficits. As the fronto-temporal, striatum, and temporal-parietal areas were involved, the unique metabolic covariant network suggests a different pathophysiology in CO-related parkinsonism.

Keywords: carbon monoxide intoxication, metabolic covariant network, nigra-striatal degeneration, parkinsonian symptoms, pre-synaptic dopamine deficit

INTRODUCTION

Suicide by inhalation of barbecue charcoal gas in Asia used to be very rare, however its use became more prominent in 2001 and increased markedly thereafter (Chang et al., 2014). Inhalation of barbecue charcoal gas is lethal and the survivors may encounter carbon monoxide (CO) intoxication. From neuroimaging analysis, diffuse white matter (WM) damages (Sohn et al., 2000; Chang et al., 2011) and deep gray matter (GM) injuries in the globus pallidus or basal ganglia (Klawans et al., 1982; Pulst et al., 1983; Lee et al., 2010) were reported. Among these seemingly unrelated areas, we recently validated that the fronto-insular-temporal brain areas represented functional network that underwent neurodegenerative processes, while the spatial extents of injury are highly predictive of the cognitive severity (Chen et al., 2015).

Survivors after CO intoxication may present with syndrome complex mixing cognitive deficits, parkinsonian features, or behavioral changes (Weaver, 1999). CO-related parkinsonism is characterized by symmetric limb rigidity, bradykinesia, gait disturbances, and postural instability (Ginsburg and Romano, 1976; Choi, 1983, 2002; Sohn et al., 2000; Hopkins et al., 2006). Different from the degenerative Parkinson's disease (PD), tremors are rarely observed in CO-related parkinsonism (Choi, 2002). In addition, satisfactory treatment with dopaminergic agonists or levodopa, which is often achieved in the early stages of PD, was less efficient in CO-related parkinsonism (Klawans et al., 1982; Tack and de Reuck, 1987; Lee et al., 2010; Chang et al., 2011). These differences highlight involvement of distinct neuronal networks in pathophysiology but the cortical hubs related to the Parkinsonian features in CO-parkinsonism are yet not known.

Using both pre- and post-synaptic dopaminergic ligands, Rissanen et al. reported presynaptic dopamine deficits in a case of CO-related parkinsonism (Rissanen et al., 2010). Since this report, the importance of structures between the pallidum and mid-brain, or the fiber integrity of pallidoreticular tract (Auer and Benveniste, 1996), were established to mediate the parkinsonian features in CO intoxication (Chang et al., 2011). Follow-up studies, using functional tracers such as 99mTc-TRODAT-1 single photon emission computed tomography (SPECT) for pre-synaptic dopamine transporter (Chang et al., 2011; Chen et al., 2012) or ^{18}F-FP-(+)-DTBZ (Chang et al., 2015) for vesicle monoamine transporter type II, repeatedly validated the importance of pre-synaptic dopaminergic deficits in CO-related parkinsonism. Other than the aforementioned structures, WM lesion loads (Sohn et al., 2000), damages of prefrontal cortical areas and caudate nucleus have been linked with parkinsonian severities in patients with CO intoxication (Chang et al., 2015). However, a systematic study evaluating the networks involved in CO-related parkinsonism is still lacking.

Abbreviations: SPECT, single photon emission computed tomography; 99mTc-TRODAT-1, 99mTc-[2-[[2-[[[3-(4-chlorophenyl)-8-methyl-8-azabicyclo [3,2,1] oct-2-yl] methyl] (2-mercaptoethyl) amino] ethyl] amino]-ethanethiolate(3-)-N2,N2,S2, S2]oxo-[1R-(exo-exo)].

Using spatial-independent component analysis (ICA), the functional connectivity between topographically distant regions can be modeled without *a priori* knowledge (Biswal et al., 1995, 2010). Meaningful metabolic covariant networks (MCN) and the consistency of MCN, showing high clinical correlations and reflecting underlying structural integrity, were reported in several ^{18}F-fluro-2-deoxyglucose positron emission tomography (PET) studies (Eckert et al., 2007; Eidelberg, 2009; Spetsieris et al., 2015). As the lesions in CO intoxications are reported to scatter in the cortex, functional approach by PET ICA modeling may help to bridge the information gap and understand the functional connections from the structural analysis.

Here, we hypothesized that the brain areas with structural lesions encountered in CO-related parkinsonism may undergo functional rewiring process and the ICA approach helps to delineate meaningful functional network. In addition, we hypothesized that the MCN might correlate with the injury caused by nigro–striatal degeneration. As the neuronal synchronization pattern may be distinct from the normal controls, we have also tested whether inter-subject MCN in CO-related parkinsonism echoes the structural-damage map and also predicts the severity of parkinsonism.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital and complied with the ethical standards established in the Declaration of Helsinki. The experiments were undertaken with the written, informed consent of each subject and their caregiver (where appropriate).

The working scheme for the network construction is shown in **Figure 1**. The proof-of-theory experiments consisted of three parts. First, voxel-wised comparisons of ^{18}F-fluoro-2-deoxyglucose PET and magnetic resonance imaging (MRI) data between 45 CO patients and 25 age- and sex-matched controls were performed to delineate the disease-specific pattern. Afterwards, using the PET images and ICA modeling of all subjects, we constructed inter-subject MCN maps. The significant inter-subject MCN maps were selected, compared with the disease-specific pattern and correlated with clinical scores. Meanwhile, using the striatum TRODAT-1 SPECT signals as the independent variable and ^{18}F-fluoro-2-deoxyglucose PET signals as the dependent variable, we constructed another map, defined as a pre-synaptic-dopaminergic-associative cortical network (Pre-DA-CN). The Pre-DA-CN here reflected cortical glucose metabolic signals that were highly parallel to the nigro–striatal degeneration process. The clinical weightings of individual cortical regions within the Pre-DA-CN were determined by correlations with Unified Parkinson's Disease Rating Scale (UPDRS) -part III motor score.

Patient Enrolment

The neurology clinic at Kaohsiung Chang Gung Memorial Hospital initiated this study in 2011. The clinical diagnosis of CO intoxication was made based on a history of a charcoal–burning suicide attempt and an elevated carboxyhemoglobin level (>10%) (Chang et al., 2012). Among the patients, none

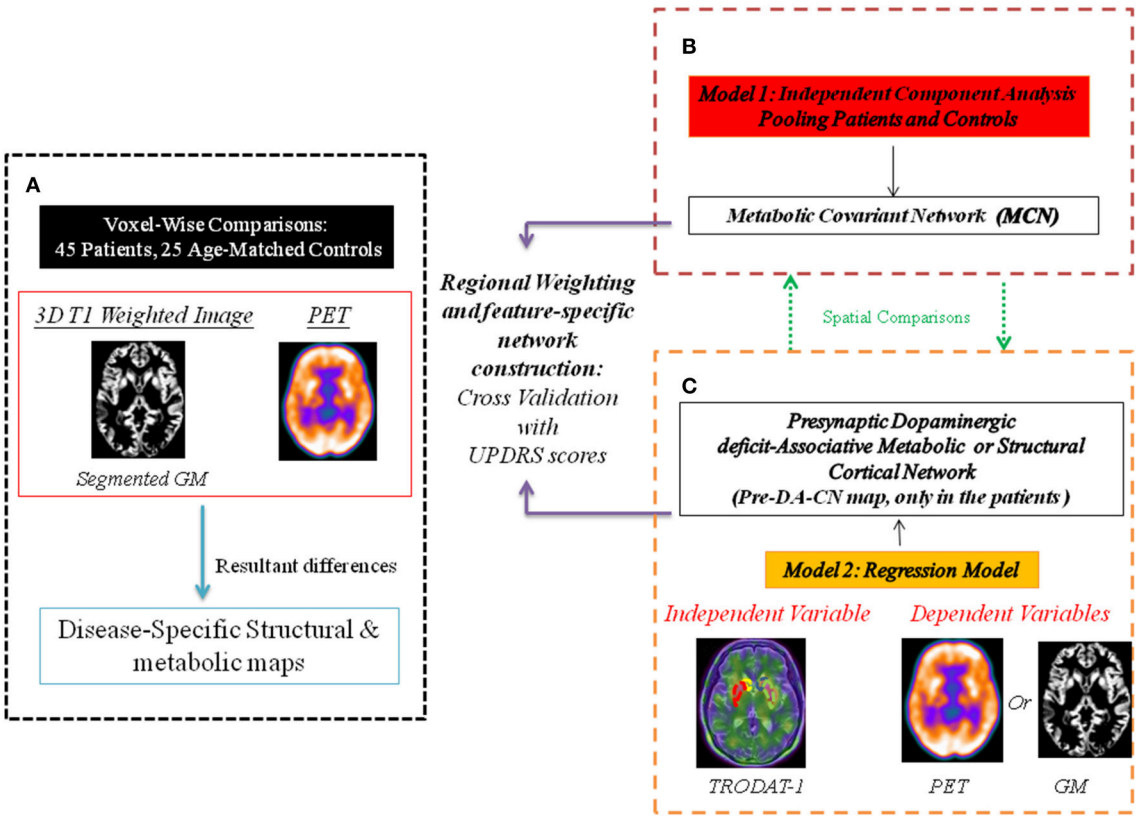

FIGURE 1 | Working scheme for the network construction. The multi-modality comparisons delineate disease-specific pattern in carbon monoxide (CO)-related parkinsonism, compared with the controls. Two constructed statistical models separate networks with different physiological meanings. PET, positron emission tomography; GM, gray matter. **(A)** The disease specific map represent differences between patients and controls. **(B)** Metabolic covariant network using independent component analysis. **(C)** Presynaptic dopaminergic deficit network represents topographies in PET or GM that are parallel to the signals of TRODAT-1.

experienced history of lung diseases while five women and seven male patients had smoking history. The exclusion criteria included a pre-existing intracranial disorder, an agitated mood, or an impaired arousal state that prevented accurate assessment of neuropsychiatric status (Chen et al., 2013). Twenty-five age-matched controls were enrolled for clinical and neuroimaging parameter comparisons.

Parkinsonism Severity Assessment and Cognitive Testing

The severity of parkinsonism was evaluated using the UPDRS-part III motor score. Eight patients received levodopa and/or treatment with dopaminergic agonists during the clinical follow-up period. For research purposes, all patients were drug-free of levodopa or dopaminergic agonists for 8 h at the time of 99mTc-TRODAT-1 neuroimaging evaluation. The score for axial features in this study was defined using the subscales of the part III score, including speech, neck rigidity, rising from a chair, posture, gait, and postural instability.

We also included the cognitive tests (Chang et al., 2011) to understand the relationships between mental status and parkinsonism severity. General intellectual function was assessed using the Mini-Mental State Examination. Verbal and non-verbal episodic memory was assessed using a modified California Verbal Learning Test-Mental Status and the Rey-Osterrieth Complex Figure Test after a 10–min delay. Specific tests to analyse executive functions included backward-span, verbal fluency, Stroop Interference, and Modified Trails B tests were also performed. For behavioral observations, we used the 12-item version of the neuropsychiatric inventory and geriatric depression score.

Structural Imaging Acquisition and Analysis

The three-dimensional T1-weighted images were acquired using a 3.0T MRI scanner (Excite, GE Medical Systems, Milwaukee, WI, USA; Chang et al., 2009). The general linear model was used to assess significant differences between groups. Age and gender were considered as covariates of no interest to exclude their possible effects on the regional GM or WM volumes. A direct comparison between the patients and controls were made to construct the differences in structural or metabolic network (**Figure 1A**). The significance threshold was set at $P < 0.01$, corrected for multiple comparisons across the entire brain (the false discovery rate) with an extended threshold of 250 voxels.

^{18}F-Fluoro-2-Deoxyglucose PET Acquisition

All ^{18}F-fluoro-2-deoxyglucose PET images were obtained using an integrated PET/CT System (Discovery ST, General Electric Medical System, Milwaukee, WI; Huang et al., 2015). Helical CT images were acquired using the following parameters: 140 kv, 170 mA (maximum), and 3.75-mm-thick sections. A single three-dimensional-mode PET/CT image of the head region was taken for 10 min and reconstructed using an ordered subsets expectation maximization algorithm (2 iterations, 30 subsets; Gaussian filter: 2 mm) with CT-based attenuation correction. The reconstructed images were characterized by a matrix size of 128 × 128 and a voxel size of 1.2 × 1.2 × 3.25 mm^3.

PET Preprocessing

PET images were first co-registered to the corresponding MR image, and individual MR images were spatially normalized to the Montreal Neurological Institute template (Müller-Gärtner et al., 1992). Each PET image was then corrected for partial volume effect (Müller-Gärtner et al., 1992) by PMOD (modified voxel-wise version with gray and WM cut-off = 0.5). The spatial normalization parameters were then applied to the corresponding partial-volume corrected PET image to obtain the final normalized PET image in the Montreal Neurological Institute domain. Another issue with ICA in PET analysis is whether the spatial covariance of signals is due to the underlying spatial variance of GM volume. To overcome inter-subject variance, we used a regression model for each subject to regress out spatial GM volume variance from the PET imaging. Finally, the spatially normalized PET images were smoothed using a Gaussian kernel of 8-mm full-width at half maximum. The inferior occipital cortex uptake was applied as the reference region.

TABLE 1 | Demographic data of the carbon monoxide intoxication patients and controls.

	Patients (n = 45)	Control (n = 25)
Age (years)	42.09 ± 10.59	40.33 ± 6.50
Gender (male/female)	16/29	8/17
Education (years)	10.88 ± 3.95	11.00 ± 3.74
Carboxyhemoglobin (%) (mean, range)	22.4, 15-68	N.A
Conscious disturbance period (day)	1.5 ± 0.5	N.A
Hyperbaric oxygen therapy (n)	24	N.A
Mini-mental state examination	21.79 ± 8.71*	28.80 ± 0.94
Memory function		
Verbal memory	5.23 ± 3.12*	8.20 ± 1.37
Visual memory	9.38 ± 5.9*	14.93 ± 2.34
Executive function test		
Digit backward scores	3.67 ± 1.75*	5.80 ± 1.27
Modified Trail-Making B test	10.0 ± 5.02*	13.67 ± 0.90
Stroop Interference test	29.74 ± 17.9*	53.7 ± 10.08
Verbal Fluency	11.49 ± 5.24*	18.80 ± 4.54
Neuro-behavior test		
Neuropsychiatric inventory total score	19.89 ± 18.51*	2.20 ± 4.54
Geriatric depression scores	8.76 ± 4.77*	2.67 ± 3.22

*Data are expressed as the mean ± standard deviation. N.A, not available; *p < 0.01 CO intoxication compared with control.*

Spatial Independent Component Analysis by PET

Spatial ICA was carried out using Multivariate Exploratory Linear Optimized Decomposition into Independent Components software package version 3.14 (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/MELODIC). The preprocessed spatial normalized PET images from the patients and controls were concatenated to form a subject series and entered into the ICA process. The resulting independent components were z-transformed and visualized using a threshold of $z > 1.96$ ($p < 0.05$; Map 1, **Figure 1B**). Differences of MCN intensities between two groups were calculated. To understand the clinical significance of the identified MCN in the patients, we also calculated the correlations between the extract MCN intensity and the clinical scores by setting the significance value at $p < 0.05$ using Bonferroni correction for multiple comparisons.

Pre-DA-CN Map

The acquisition procedure of 99mTc-TRODAT-1 followed a previously published protocol (Chang et al., 2011). The ratios of specific to non-specific striatal 99mTc-TRODAT-1 binding in the caudate, putamen, and striatum regions were calculated. As there is a lack of laterality in pre-synaptic dopamine deficits (Chang et al., 2011), mean striatal 99mTc-TRODAT-1 binding ratio, calculated by averaging the values from the left and right hemispheres, was used for regression modal analysis.

The striatum TRODAT-1 uptake ratios were entered as covariates of interests into the correlation analysis matrix in preprocessed PET or structural images using the Statistical Parametric Mapping software package version 8 (http://www.fil.ion.ucl.ac.uk/spm; **Figure 1C**). The significance threshold was set at $p < 0.01$, corrected for multiple comparisons across the entire brain (the false discovery rate) with an extended threshold of 250 voxels. The resulting map was considered as the metabolic or structural Pre-DA-CN map. For PET, the standard uptake value ratio within each significant volume of interest using an automated anatomic labeling template (Tzourio-Mazoyer et al., 2002) was calculated and correlated with the clinical parameter to explore the clinical significance.

Statistical Analysis

The data were presented as mean ± standard deviation. Spearman correlation was used to explore the relationships between the continuous variables. All statistical analyses were performed using the Statistical Product and Service Solutions software package (version 11.0 for Windows; SPSS, Chicago, IL, USA) and Bonferroni correction for multiple comparisons. The $p < 0.05$ (two-tailed) was considered statistically significant.

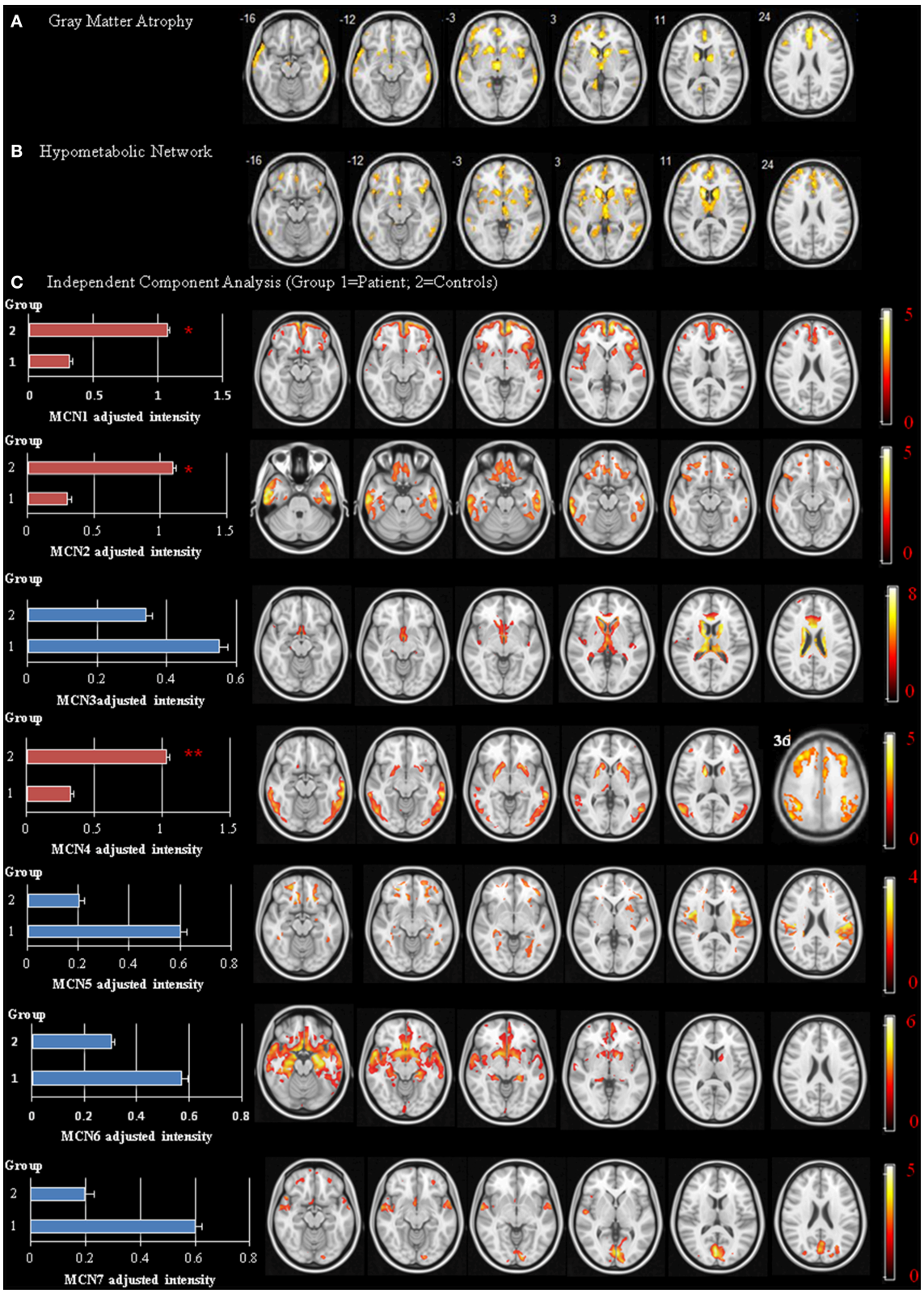

FIGURE 2 | Disease-specific network showing significant differences between the patients and controls. Voxel-based morphometry of gray matter (GM) volume **(A)** and positron emission tomography (PET) signals using occipital cortex as reference region **(B)** Independent Component Analysis of PET **(C)** Image results overlay on the T1 template and color bar represents *t*-value ranges. MCN, metabolic covariant network. $^{*}p < 0.05$, $^{**}p < 0.01$.

RESULTS

Demographic Data and Disease-Specific Maps

Forty-five patients and 25 age-matched controls completed the study, and their demographic data are shown in **Table 1**. The mean interval from CO intoxication to the study was 4.8 ± 0.8 months. Imaging differences between the patients and controls are shown in **Figure 2**. Regions showing greater GM atrophy in the patients were located in the medial and lateral prefrontal cortex, lateral temporal cortex, caudate, and thalamus (**Figure 2A**). PET analysis revealed lower cortical glucose metabolism in the patients. The cortical hubs included the medial and lateral prefrontal cortex, caudate, anterior putamen regions, thalamus, temporal-parietal junction, and precunesus (**Figure 2B**). The topographies of PET hypometabolism and GM atrophy were highly coherent.

Striatum TRODAT-1 and PET Analysis

Compared with the controls, the patients showed significantly lower striatal TRODAT-1 and PET signals ($p < 0.001$) suggestive of pre-synaptic dopaminergic deficits. In the patients (**Figure 3A**), the signal uptake ratios of TRODAT-1 were symmetrically distributed in the caudate (left 1.21 ± 0.32, right 1.24 ± 0.33), putamen (left 1.81 ± 0.43, right 1.78 ± 0.42), and striatum (left 1.51 ± 0.34, right 1.51 ± 0.34). For PET, the signal uptake ratios were also symmetrically distributed in the caudate (left 0.57 ± 0.16, right 0.53 ± 0.16), putamen (left 0.90 ± 0.12, right 0.88 ± 0.12), and striatum (left 0.73 ± 0.13, right 0.70 ± 0.12, **Figure 3B**) while the patterns echoed those in TRODAT-1 signals. In the striatum, there was a significant linear correlation between the TRODAT-1 and positron emission tomography signals ($\rho = 0.474$, $p = 0.001$, $R^2 = 0.293$, **Figure 3C**) or TRODAT-1 and mean diffusivity values ($\rho = -0.322$, $p = 0.031$, $R^2 = 0.23$, **Figure 3D**). There was a lack of correlation between initial carboxyhemoglobin levels and the striatum TRODAT-1 ($\rho = -0.14$, $p > 0.05$), or PET signals ($\rho = -0.1$, $p > 0.05$). Correlations between the caudate and putamen TRODAT-1 signals and parkinsonism total or sub-domain motor scores are listed in Supplementary Table 1.

Spatial ICA Map and Its Clinical Significance

The spatial ICA yielded seven independent MCN, of which only three were considered significantly different between the patients and the controls (**Figure 2C**, MCN1, 2, and 4). For each component, the component intensity showing significant differences between two groups were extracted and their coordinates of peak activation were shown in Supplementary Table 2. The three significant MCNs all correlated with the mini-mental state examination and total UPDRS scores (**Figure 4**).

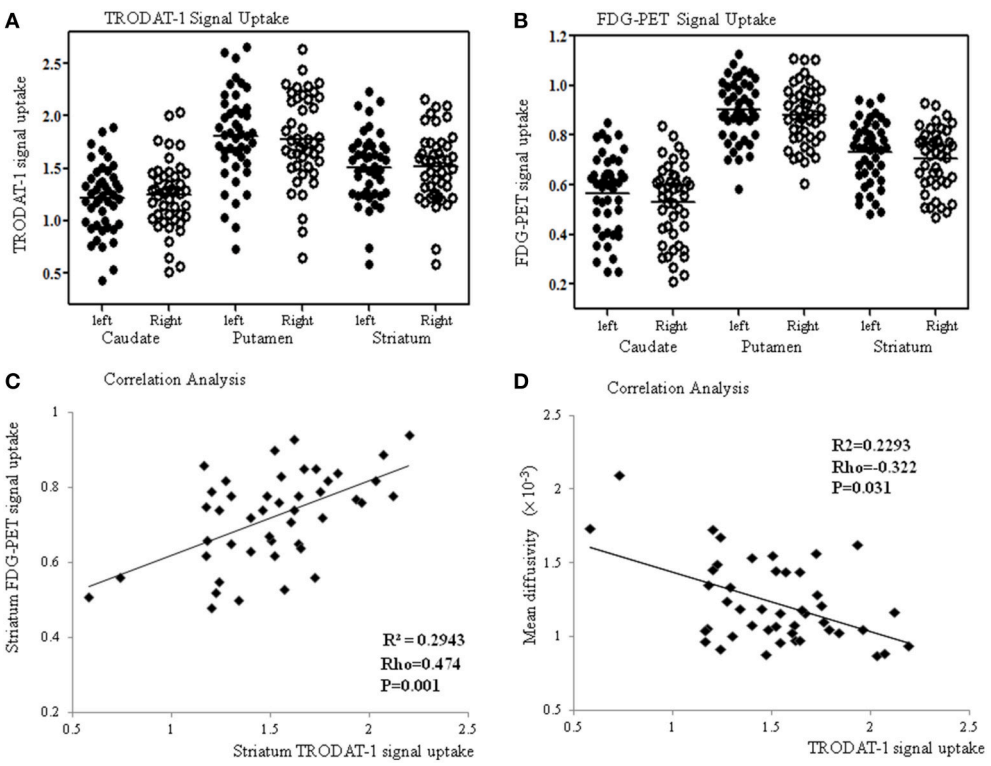

FIGURE 3 | The TRODAT-1 signals (A) or ^{18}F-fluoro-2-deoxyglucose positron emission tomography (FDG-PET) signals (B) are symmetrically distributed in the caudate, putamen and striatum. Significant correlations are found between the striatal FDG-PET and TRODAT-1 signals **(C)** or striatal mean diffusivity values and TRODAT-1 signals **(D)**.

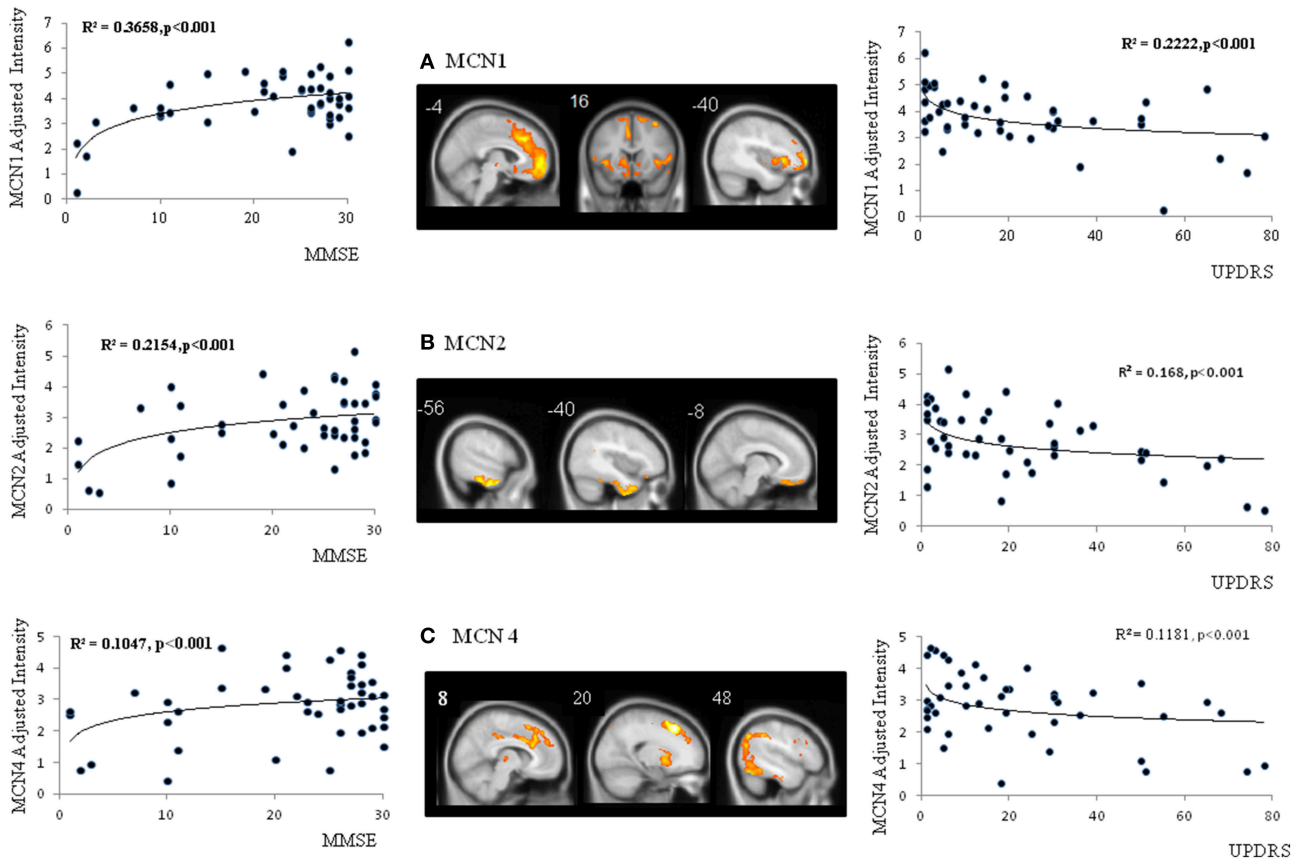

FIGURE 4 | Spatial maps of three significant metabolic covariant networks (MCN) (A–C) and correlations between MCN adjusted intensity and clinical parameters. MMSE, mini-mental state examination; UPDRS, Unified Parkinson's Disease Rating Scale.

Features of the MCNs supported our initial hypothesis that the structural lesions encountered in CO-related parkinsonism may undergo functional rewiring process and the ICA approach helps to delineate meaningful functional network. For TRODAT signal correlations, MCN3 showed significant correlations with TRODAT signals in the caudate ($p = 0.021$) and MCN4 with TRODAT signals in the putamen ($p = 0.022$).

Pre-DA-CN Map

The metabolic Pre-DA-CN map (**Figure 5A**) included the medial cerebral peduncle, basal striatum, thalamus, caudate, posterior putamen, anterior insular, anterior cingulate, and dorsolateral prefrontal cortex. As the patterns of MCN and Pre-DA-CN map were highly spatially coherent, our hypothesis that the MCN might reflect injury from nigro-striatal degeneration was validated.

Regions showing correlation with TRODAT-1 signals were explored (**Figures 5B–E**). The pre-synaptic dopaminergic signals showed positive correlations with WM fractional anisotropy (**Figure 5B**). There were inverse correlations between pre-synaptic dopaminergic signals with mean diffusivity, axial diffusivity, and radial diffusivity maps in the thalamus (**Figure 5C**). Cortical volumes that correlated with TRODAT-1 signals included the medial prefrontal, basal striatum, anterior insular, caudate, and thalamus (**Figure 5D**). The TRODAT-1 signals correlated with WM volume in the midbrain, thalamus, and prefrontal subcortical areas (**Figure 5E**).

Feature-Specific Network with Clinical Weightings among the Pre-DA-CN Map

Focusing on the metabolic Pre-DA-CN map, we further explored the clinical significance of individual regions in determining the features of parkinsonism (**Figure 6A**), cognitive, or behavior scores (**Figure 6B**). The axial feature scores were related to the PET standard uptake value ratio in the putamen, thalamus, insular, ventral medial prefrontal, and superior frontal regions (**Figure 6A**). Among these, the pregenual prefrontal or superior frontal regions predicted most of the UPDRS subscores.

For the cognitive and behavior data (**Figure 6B**), the verbal memory scores were related to the standard uptake value ratio of anterior cingulate cortex, thalamus, insular, ventral striatum, and superior frontal cortical regions, while the visual memory scores were related to the standard uptake value ratio of putamen. A number of regions within the anterior cingulum-striatum-frontal

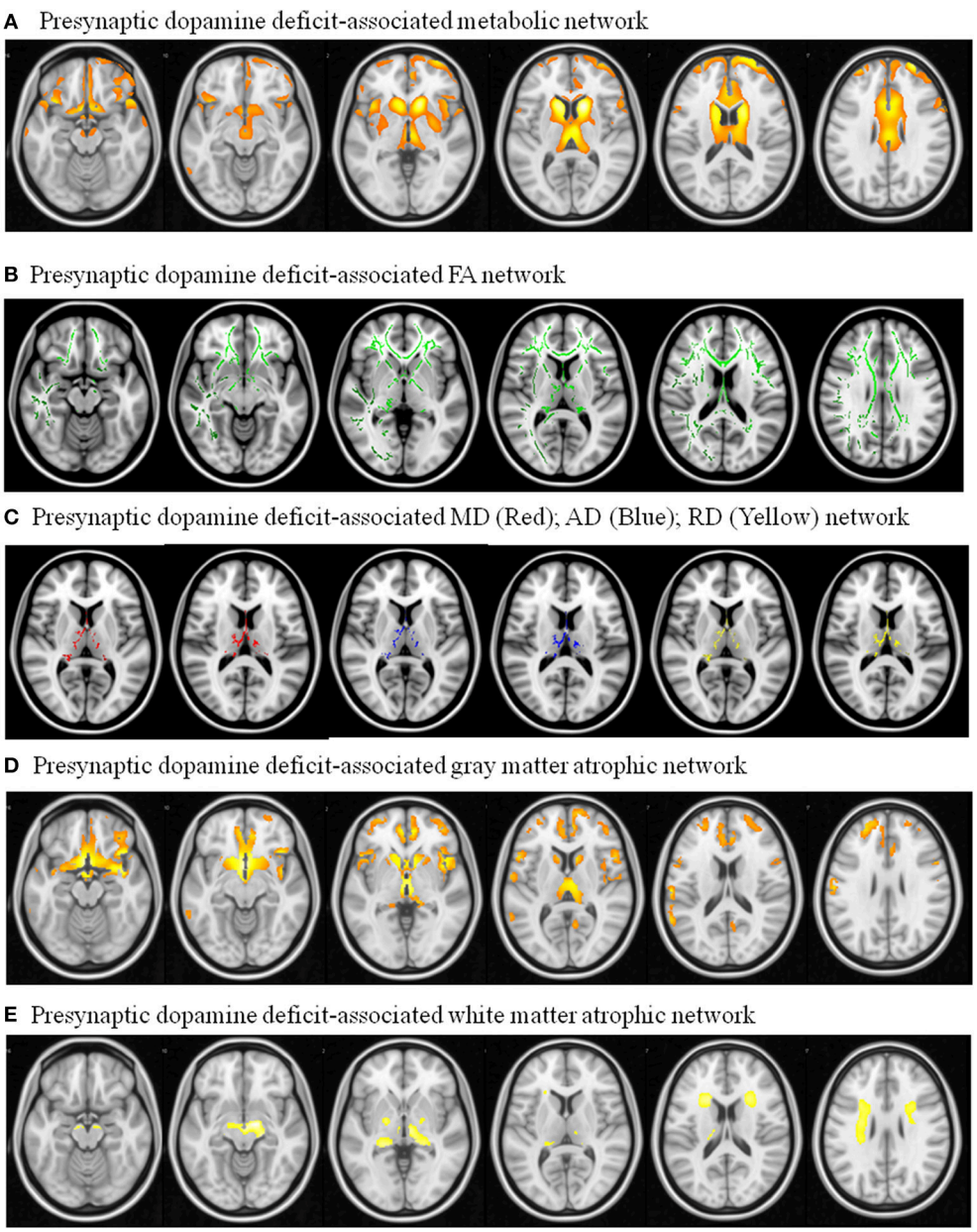

FIGURE 5 | Pre-synaptic dopaminergic deficit-associated network with dependent variable using (A) ^{18}F-fluro-2-deoxyglucose positron emission tomography ; (B) Fractional anisotropy (FA); **(C)** mean diffusivity (MD), axial diffusivity (AD), radial diffusivity (RD); **(D)** 3D T1-weighted segmented gray matter image; **(E)** 3D T1-weighted segmented WM image. Image results overlay on the T1 template.

regions correlated significantly with the executive function test scores. In comparison, none of the PET signals correlated with neurobehavior scores.

DISCUSSION

Major Findings

In this study, CO-related parkinsonian networks were constructed and the clinical significance of these networks from disease-specific, motor severity-specific, and symptom-specific levels were explored. This analysis revealed three major findings. The first is that the medial and lateral prefrontal-caudate-thalamus regions represented CO-parkinsonism specific network (**Figures 2A,B**). The second, we identified 3 MCNs (**Figure 2C**) that distinguish the patients from controls. These 3 MCN not only overlapped spatially with the disease-specific map, but this spatial intensity also correlated with total UPDRS scores (**Figure 4**). The consistency between metabolic Pre-DA-CN (**Figure 5A**) map and the MCN in the prefrontal-subcortical

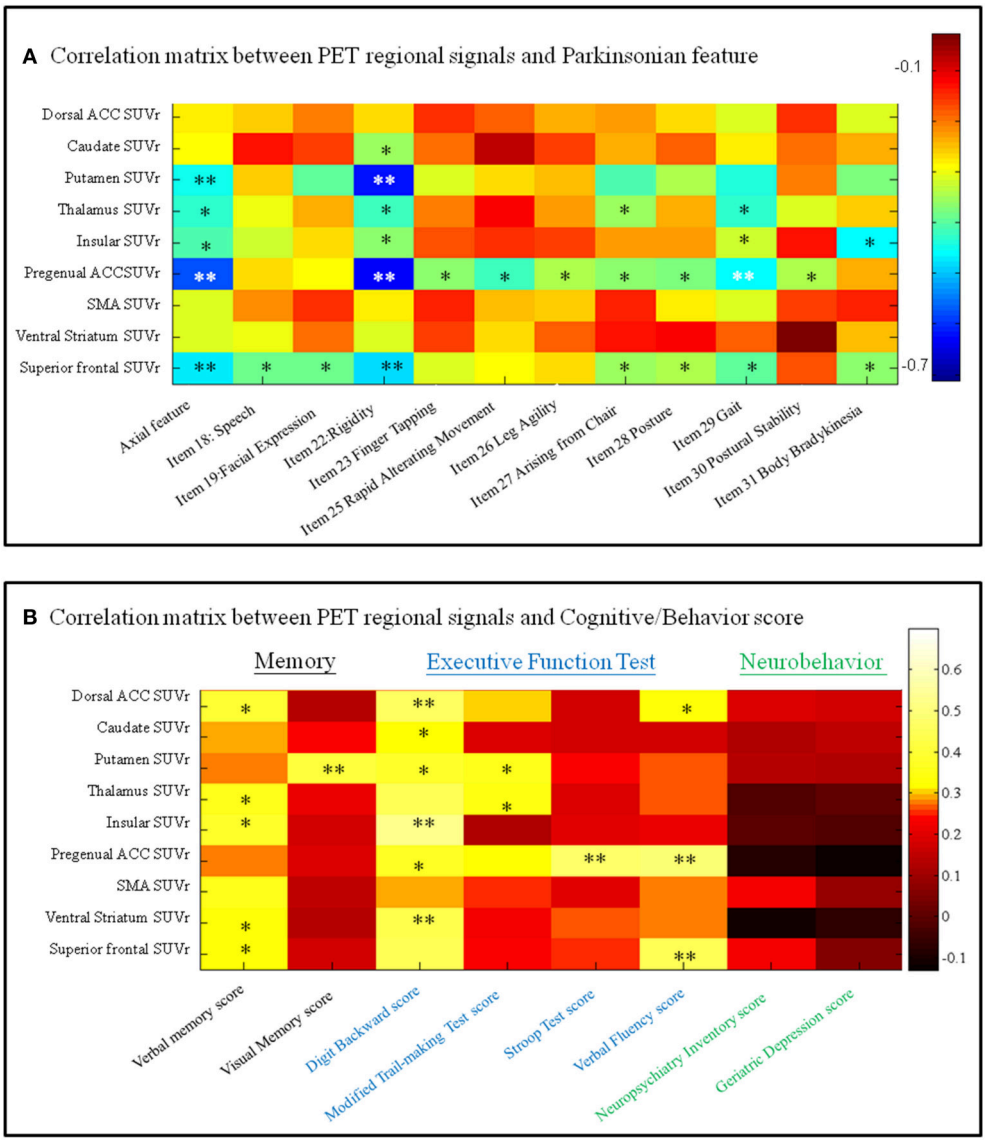

FIGURE 6 | Correlation matrix between regional imaging parameters and (A) Unified Parkinson's Disease Rating Scale items or (B) cognitive and neurobehavior scores. SUVr, standard uptake value ratio; ACC, anterior cingulate cortex; SMA, supplementary motor area. *$p < 0.05$, **$p < 0.01$. Color bar, Pearson correlation coefficient value. PET, positron emission tomography.

regions provided evidence of cortical rewiring processes after nigrostriatal degeneration. Finally, the correlation between regional standard uptake value ratio within the Pre-DA-CN and UPDRS scores offered the clinical weightings of each area (**Figure 6**). The combined analysis with clinical correlations may offer insights into symptomatology prediction in CO-related parkinsonism and validate the functional rewiring process after the structural damages.

Metabolic Pre-DA-CN Map in Relation to Nigrostriatal Disruptions

Although previous studies validated the nigrostriatal damages in CO-related parkinsonism (Chang et al., 2011, 2015), our Pre-DA-CN map suggested that injuries of prefrontal–basal ganglia cortical regions were in parallel with the nigrostriatal degeneration. Like dopaminergic imaging in other PD studies (Antonini et al., 1998; Mure et al., 2011), the Pre-DA-CN correlate mainly with bradykinesia, gait disturbance, and rigidity, rather than tremor. These observations suggest that parkinsonian cortical network in CO-related parkinsonism were related to both nigrostriatal deficits and pre-synaptic dopaminergic projection.

To facilitate the discussion of regional weighting of the Pre-DA-CN and the interactions between cognition and motor features, we constructed a model from our results (**Figure 7**). Based on the clinical symptom-segregations, the regions that jointly explained the parkinsonian and cognitive features included the thalamus, caudate, putamen, pregenual anterior cingulate cortex, and superior frontal regions, while

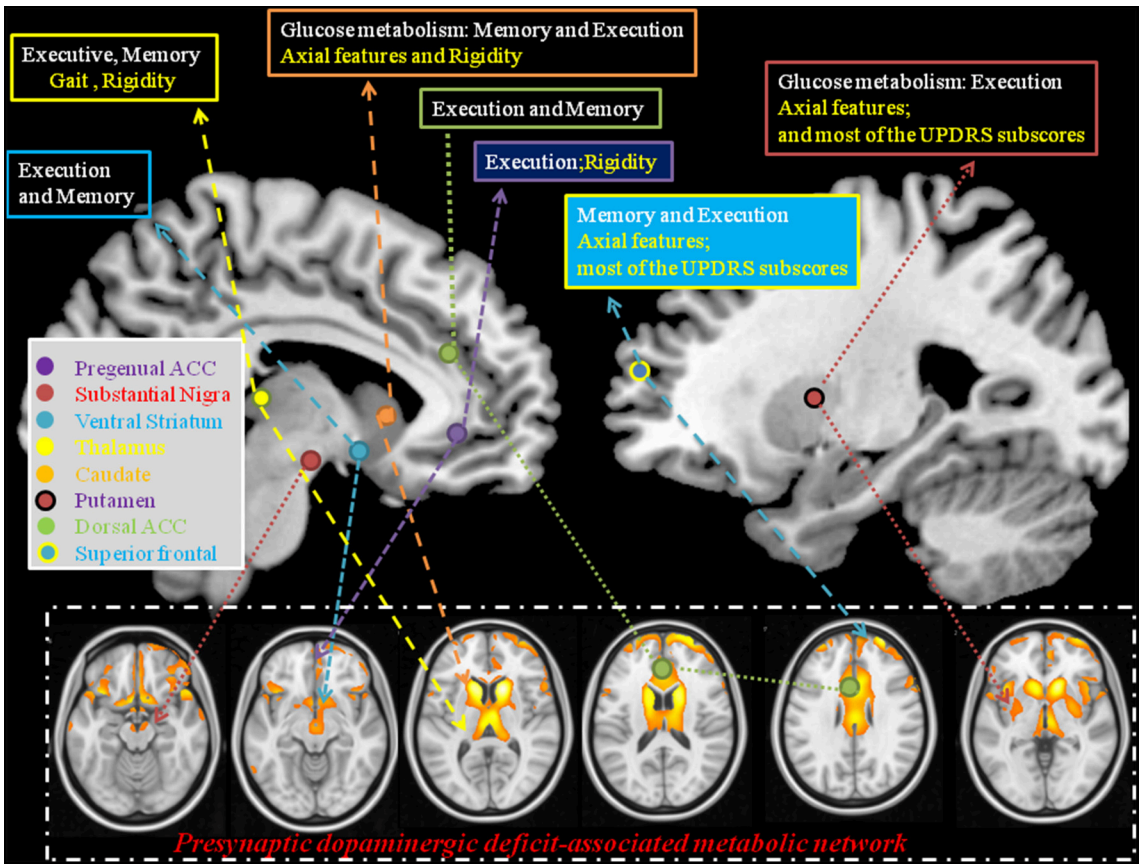

FIGURE 7 | Explanatory model using statistical analysis results from the pre-synaptic deficit-associative metabolic network with symptom segregation. The results highlight the clinical roles of thalamus, caudate, pregenual ACC, superior frontal, and putamen in mediating cognitive and Parkinsonian features. ACC, anterior cingulate cortex; Text in white, cognitive domains; Text in yellow, parkinsonism domains; UPDRS, Unified Parkinson's Disease Rating Scale.

the ventral striatum and dorsal anterior cingulate cortex were related to memory and executive performance. All the aforementioned regions coincided with the cortical projection zones of mesolimbic and mesocortical dopamine pathways. Whether they also demonstrated post-synaptic dopaminergic deficits were not explored in this study. However, the parallel relationships between the cortical PET and the striatal TRODAT-1 signals possibly indicate a common pathophysiological mechanism triggered by CO intoxication (Plum et al., 1962; Lapresle and Fardeau, 1967).

The clinical weightings of individual area in Pre-DA-CN map were also explored here. The identified ventral pallidum, mediodorsal thalamus, and medial prefrontal cortex, part of the mesolimbic system, are well-known to mediate motor function. Specific to the anterior cingulate cortex, we found symptom differences between the ventral and the dorsal regions. The dorsal anterior cingulate cortex has connections with the dorsal caudate, ventral striatum, and nucleus accumbens and is involved in the motivational aspects of movement (Soriano-Mas et al., 2013). The unique contribution of the anterior cingulate cortex to motor function is well known due to its direct efferent projection to the motor system with recruitment of thalamocortical projections (Minciacchi et al., 1986). The projections to the skeletomotor and autonomic nervous systems also regulate integrated motor responses and motor initiation (Devinsky et al., 1995) which may explain the relationships between rigidity scores and ventral part of anterior cingulate cortex signals.

MCN Reflect General Cognitive and Parkinsonism Severity

Spatial covariance analysis has been used extensively to detect network-based abnormalities in a variety of neurodegenerative parkinsonian disorders, including PD (Eidelberg et al., 1994; Eckert et al., 2007), multiple system atrophy, and progressive supranuclear palsy (Eckert et al., 2008). Applying this method to resting-state PET scan from CO-related parkinsonism patients has revealed an abnormal disease-related spatial covariance pattern. The disease-related pattern is characterized by a reduction of covariance signals involving elements of the fronto-insular-basal ganglia-temporal-parietal circuitry. The patterns also show spatial coherence with structural damage map.

The disease related MCN consist of the core hubs of the default mode network (Greicius et al., 2004), basal ganglia

networks (Horwitz et al., 1984), salience, and executive networks (Seeley et al., 2007). Why these cortical hubs shared co-activation pattern in CO-related parkinsonism patients is not well understood. Based on the physiological meaning of MCN, the topography may reflect neural synchronization in energy consumption. As these images were performed at the disease phase, the MCN seen in CO-related parkinsonism is speculated to reflect functional rewiring processes. As the topography of the identified MCN mirrors that of disease-damage map, connectivity changes may result from lesions of the GM or WM. Therefore, further studies are needed to investigate whether dysfunction of these cortical hubs already existed at the acute phase and could predict the occurrence of delayed neuropsychological sequelae.

LIMITATIONS

There are several limitations to this study. First, the reconstructed maps represented reorganization process at the chronic phase. A longitudinal study design is sneeded to investigate the predictive roles of these networks at the acute stage. Second, as the study did not include a post-synaptic dopaminergic tracer in the analysis, the metabolic, or structural Pre-DA-CN map here only indicates a parallel relationship with the pre-synaptic dopaminergic deficits. Future studies may include validation of the Pre-DA-CN map using a post-synaptic dopamine tracer such as 11C-raclopride. Finally, there were patients having smoking history that may also contribute to the cortical rewiring processes although the significance may be minor than the acute exposure to CO.

CONCLUSION

In conclusion, the PET spatial covariance network and the nigrostriatal degeneration cortical maps overlap considerably in the prefrontal-caudate-thalamus axis that echoes the disease-related damage patterns. Based on the significant clinical-imaging parameter correlations, our study results add to the literature that the parkinsonian features in CO intoxication patients were mediated by spatial-segregated but functionally-integrated network in the midbrain-basal ganglia-cortical axis.

AUTHOR CONTRIBUTIONS

All the authors contributed equally to the conceptualization, design, and outline of this manuscript. CC collected the literature, created the figures, and provided the initial draft of the review. SH, Chun-CL and Chen-CL offered valuable clinical insight and ensure clinical accuracy of the manuscript. JH, WC, CH, YC, and NC provided feedback throughout the entire writing process and were instrumental during the editing process.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2016.00187

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Mitochondria-controlled signaling mechanisms of brain protection in hypoxia

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The article is focused on the role of the cell bioenergetic apparatus, mitochondria, involved in development of immediate and delayed molecular mechanisms for adaptation to hypoxic stress in brain cortex. Hypoxia induces reprogramming of respiratory chain function and switching from oxidation of NAD-related substrates (complex I) to succinate oxidation (complex II). Transient, reversible, compensatory activation of respiratory chain complex II is a major mechanism of immediate adaptation to hypoxia necessary for (1) succinate-related energy synthesis in the conditions of oxygen deficiency and formation of urgent resistance in the body; (2) succinate-related stabilization of HIF-1α and initiation of its transcriptional activity related with formation of long-term adaptation; (3) succinate-related activation of the succinate-specific receptor, GPR91.This mechanism participates in at least four critical regulatory functions: (1) sensor function related with changes in kinetic properties of complex I and complex II in response to a gradual decrease in ambient oxygen concentration; this function is designed for selection of the most efficient pathway for energy substrate oxidation in hypoxia; (2) compensatory function focused on formation of immediate adaptive responses to hypoxia and hypoxic resistance of the body; (3) transcriptional function focused on activated synthesis of HIF-1 and the genes providing long-term adaptation to low pO_2; (4) receptor function, which reflects participation of mitochondria in the intercellular signaling system via the succinate-dependent receptor, GPR91. In all cases, the desired result is achieved by activation of the succinate-dependent oxidation pathway, which allows considering succinate as a signaling molecule. Patterns of mitochondria-controlled activation of GPR-91- and HIF-1-dependent reaction were considered, and a possibility of their participation in cellular-intercellular-systemic interactions in hypoxia and adaptation was proved.

Keywords: hypoxia, reprogramming of respiratory chain function, HIF-1a, GPR91, complexes I and II

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Mitochondrial Signaling Function and the Body Vital Activity

Oxygen dependence and capability for maintaining the oxygen homeostasis are the properties of all higher organisms whose vital activity is related with aerobic energy production due to functioning of the respiratory chain in the inner mitochondrial membrane. Inhaled air reflects the state and oxygen demand of mitochondria (Balaban, 1977; Chandel and Schumacker, 2000; Duchen et al., 2003; Duchen, 2004; Gnaiger, 2005;

Das, 2006; Devin and Rigoulet, 2007; Kann and Kovacs, 2007; Wheaton and Chandel, 2011).

In mammals, up to 98% of body consumed oxygen is related with oxidative phosphorylation, a process occurring in the respiratory chain, where O_2 is the final acceptor of electrons delivered by NADH and flavoproteins (Silver and Erecinska, 1998).The electromotive force generated in this process is used for formation of membrane potential and ATP synthesis, which are required for various energy-dependent reactions responsible for maintaining the cell vitality.

Mitochondria, being oxygen-dependent, are a target for hypoxia/ischemia. Shortage of cell environmental oxygen decreases aerobic synthesis and content of high-energy compounds (ATP, CP) and reduces the membrane potential required for maintaining energy demands and the osmotic balance. These factors, in turn, lead to depression of multiple energy-dependent reactions involved in ion transport, electrogenic and receptor cell function, muscle contractions, respiration, etc. The most typical disorders are membrane depolarization, uncontrolled Ca^{2+} entry through voltage-dependent Ca^{2+} channels, activation of Ca^{2+} -dependent phospholipases and proteases, uncontrolled swelling of cells, hydrolysis of most cell components, and, finally, necrosis. Thereby, mitochondrial dysfunction induced by hypoxia may initiate development of various abnormalities and even cause fatality (Acker, 1994; Lukyanova, 1997, 2014; Rumsey et al., 1999; Wenger, 2000; Peers and Kemp, 2001; Lutz and Prentis, 2002; Michiels, 2004; Gnaiger, 2005; Heerlein et al., 2005; Larsen et al., 2006; Seppet et al., 2009; Wheaton and Chandel, 2011; Lukyanova et al., 2013).

Brain is one of the most oxygen-dependent organs (Siesjo, 1978; Nicholls and Budd, 2000; Bickler and Donohoe, 2002; Neubauer and Sunderram, 2004; Kann and Kovacs, 2007). Brain uses up to 20% of consumed oxygen, although the brain weight constitutes only 2% of body weight (Siesjo, 1978; Silver and Erecinska, 1998). Brain is characterized by high expenditure of energy and low energy reserve. Furthermore, maintenance of energy-dependent process takes 80% of ATP produced in neuronal mitochondria. For this reason, the brain is extremely sensitive to hypoxia, particularly specific brain regions, such as the cortex and hippocampus. All this makes essential the insight into functioning of the brain mitochondrial apparatus in the conditions of insufficient oxygen supply.

According to current concepts, mitochondria are an evolutionary product of endosymbiosis, a process that has occurred 2–4 billion years ago. This process involved fusion of two akaryocytes, an anaerobic bacterial cell, the "host," and a proteobacterium symbiont, which possessed a respiratory apparatus including elements of the tricarbonic acid cycle (TAC), a respiratory chain, and oxidative phosphorylation (Margulis, 1981; Alberts et al., 1989; Gray et al., 1999, 2001; Martin et al., 2001; Henze and Martin, 2003; Duchen, 2004; Gray, 2012). In this symbiosis, the future symbiont mitochondrion (promitochondrion) provided the host cell with energy, which was produced by a very economical, aerobic way, while the host cell, in turn, synthesized metabolites necessary for the mitochondrion. During the development of endosymbiotic interrelations, proteobacteria transferred many genes to the host cell nucleus, which had formed due to the increased energy efficiency. This process resulted in simplification of the promitochondrion endosymbiont, which evolved from an independent microorganism into a cell organelle. In this way, the symbiont cells turned into mitochondria whereas host cells turned into eukaryocytes. Due to the endosymbiotic relations, the pro-eukaryocyte has received not only energetic advantages but also a chance for survival in the conditions of gradual oxygen accumulation in the atmosphere, which had initially contained very little oxygen, less than 0.1%. Before oxygen enrichment of the atmosphere, all forms of life that existed in the biosphere of that time, were anaerobic (Margulis, 1981; Alberts et al., 1989; Gray et al., 1999, 2001; Martin et al., 2001; Henze and Martin, 2003; Duchen, 2004; Gray, 2012).

Current mitochondria are unique cytoplasmic organelles characteristic of eukaryotes, i.e., the organisms with cells containing formed, membrane-confined nuclei. Due to their origin, mitochondria possess three distinctive features of structural organization, which differ it from all other intracellular organelles: (1) presence of respiratory chain enzymes designed for aerobic energy production and arranged into four complexes. These complexes are capable for uniting into supercomplexes (respirasomes), which stabilizes the electron transport chain performance, increases the electron transport velocity, may involve substrate channeling, and restricts generation of reactive oxygen species as a by-product (Acín-Pérez et al., 2004; Schagger et al., 2004; McKenzie et al., 2006; Schäfer et al., 2006; Starkov, 2008); (2) presence of its own genome, which allows renewing components of respiratory chain complexes, the most functionally important proteins, independently on the nucleus genome in the conditions of intensive functional loads; and (3) capability for motion, including division, fusion, and intracellular traveling (Kuznetsov et al., 1994, 2004; Gray et al., 1999, 2001; Kaasik et al., 2001; Martin et al., 2001; Henze and Martin, 2003; Chen and Chan, 2005; Kuznetsov and Margreiter, 2009; Gray, 2012; Van der Bliek et al., 2013). Due to the latter property known in scientific literature as "mitochondrial dynamics," mitochondria can form functional complexes with the endoplasmic reticulum and cytoskeletal structures (*intracellular functional energetic units, mitochondrial reticulum*). These complexes provide energy metabolism in local energy-transportation networks. In these complexes, ADP diffusion is facilitated, and a system of channels for metabolite exchange is available. The capability of mitochondria for structural remodeling and metabolic reprogramming is a basis mechanism inseparably linked with mitochondrial energy production, which provides interaction of mitochondria with each other and with other cell structures and systems. Therefore,

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; BC, brain cortex; C-I, II, III, IV, mitochondrial complexes I, II, III, IV; GABA, gamma-aminobutyric acid; GPR91, succinate-related guanine nucleotide - binding protein-coupled receptor; HBH, hypobaric hypoxia; HIF, hypoxia-inducible factor; HRE, hypoxia response element; HR, high-resistance rats; LR, low-resistance rats; NAD, NADH, nicotine adenine nucleotides; PC, phosphocreatine; PDH – HIF prolyl-hydroxylase; PDK, pyruvate dehydrogenase kinase; SDH, succinate dehydrogenase.

in the process of evolution, the mitochondrial major function of aerobic energy production has allowed mitochondria to become involved in *regulation of various physiological functions* by providing energy to most of intracellular processes necessary for the body vital activity (Kaasik et al., 2001; Appaix et al., 2003; Chen and Chan, 2005, 2009; Schäfer et al., 2006; Kuznetsov and Margreiter, 2009; Van der Bliek et al., 2013).

Mitochondria actively participate in *cell metabolism*. They contain many key, limiting enzymes for fatty acid oxidation, steroid biosynthesis, heme synthesis, insulin secretion in β-cells (MacDonald et al., 2005) and gastric juice secretion (Kennedy and Lehninger, 1949; Gnaiger et al., 2000; Duchen et al., 2003; Duchen, 2004; Gnaiger, 2005). Mitochondria participate in regulation of cell redox potential (Meister, 1995) and protein import (Wiedemann et al., 2004). In addition, mitochondria contribute to intracellular signaling and regulation with a central role in keeping homeostatic cell ionic composition (Chandel, 1990; Chandel and Schumacker, 2000; Butow and Avadhani, 2004; Darley-Usmar, 2004). A system of regulatory interaction related with Ca^{2+} and K^{+}- metabolism exists between mitochondria and the endoplasmic reticulum (Gunter and Gunter, 1994; Hansford and Zorov, 1998; Liu et al., 1998; Rizzuto et al., 1998; Fiermonte, 1999; Da Silva et al., 2003).

A system exists that is connected to Ca^{2+} metabolism and involves regulatory interactions between mitochondria and the endoplasmic reticulum as well as between activation of G-coupled surface receptors, the phosphatidyl-inositol-3-kinase (PI3K) cascade, eNOS, guanylyl cyclase, and protein kinase G (PKG), and performance of the mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channel (Wang and Semenza, 1993; Pek and Lutz, 1998; Bernaudin et al., 2004; He et al., 2004; Murphy, 2004; Fan et al., 2010). Activated PKG opens the mitoK_{ATP} channel, which results in increased ROS production followed by activation of other kinases. In this system, PKG is the terminal cytosolic component of the terminal signaling pathway transmitting the cardioprotective signal from the cytosol to the inner mitochondrial membrane through the protein kinase C (PKC)-dependent pathway (Wang and Semenza, 1993; Zhu and Bunn, 1999; Stroka et al., 2001; Semenza, 2002; Bruick, 2003; Butow and Avadhani, 2004; Murphy, 2004). In addition, mitochondria participate in cell-to-cell interactions and systemic regulation (Zhu and Bunn, 1999; Chandel and Schumacker, 2000; Nicholls and Budd, 2000; Nishimura et al., 2001; Brunk and Terman, 2002; Butow and Avadhani, 2004; Kuznetsov et al., 2004; Michiels, 2004; Felty and Roy, 2005; MacDonald et al., 2005; Lukyanova et al., 2008a, 2011). Mitochondria are the major regulators of the *oxygen homeostasis in the body*. At the *systemic* level, mitochondria determine the concentration gradient for oxygen delivered from the environment to the cell and represent the final step of interaction with molecular oxygen (Brunk and Terman, 2002; Lukyanova et al., 2008a, 2011). Due to this function, which determines both viability and vital activity of aerobic organisms, evolution has created very sophisticated physiological systems for oxygen delivery to mitochondria and maintenance of optimum oxygenation in cells (act of breathing; pulmonary system of oxygen transportation; cardiovascular circulatory system; blood mass-transfer system; red cells; and hemoglobin) (Zhu and Bunn, 1999; Nicholls and Budd, 2000; Prabhakar and Overholt, 2000; Di Lisa and Ziegler, 2001; Peers and Kemp, 2001; Brunk and Terman, 2002; Voos and Rotgers, 2002; Da Silva et al., 2003; Duchen, 2004; Kuznetsov et al., 2004; Michiels, 2004; Devin and Rigoulet, 2007).

Organization of the digestion system, including consumption and subsequent stepwise enzymic processing of the food, is also dictated primarily by the need for substrate supply to reactions of mitochondrial oxidation and oxidative phosphorylation (Michiels, 2004). The signaling function of mitochondria is related with such processes as growth (Felty and Roy, 2005), aging (McCarter, 1995; Brunk and Terman, 2002), response to temperature (thermogenesis), apoptosis (Kroemer et al., 1998; Nishimura et al., 2001), insulin secretion in β-cells (MacDonald et al., 2005), and formation of adaptive responses (Bruick, 2003).

Mitochondria provide energy for physiological functions involved in the body vital activity. These functions include, first of all, maintenance of ion gradients in excitable tissues; accumulation of vesicular secret; maintenance of hormonal and neurotransmitter functions; contractility of the heart and vascular, pulmonary and gastrointestinal smooth muscles; fertilization and embryogenesis; and adaptation to stresses (Zhu and Bunn, 1999; Nicholls and Budd, 2000; Di Lisa and Ziegler, 2001; Peers and Kemp, 2001; Voos and Rotgers, 2002; Da Silva et al., 2003; Duchen, 2004; Kuznetsov et al., 2004; Devin and Rigoulet, 2007; Seppet et al., 2009).

Therefore, the mitochondrial respiratory chain in involved not only in the intracellular signaling, but also transmembrane and intercellular signaling. Mitochondria themselves function as active signaling organelles, which contribute to information transmission in various intracellular signaling pathways, and play a key role in most important regulatory physiological processes.

Regulatory Role of Mitochondria in Hypoxia

The body response to hypoxia includes various adaptive reactions, which facilitate elimination of functional and metabolic disorders typical for hypoxia and focus primarily on preservation of the mitochondrial function. In this process, two types of mechanisms are used: (a) an urgent compensatory mechanism designed for mobilization of immediate responses to prevent or eliminate the disorders induced by acute hypoxia; these responses provide fast recovery of the body in the posthypoxic period; and (b) delayed mechanisms of adaptation to hypoxia, which develop within a longer period and enhance non-specific resistance to oxygen shortage. These mechanisms are based on regulatory reprogramming of mitochondrial complex function.

The mitochondrial respiratory chain consists of a series of electron carriers that function as redox pairs and that are mainly prosthetic groups of integral proteins. There are four electron transfer or respiratory complexes (complexes I–IV), each capable of catalyzing electron transfer in a partial reaction of the respiratory chain. Complex I (NADH-ubiquinone oxidoreductase) (C-I) is a multisubunit complex that possesses the NADH-ubiquinone oxidoreductase activity. This largest

component of the respiratory chain consists of at least 46 protein subunits (46–49) (Grivennikova et al., 2001). Seven of which are encoded by the human mitochondrial genome. Additionally, C-I contains stably bound cofactors, including flavin mononucleotide and eight iron-sulfur clusters. C-II (succinate-ubiquinone oxidoreductase) mediates the succinate dehydrogenase (SDH) activity. C-III is ubiquinol-cytochrome c reductase, while C-IV displays the cytochrome c oxidase activity. The electrons resulting from the oxidative process are transferred from C-I and C-II to C-III through ubiquinone and from C-III to C-IV through cytochrome ***c*** as shuttles. Electron transfer through complexes I, III, and IV is coupled to proton pumping, creating the electrochemical gradient used by complex V (ATP synthase) to synthesize ATP from ADP and in organic phosphate (Genova et al., 1995; Brandt, 1997; Briére et al., 2004; Schagger et al., 2004; Schäfer et al., 2006; Lenaz and Genova, 2007).

In the normoxic conditions, ***r***espiratory complexes are assembled into supercomplexes. Complexes I, III, and IV exist together as supramolecular assemblies called respirasomes. The likely functions of the supercomplexes are facilitating the electrons transfer between complexes and minimizing the risk of releasing electrons. The assembly of a respirasome might have advantages in substrate channeling of quinones and/or cyt *c*, sequestration of reactive intermediates, and stabilization of individual complexes. In humans, it was shown that supercomplexes are essential for C-I stability (Acín-Pérez et al., 2004; Schagger et al., 2004; Althoff et al., 2011; Chen et al., 2012; Moreno-Lastres et al., 2012; Winge, 2012). In contrast to the organized electron flow by respiratory supercomplexes, mitochondrial C-II (SDH) can function as an independent enzyme whose activity is limited only by substrate availability (Hawkins et al., 2010).

In normoxia, performance of the respiratory chain usually depends on oxidation of NAD-dependent substrates, the major supplier of reduction equivalents to the respiratory chain through C-I (**Figure 1A**). Contribution of this pathway, as estimated by oxygen consumption, predominates in intact cells. In brain, this contribution may reach 90% relative to the total mitochondria oxygen consumption (Lukyanova, 1997, 2014; Lukyanova et al., 2009a, 2013). Nevertheless, in these conditions, a part of mitochondrial respiration is related with the activity of an alternative pathway of electron transport through C-II and oxidation of succinate, a TAC cycle product found in relatively low concentrations (0.2–0.4 mM) in the normoxic mitochondrial matrix (Hems and Brosnan, 1970; Komaromy-Hiller et al., 1997). In normoxia, the ratio of the two oxidation pathways (NAD-dependent and succinateoxidase) depends primarily on properties of the major C-I and C-II enzymes, NADH-ubiquinone oxidoreductase and SDH, respectively. Their kinetic characteristics are tissue-specific and differ in animals with different hypoxia tolerance (Lukyanova, 1988, 1997, 2005; Dudchenko et al., 1993; Dudchenko and Luk'yanova, 1995, 1996; Lukyanova and Dudchenko, 1999; Lukyanova et al., 2008b, 2009a,b).

Hypoxia induces regulatory reprogramming of the respiratory chain, including reversible suppression of the C-1 electron transport function and compensatory activation of C-II Lukyanova, 1997; Lukyanova et al., 2008a, 2013). The dissociation of C-I from the large supercomplexes occurs under the hypoxic conditions, when succinate accumulates as a substrate for C-II (**Figure 1B**) (Sanborn et al., 1979; Acín-Pérez

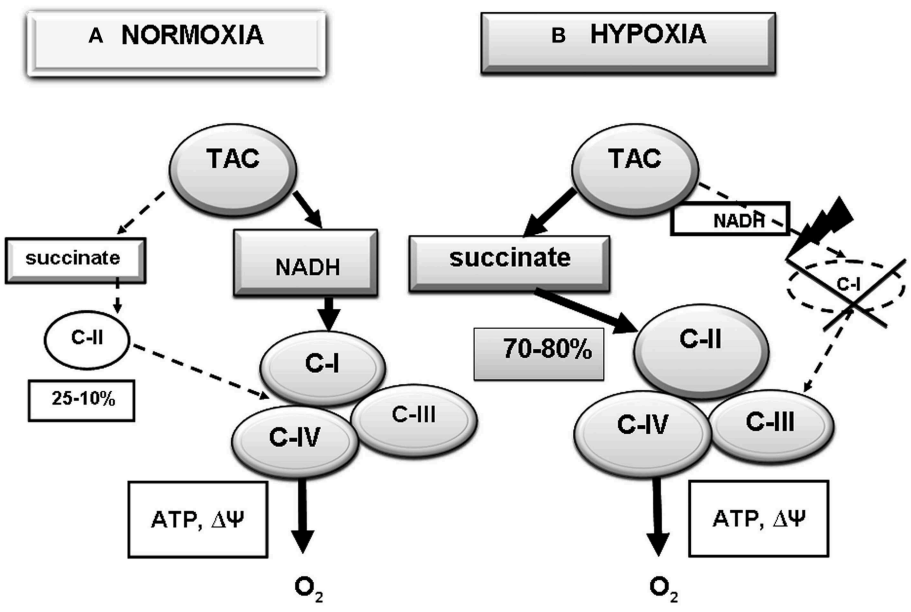

FIGURE 1 | Reprogramming of the respiratory chain function and switching from oxidation of NAD-related substrates (Complex I, C-I) in normoxia (A) to succinate oxidation (C-II) in hypoxia (B). (A) Normoxia; high activity of C-I (70–80% of mitochondrial respiration), low activity of C-II (25–10% of mitochondrial respiration); **(B)** hypoxia; depression of C-I, activation of C- II (90–75% of mitochondrial respiration).

et al., 2004; Althoff et al., 2011; Chen et al., 2012; Moreno-Lastres et al., 2012; Winge, 2012).

Hypoxia is associated with activation of succinate dehydrogenase and succinate oxidation and with increased contribution of the latter to respiration and energy production (Acín-Pérez et al., 2004; Lenaz and Genova, 2007; Althoff et al., 2011; Chen et al., 2012; Moreno-Lastres et al., 2012). The contribution of succinate oxidase oxidation may reach 70–80% (Lukyanova et al., 2008a, 2009a). Under these conditions, mitochondrial C-II can function as an independent enzyme whose activity is limited only by the substrate availability. The C-II-driven electron flow is the primary way of mitochondrial membrane polarization under the hypoxic conditions and that lack of the C- II substrate succinate resulted in reversible membrane potential loss that could be restored rapidly by succinate supplementation (Nowak et al., 2008; Hawkins et al., 2010). The inhibition of mitochondrial C-II also leads to mitochondrial depolarization and mimics hypoxia in cells. The effect of reversible inactivation of the C-I electron transport function in hypoxia is the first stage in the development of hypoxia-induced mitochondrial disorders, which correlate with functional and metabolic phase changes observed at the systemic level (Lukyanova, 1997; Lukyanova et al., 2009b, 2013; Lukyanova, 2014).

At present time, much experimental evidence is available in support of hypoxia-induced disorders in the C-I electron transport function. These disorders persist and even increase in the early posthypoxic period (first 30 min to 2 h of reoxygenation) and are known as *mitochondrial dysfunction* (Rouslin and Millard, 1980; Lukyanova, 1988; Genova et al., 1995; Pitkanen and Robinson, 1996; Robinson, 1998; Chávez et al., 2000; Kunz et al., 2000; Weinberg et al., 2000; Maklashinas et al., 2002; Da Silva et al., 2003; Feldkamp et al., 2004; Sadek et al., 2004; Regard et al., 2008; Lukyanova et al., 2009a).

Also, much evidence exists for a special role of succinate in tissue oxygen metabolism at early hypoxia. Thus, tissue and blood content of succinate was shown to increase by an order of magnitude up to 4–7 mM already in the first 30 min of hypoxia, and to continue growing through early reoxygenation, which allowed some researchers to consider succinate a *hypoxia marker molecule* (Hems and Brosnan, 1970; Hochachka and Dressendorfer, 1976; Taegmeyer, 1978; Hohl et al., 1987; Komaromy-Hiller et al., 1997; Hochachka and Somero, 2001; Kushnir et al., 2001).

The activation of succinate oxidase oxidation in these conditions should be regarded *as an evolutionarily formed, urgent, protective, regulatory, and compensatory mechanism,* which occurs in most tissues *under any form of oxygen shortage* and provides preservation of the aerobic energy production during early disorders of the oxygen homeostasis (Lukyanova, 1997; Lukyanova et al., 2009a, 2013). If this switch does not happen, the uncompensated hypoxic dysfunction of C-I results in acute de-energization (decreased membrane potential; loss of ATP and changes in the adenine nucleotide pool; and respiration disorders due to oxidation of NAD-dependent substrates, the electron donators for C-I) (Dudchenko et al., 1993; Dudchenko and Luk'yanova, 1996; Lukyanova, 1997, 2005; Lukyanova and Dudchenko, 1999; Lukyanova, 2005; Lukyanova et al., 2009a).

All this taken together precedes changes in other functional and metabolic parameters that control the cell vital activity, including condensation of the mitochondrial matrix; disturbed calcium and potassium homeostasis; disordered expression of the mitochondrial genome; generation of reactive oxygen species; loss of CoQ; exit of cytochrome c to the intermembrane space; apoptosis; and impaired capability of cells for adaptation to low pO_2. These processes are associated with an increased lactate/pyruvate ratio; changes in the cell redox potential; metabolic acidosis; and impairment of various energy-dependent processes (such as the electrogenic function of electro-excitable cells and anabolic processes, including urea synthesis, phase II biotransformation reactions, etc.

Features of Respiratory Chain Reprogramming in Hypoxic Brain Cortex

The described above hypoxia-induced changes in the performance of respiratory chain are universal and occur in all tissues, although they are tissue-specific. This process is particularly important for the brain, since the major energetic substrate of oxidation in brain is glucose, which is metabolized to pyruvate in the process of glycolysis. In the aerobic conditions, pyruvate is oxidized in TCA cycle reactions in the NAD-dependent pathway. In the normoxic brain cortex (BC), this oxidation pathway may use up to 90% of consumed oxygen (Lukyanova, 1997). However it should be taken into account that pheno- and genotypic features of the body response to hypoxia are critically important[1]. Furthermore, individual differences in the response to hypoxia manifest themselves not only at the systemic level but also at the cellular and subcellular levels.

Thus, processes of oxidative phosphorylation were shown to be fundamentally different in BC mitochondria of intact hypoxia high-resistant (HR) and low-resistant (LR) rats. In BC of normoxic LR, the baseline similarity of oxidative phosphorylation efficiency is due to a higher rate of phosphorylating respiration, which indicates less economy of this process at baseline. In addition, during oxidation of NAD-dependent substrates by LR BC mitochondria, the velocity of electron transport in the respiratory chain is maximum and may exhaust its reserve capacity, should the functional load increase; this is not observed in the brain of HR animals (Luk'yanova et al., 1991; Dudchenko et al., 1993; Lukyanova et al., 2009a,c).

[1] Any population of animals is known to contain animals with dissimilar tolerance to hypoxia, which correlates with features of the higher nervous system and the functional metabolic profile (Lukyanova, 1997; Lukyanova et al., 2008b, 2009a,b). This dissimilarity is based on differences in the state of the central nervous system, neuro-humoral regulation, stress-activating, and stress-limiting systems, oxygen transporting function of blood, and the state of membranes and receptors. Low-resistant (LR) animals are considered to have a weak type of the nervous system, increased excitability and emotional reactivity, less developed internal inhibition, and high exhaustibility of excitation. LR animals respond to hypoxia with agitation and high locomotor activity. In LR rats, acute hypoxia results in a greater variety of functional and metabolic parameters than in high-resistant (HR) rats. In contrast, HR to hypoxia animals have reduced excitability and anxiety, milder aggressiveness, more pronounced internal inhibition, low sensitivity to any provocative factors, and tendency to social domination. Acute hypoxia induces an inhibitory response in HR rats (Goryacheva et al., 1993; Livanova et al., 1992; Lukyanova, 1997, 2005; Lukyanova et al., 2009c).

Differences in the oxidative capacity of the NAD-dependent substrate region in the BC respiratory chain of HR and LR animals reflect peculiarities of kinetic characteristics for some of respiratory chain enzymes. Thus, in the normoxic BC of LR rats, maximum activity and K_M values (NADH) of NADH-cytochrome c reductase, the main C-I enzyme, are significantly lower than in the BC of HR rats (Dudchenko et al., 1993; Dudchenko and Luk'yanova, 1995, 1996; Lukyanova, 1997, 2005, 2014; Lukyanova et al., 2013). Similar, though less pronounced differences in values of kinetic parameters are typical for cytochrome oxidase in BC of HR and LR animals. Therefore, in the LR BC, these enzymes faster become saturated with the enzyme-specific substrates (NADH or cyt-*c*) and slower oxidize them, which may result in lower activity and faster inactivation of C-I (Dudchenko and Luk'yanova, 1995, 1996; Luk'yanova et al., 1995).

The differences in kinetic parameters of respiratory chain enzymes remain also in oxygen-deficient BC of HR and LR animals. For example, in the neocortex of LR rats, any hypoxia exposure induced opposite changes in K_M values of both complex enzymes—increased NADH-ubiquinone oxidoreductase (C-I) K_M and decreased SDH K_M (C-II). This process reflects reduced and increased efficiency of the enzyme in the first and the second instances, respectively. For this reason, the suppression of BC C-I activity occurs earlier and is more marked in LR rats than in HR rats. Therefore, in different forms of hypoxic exposure, the switching of oxidation pathways for respiratory chain substrates from the NAD-dependent pathway to the succinate pathway is due to kinetic reasons (Luk'yanova et al., 1995). The existence of different kinetic properties of respiratory chain enzymes in HR and LR rat BC, a target for hypoxia, suggests that this phenomenon is genetically predetermined, and the energy metabolism is involved as a major factor determining formation of individual resistance.

These features of C-I and C-II responses to hypoxia correlate with changes in ATP content. Thus, evaluating the dependence of intracellular ATP level on pO_2 showed that a decrease in ATP concentration in the LR rat BC began at higher pO_2 values and was much more pronounced than in HR rats (Dudchenko et al., 1993; Dudchenko and Luk'yanova, 1995; Lukyanova and Dudchenko, 1999). The hypoxic reprogramming of respiratory chain substrate region not only prevents or alleviates disorders of ATP synthesis and normalizes parameters of the adenylate pool but also beneficially influences vital functions, stabilizes and normalizes pH, eliminates acidosis typical for hypoxia (Maevsky et al., 2001), and enhances resistance to the oxygen shortage (**Figure 1**) (Taegmeyer, 1978; Weinberg et al., 2000; Lukyanova, 2005; Lukyanova et al., 2013; Lukyanova, 2014). This process occurs rather rapidly. Changes in kinetic parameters of C-I and C-II major enzymes were observed already at 30 min of various hypoxic exposures (Lukyanova et al., 2008b).

Analyzing effects of different hypoxic regimens on the respiratory chain substrate region showed that regulatory rearrangements of the respiratory chain performance were qualitatively similar in all studied instances. These rearrangements are aimed at activation of the succinate oxidase pathway (C-II), which is energetically more efficient in the hypoxic conditions (Hawkins et al., 2010). When changes in the kinetic parameters of succinate oxidase oxidation were minor or absent, the development of resistance was difficult (Lukyanova et al., 2009a).

Qualitatively different changes in kinetic properties of BC mitochondrial enzymes occur in long-term hypoxic exposures, which provide formation of delayed adaptation (lengthy stay in mountains, hypoxia courses in different regimens). An undoubted sign of adaptation to high altitude hypoxia is increased mitochondrial mass in tissues. The increase in mitochondrial mass during long-term adaptation to hypoxia correlated with activated synthesis of nucleic acids and mitochondrial proteins to provide potentiation of the mitochondrial apparatus. The increase in brain total protein was associated with simultaneous increases in cytochromes, including cytochrome aa_3, which changed in the same way in BC of both HR and LR animals. The cytochrome *aa* content per unit tissue was increased and correlated with the increase in mitochondrial mass and was decreased per unit mitochondrial protein (Dudchenko and Luk'yanova, 1996; Lukyanova and Dudchenko, 1999), which indicated a decreased amount of respiratory transporters in the respiratory chain cytochrome region. Furthermore, the decrease in oxidation rate of NAD-dependent substrates was greater in the LR rat brain than in HR rats.

However, the phosphorylation efficiency estimated by the ATP/O ratio was higher after than before long-term adaptation to hypoxia (Luk'yanova et al., 1990, 1991; Lukyanova et al., 2009b). The increased efficiency of NAD-dependent substrate oxidation together with the reduced electron transport function and the increased amount of mitochondria suggest economization of the energy production process in the brain of adapted rats. Furthermore, the velocity of electron transport in the respiratory chain was no longer limit as it had been before the adaptation, and a "respiratory activity reserve" appeared. In contrast, the "physiological range of respiratory activity" decreased. All these processes were more pronounced in the BC of LR rats. Taken together, these data suggest that long-term adaptation to hypoxia restored the leading role of NAD-dependent oxidation in the energy metabolism. Therefore, preserving high activity of specifically these pathways is essential for development of individual brain resistance to oxygen shortage. On the contrary, the participation of succinate oxidase pathway in energy metabolism during long-term adaptation to hypoxia gradually declined, particularly in the brain of LR animals, and the use of this pathway as a compensatory mechanism became restricted (Lukyanova and Dudchenko, 1999; Lukyanova, 2005).

Kinetic properties of mitochondrial enzymes also changed in the process of long-term adaptation to hypoxia. In the BC of LR rats adapted to hypoxia, Vmax values for NADH cytochrome *c* reductase and cytochrome oxidase 1.5–2.5 times increased while K_M (cyt *c*) values decreased, and the Vmax values approached or exceeded those for HR rat BC. In other words, in the LR BC, activities of these enzymes increased during adaptation to hypoxia whereas their substrate (NADH and cyt *c*) affinity decreased (Lukyanova et al., 2008a). In the BC of HR

rats, parameters of these enzymes remained unchanged or even decreased.

The physiological significance of such transformation may be that new isoforms of mitochondrial enzymes with new features emerge in the LR BC during adaptation. These features allow the enzymes to function in a broader range of their substrate (NADH or reduced cyt *c*) concentrations and at higher rates. The reduction degree of pyridine nucleotides and cytochromes, specifically cyt *c*, increases in hypoxia. Therefore, emergence of new kinetic properties in NADH cytochrome *c* reductase and cytochrome oxidase may provide more effective performance of these enzymes in long-term oxygen shortage. This may result in higher resistance of LR BC mitochondria to acute hypoxia.

Therefore, the economization of energy production characteristic of long-term adaptation to hypoxia is due to emergence of a new mitochondrial population with new properties, including the decreased content of respiratory transporters in the respiratory chain terminal region and the lower oxidizing capacity of these transporters, which, however, work more efficiently due to the increase deficiency of oxidative phosphorylation and the increased amount of mitochondria in the cell. On the whole, both these processes focus on replenishment of ATP losses, which should have occurred in these conditions.

However, in long-term adaptation to hypoxia, the significance of succinate oxidase oxidation gradually declines. In this process, the electron transport function of the NAD-dependent oxidation pathway gradually recovers, and the efficiency of C-I performance increases (Lukyanova et al., 2008a,b, 2009a). This may be related with the adaptation-induced emergence of new isoforms of the major complex I enzyme with new kinetic properties, which enhances the complex efficiency in high reduction of the pyridine nucleotide pool. Maintaining the high activity of C-II in these conditions may hamper the process.

Therefore, succinate contributes to formation of both immediate and delayed mechanisms of adaptation and resistance to hypoxia.

Brain Mitochondria and HIF-1 Transcriptional Activity in Hypoxia

According to current concepts, the leading role in development of adaptation to hypoxia belongs to hypoxia-inducible factor 1 (HIF-1), a *specific protein factor induced by hypoxia*. This factor discovered in the early 1990s (Wang and Semenza, 1993; Semenza, 2002, 2007, 2009) functions as the major regulator of oxygen homeostasis. HIF-1 is a mechanism that the body uses to respond to hypoxia by controlling expression of proteins responsible for oxygen delivery to cells, i.e., HIF-1 mediates cell adaptive responses to changes in tissue oxygenation.

HIF-1 is a heterodimeric redox-sensitive protein consisting of two subunits, the cytoplasmic inducible, oxygen-sensitive **α** subunit (Semenza, 2002, 2007, 2009), which is expressed in practically all mammalian cells, and the constitutive **α** subunit. The HIF-1 activity depends primarily on the HIF-1α subunit whose synthesis is controlled by the MAPK and P13K signaling systems activated by the tyrosine kinase receptor. The receptor agonists include tyrosine hydroxylase, cytokines, growth factors (such as insulin-like factor), and succinate. Normally, the intracellular level of HIF-1α subunit is low because this subunit undergoes proteasomic degradation in oxygen-dependent reactions of prolyl hydroxylation and ubiquitination. Hypoxia creates prerequisites for inactivation of prolyl hydroxylase reactions and thereby provides HIF-1α stabilization and accumulation, induction of HIF-1α transcription and translocation to the nucleus, HIF-1α heterodimerization with the HIF1 β/ARNT subunit, formation of the active transcription complex HRE, expression of HIF-1 dependent target genes, and synthesis of protective, adaptive proteins (Semenza, 2002, 2007, 2009; Kim et al., 2006).

Our investigations have shown that under hypoxic preconditioning, neither free-radical processes nor cytokines and NO perform the function of signaling mechanisms for immediate adaptation responsible for the accumulation of HIF-1α in the early posthypoxic period, and they are likely to be only secondary messengers playing an important role in the formation of delayed adaptation (Kirova et al., 2013, 2014; Lukyanova, 2014).

At the same it is known that oxygen-dependent process of HIF-1α prolyl-hydroxylation and proteasomic degradation occurring in the cytosol of normoxic cells is coupled with utilization of the NAD-dependent substrate of TAC cycle, α-ketoglutarate, while another TAC cycle substrate, succinate, is an allosteric inhibitor of this process (Semenza, 2002, 2007, 2009; Hewitson et al., 2007). Hypoxia inhibits the malate-aspartate bypass, which provides α-ketoglutarate to the cytosol, whereas succinate synthesis is intensified. This creates prerequisites (along with O_2 and Fe^{2+} shortage) for inactivation of prolyl hydroxylase reactions and HIF-1α stabilization, accumulation and potentiation of HIF-1α transcriptional activity.

Now it is proved that functioning of the mitochondrial respiratory chain is coupled with the hypoxia-induced transcriptional expression of HIF-1α. It was shown that even a partial (20%) suppression of C-II activity almost completely inhibited the hypoxic induction of HIF-1α. However it recovered in the presence of succinate (Vaux et al., 2001; Paddenberg et al., 2003; Napolitano et al., 2004; Selak et al., 2005; Hewitson et al., 2007; Koivunen et al., 2007).

We have also shown, that induction of HIF-1α requires a low C-1 activity and a high C-II activity, i.e., potentiation of succinate oxidation (Lukyanova et al., 2008b, 2009b, 2011; Kirova et al., 2013, 2014; Lukyanova, 2014). If that is the case, a relationship should exist between activation of the succinate oxidase oxidation pathway and HIF-1α formation in hypoxia (**Figure 2**).

However, it should be kept in mind that excessive tissue accumulation of succinate in pathological conditions related with impairment of the SDH oxidative function or deficiency of this enzyme may result in excessively high tissue content of HIF-1α and, eventually, uncontrolled potentiation of proliferation, encephalomyopathy, and tumors (Chávez et al., 2000). Thus, succinate dehydrogenase mutations were shown to induce renal, gastric, and thyroid carcinoma, and degeneration of striatal spiny neurons (Huntington's disease) (Baysal, 2003; Selak et al., 2005).

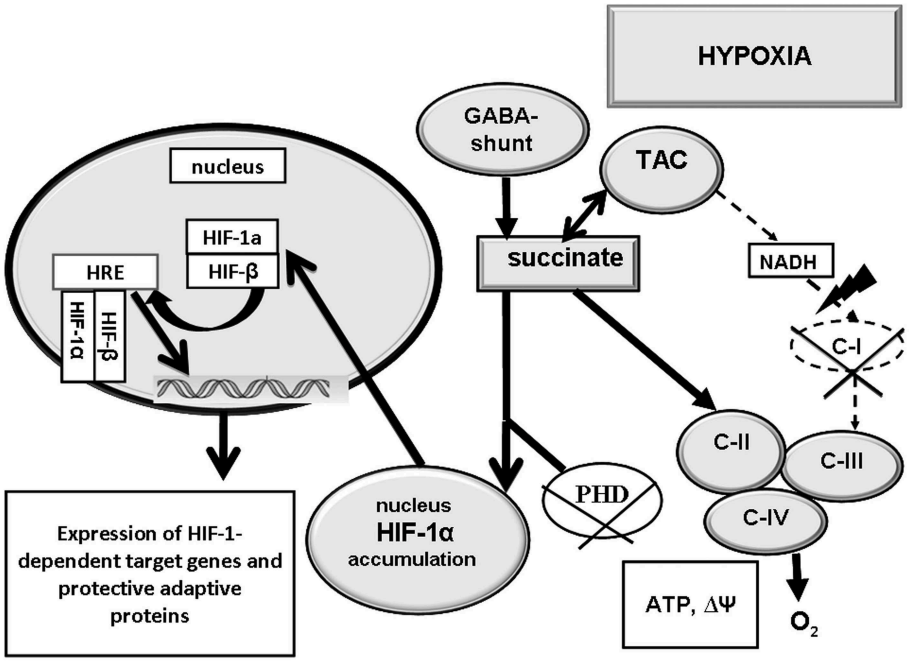

FIGURE 2 | Interaction of succinate oxidase-mediated oxidation (C-II) and HIF-1α transcriptional activity in hypoxia. Activation of C-II contributes to inhibition of prolyl hydroxylase-mediated reactions (PHD) HIF-1α accumulation and translocation to the nucleus, and expression of HIF-1α-dependent adaptation genes. C-II, C-III, C-IV - mitochondrial enzyme complexes; TAC, tricarbonic acid cycle; PHD, prolyl hydroxylase-mediated reaction.

Brain Expression of the Succinate-Dependent Receptor, GPR91, in Hypoxia

Hypoxic reprogramming and switching of the respiratory chain to the succinate oxidase oxidation pathway creates prerequisites for induction of another succinate oxidase signaling pathway, the expression of GPR91 receptor. In 2004, it was shown that succinate, a substrate for the TAC and SDH, the respiratory chain enzyme (C-II), was a specific ligand of GPR91, a metabotropic, purinergic, G protein-coupled receptor from the P2Y family localized in the plasma membrane (He et al., 2004). Succinate-stimulated expression of GPR91 is coupled with activation of Gq/11, G_o and G_i signaling pathways (He et al., 2004; Vargas et al., 2009). The Gq/11 subfamilies activate phospholipase Cβ, which converts phosphatidylinositol-4,5,-bisphosphate into inositol-1,4,5-trisphosphate and diacylglycerol (Fraser, 2008). Members of the Gi family, including, Go, activate a variety of phospholipases and phosphodiesterases, and promote opening of several ion channels. Gi family members can inhibit a subset of enzymes, thereby controlling the intracellular concentrations of cAMP (Hamm, 1998). Inhibition of Gi causes strong impairment of lymphocyte migration *in vitro* (Kaslow and Burns, 1992), suggesting that signaling through the Gi is involved in cell motility processes.

The succinate-dependent receptor, GPR91, is identified in more than 20 tissues (Stroka et al., 2001). The list of GPR91 localizations have been continuously expanding. The role of GPR91 is best studied for kidneys where the succinate-dependent expression of GPR91 involves the renin-angiotensin system and promotes development of renovascular hypertension, which is closely associated with atherosclerosis, diabetes, and renal insufficiency (He et al., 2004; Correa et al., 2007; Sadagopan et al., 2007; Sapieha et al., 2008; Toma et al., 2008; Vargas et al., 2009; Kermorvant-Duchemin et al., 2010; Deen and Robben, 2011). This activation is a part of a kidney-specific, paracrine signaling pathway initiated by high glucose concentrations. The GPR91 signaling cascade in kidneys includes local accumulation of succinate and expression and internalization of GPR91 in endothelial cells of kidney tubules. In the process of signal transduction, increased endothelial Ca^{2+} and formed NO and prostaglandin E2 exert a paracrine effect on adjacent renin-producing cells. This cascade can modulate renal function and facilitate elimination of metabolites by hyperfiltration.

The succinate-dependent GPR91 expression was found in the rodent retinal ganglion cell layer (Sapieha et al., 2008); in ischemic dendrite cells where GPR91 also functions as a trigger for Ca^{2+} mobilization and induction of proinflammatory cytokines (Rubic et al., 2008); in penumbra neurons and astrocytes of infracted brain where GPR91 stimulates an increase in microvessel density (Hamel et al., 2014); in hemopoietic progenitor cells where GPR91 stimulates their growth (Hakak et al., 2009). In hepatic ischemia, GPR91 is expressed only in hepatic stellate cells (Correa et al., 2007). In this case, the succinate-dependent effect is not related with hypertension. The receptor transforms the signal of increased extracellular

succinate concentration into an intracellular signal, which provides activation of stellate cells in response to liver damage.

Information about the functional role of GPR91 is practically limited to these reports, which, however, do not answer the questions, what place the receptor occupies in the body response to hypoxia, and how GPR91 interacts with other signaling systems. According to our data, in the normoxic conditions, the succinate dependent receptor, GPR91, was present in all studied aerobic tissues. However GPR91 was absent in mitochondria-free red cells, which indirectly evidenced GPR91 dependence on mitochondria. The highest density of GPR91 was observed in the myocardium where the GPR91 density was 1.7 times higher than in BC and 2.7 times higher than in liver and kidneys (Lukyanova et al., accepted).

We have also shown that the *immediate* GPR91 expression response to a single hypoxic exposure is tissue-specific, depends on the intensity and duration of the hypoxic exposure, and its intensity does not correlate with the baseline receptor concentration. Thus, the baseline GPR91 concentration was the highest in the myocardium. However, the intensity of GPR91 expression induced by mild and moderate hypoxia was low and increased slowly. The GPR91 baseline concentration in the BC was half the myocardial concentration. Nevertheless, the GPR91 induction was observed in the BC in a broader range of low O_2 in the inhaled air than in the myocardium and liver and was highly intensive (Lukyanova et al., accepted). These facts indicate higher reactivity and sensitivity to oxygen shortage of this succinate-dependent signaling system in the BC than in other studied organs, which may reflect a special significance of this system to the brain under the hypoxic conditions. $F_{IO2} = 14\%$ is a threshold value for GPR91 induction in the BC. In other tissues, GPR91 expression is not induced by these oxygen concentrations in inhaled air.

As distinct from a single, hypoxic session, repeated 60-min exposures (moderate hypoxia) induced a *phase of delayed* GPR91 expression in the BC, which formed regardless of whether the immediate phase of receptor expression was induced. The delayed phase developed following 5–8 sessions and reached a peak at 8–12 days. The delayed phase contemporized with development of delayed, non-specific tolerance of the body (Lukyanova et al., 2009a; Lukyanova et al., accepted). In none of the studied tissues, the phase of delayed GPR91 expression developed after a single hypoxic exposure.

Therefore, the succinate-dependent receptor, GPR91, is involved in the mechanism of formation of both immediate and delayed adaptation to hypoxia. The immediate GPR91 induction was the most intensive in hypoxic BC, which implied a high significance of this signaling pathway for the brain functioning. The fact that the phase of delayed GPR91 expression did not develop after a single hypoxic exposure and required repeated hypoxic sessions suggested that the mechanism of delayed phase formation did not depend on the immediate GPR91 expression but was mediated by other processes developing during a later phase.

The typical for BC immediate hypoxic expression of GPR91 may reflect a local increase in tissue succinate consistent with the hypoxic exposure and followed by succinate binding to the receptor as a paracrine signal. In the brain, the GABA bypass (Roberts cycle) may be a source of succinate in hypoxia. This brain-specific cycle consists of successive biochemical reactions activated by hypoxia/ischemia. In these reactions, gamma-aminobutyric acid (GABA) transforms to succinic acid, a C-II (SDH) substrate, through an intermediate step of succinic semialdehyde (**Figure 3**).

We have tested a possibility of using succinate formed in this cycle in the BC as a specific ligand for the GPR91 receptor. When the activity of glutamate decarboxylase, an enzyme responsible for GABA synthesis, modulation of the GABA bypass activity, and local endogenous formation of succinate, was inhibited with thiosemicarbazide (TSC), the immediate GPR91 expression was not observed in the BC under moderate hypoxia (Lukyanova et al., accepted). However, when TSC was administered together with succinate-containing agents (mexidol, proxipin, sodium succinate), the tissue receptor density recovered and was only slightly lower than the receptor density without the GABA inhibitor. These data demonstrate dependence of the GPR91 expression in the BC on succinate formed in hypoxia-induced reactions of the GABA bypass. This dependence, in its turn, suggests that in the BC, the induction of the succinate-dependent receptor, GPR91, performs a specific function in hypoxia. This function is related with the bypass performance and focused on elimination of glutamate excitotoxicity and maintenance of aerobic oxidation due to activation of the succinate-dependent pathway catalyzed by C-II. Therefore, the BC-specific immediate hypoxic expression of the succinate-dependent receptor, GPR91, is related with activity of the GABA bypass, which functions as a source of succinate both for the receptor and the respiratory chain.

Pharmacological Methods for Protection of the Brain Respiratory Chain in Hypoxia

Prevalence of pathologies related with the disturbed function of C-I in both normoxic and hypoxic conditions, defines the exceptional importance and social significance of protecting the respiratory chain from oxygen shortage and protecting the body from the accompanying energy deficiency.

At present, two approaches to solving this problem exist—pharmacological and physical-therapeutic. The first approach (energotropic therapy) is based on the use of drugs containing an active substance that can interact with the mitochondrial respiratory chain and activate C-I bypassing oxidation pathways while the respiratory chain cytochrome region remains intact. This provides fast reversal of the energy deficiency induced by dysfunction of the NADH-dependent region.

The second approach (hypoxic therapy) is based on repeated exposures to mild hypoxia regimens (hypoxic preconditioning). These exposures induce a variety of adaptive responses, including formation of new isoforms of C-I enzymes with new redox properties, which allow C-I to function in the hypoxic conditions. Non-specific stress resistance of the body increases simultaneously.

The leading place among energotropic medicines belongs to succinate-containing drugs which have been successfully used

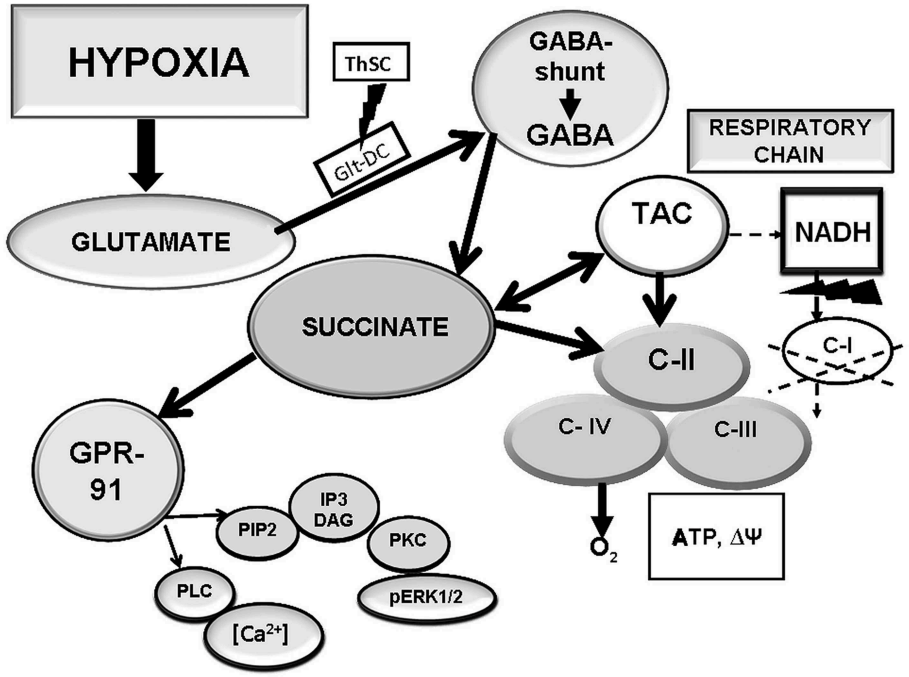

FIGURE 3 | Interaction of the respiratory chain and the GABA bypass aimed at formation of succinate, a GPR91 ligand, in hypoxic BC.

as effective antihypoxic and adaptogenic compounds. The most effective of these drugs are structural derivatives of vitamin B_6, which belong to 3-oxypyridine derivatives (mexidol, proxipin). These drugs are used in the early phase of acute disorders related with oxygen shortage (Luk'yanova et al., 1990; Kondrashova, 1993; Lukyanova and Dudchenko, 1999; Lukyanova et al., 2009a,b,c; Lukyanova, 2005; Nowak et al., 2008).

All succinate-containing drugs are very rapidly absorbed in the setting of different types of hypoxia/ischemia. They exert stabilizing or restoring effect on intracellular ATP as soon as in 15 min after administration. Studies of their mechanism showed that the respiratory chain uses succinate incorporated in the drug structure as an energy substrate. This process stimulates respiration and switches the electron flow from C-I to C-II (succinate monopolization of the respiratory chain proven by the increased respiration sensitivity to malonate and the reduced rotenone-sensitive respiration due to oxidation of NAD-dependent substrates) (Lukyanova and Dudchenko, 1999). Thus, succinate-containing drugs are succinate donors for the respiratory chain. In the hypoxic conditions, they function as antihypoxants potentiating activation of the succinate oxidase oxidation pathway to facilitate recovery and normalization of aerobic energy production (Lukyanova et al., 2009a).

Energotropic and antihypoxic effects of succinate-containing compounds are associated with (1) pronounced antioxidant properties; (2) modification and resynthesis of phospholipids, which decrease the membrane ionic permeability and K+ outflow from mitochondria by the concentration gradient; (3) normalizing effect on calcium metabolism; (4) catecholamine-mimetic, antiteratogenic, antitoxic, hepatoprotective, antiketogenic, and anticholesterolemic effects; (5) removal of excessive acetyl-Co-A associated with decreases in excessive lipids and their metabolites; (6) reduction and normalization of pH and elimination of metabolic acidosis (Kondrashova, 1993; Maevsky et al., 2001).

Along with the energotropic and antihypoxic effects, succinate-containing compounds beneficially influenced multiple vital functional parameters in the conditions of hypoxia and ischemia. These drugs reduced the death rate, recovered the body capability for gaining weight, decreased severity of neurological disorders and aggression typical for hypoxia, exerted antistress and normalizing effects on locomotor, exploratory, and emotional activities of animals (Luk'yanova et al., 1990; Lukyanova, 2005; Lukyanova et al., 2009a,b). Proxipin and mexidol are characterized by a broad range of pharmacological actions. In addition to pronounced antioxidant and psychotropic properties, they are able to increase resistance of the body to different types of hypoxia, reduce ATP losses in ischemic brain and myocardium, and normalize the process of oxidative phosphorylation (Lukyanova, 1997, 2005; Lukyanova and Dudchenko, 1999; Lukyanova et al., 2009b).

It should be also kept in mind that beneficial effects of succinate-containing drugs are supplemented with their modulating effects on the HIF-1α transcriptional activity and the coupled expression of genes for immediate and delayed adaptation, and involvement of critical intracellular signaling pathways via the succinate-dependent receptor, GPR91.

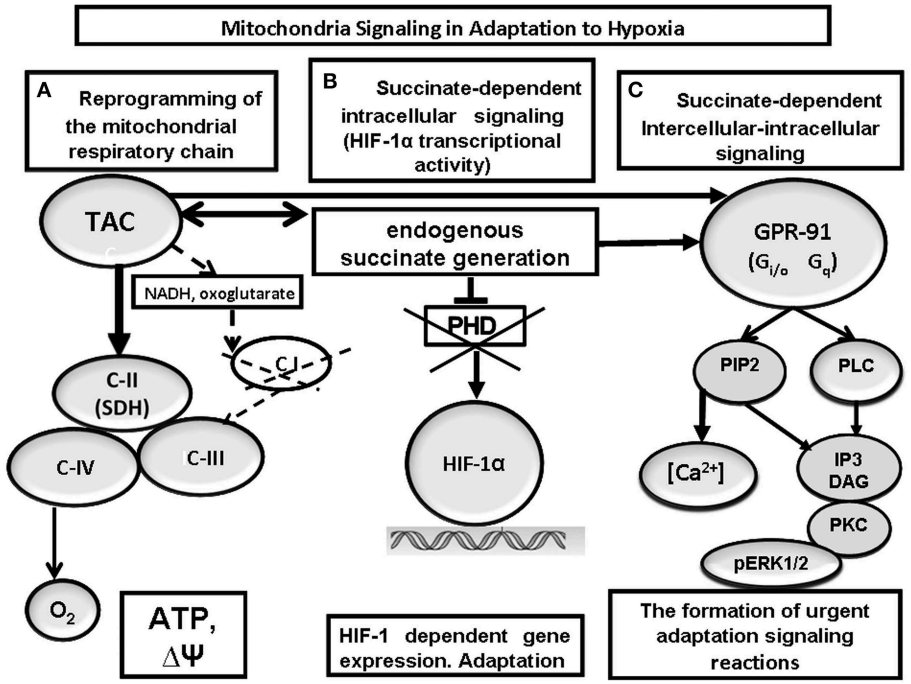

FIGURE 4 | Mitochondrial signaling in cell-to-cell interactions at hypoxia. Reprogramming of the mitochondrial respiratory chain function in hypoxia is a regulatory and compensatory mechanism, which provides: (1) activation of succinate oxidation, a more efficient pathway of energy production in the conditions of rapidly progressing oxygen shortage **(A)**; (2) succinate-dependent PDH inhibition, subsequent stabilization of HIF-1α, and initiation of HIF-1α transcriptional activity **(B)**; (3) succinate-dependent activation of the GPR91 signaling pathway and related signaling pathways, stimulation of HIF-1α synthesis and transcriptional activity **(C)**.

All this provides that the succinate therapy used in the early phase of acute disorders, i.e., during formation of immediate adaptive mechanisms (first 1–3 days of global cerebral ischemia, stroke, myocardial infarction, acute heart failure, traumatic shock, recovery after heart arrest, early postoperative period, after anesthesia, etc.,) exerts a pronounced protective, antihypoxic effect, and increases ATP in tissues.

Conclusion

The data provided above show that the mitochondrial respiratory chain not only directily contribiutes to the development of both early and late adaptive responses in the conditions of hypoxia but is also involved in a sophisticated system of intra- and intercellular signaling, which provides formation of a systemic response to oxygen shortage.

The response to hypoxia initially mediated by C-I inhibition and C-II activation at the subcellular (mitochondrial) level triggers a cascade of succinate-dependent, interacting regulatory reactions, which develop at both cellular and systemic levels and regulate the maintenance of oxygen homeostasis in the body (**Figure 4**).

Transient, reversible, compensatory activation of respiratory chain complex II is a major mechanism of immediate adaptation to hypoxia necessary for (1) succinate-related energy synthesis in the conditions of oxygen deficiency and formation of urgent resistance in the body; (2) succinate-related stabilization of HIF-1α and initiation of its transcriptional activity related with formation of long-term adaptation; (3) succinate-related activation of the succinate-specific receptor, GPR91.

This mechanism participates in at least four critical regulatory functions: (1) *sensor* function related with changes in kinetic properties of C-I and C-II in response to a gradual decrease in ambient oxygen concentration; this function is designed for selection of the most efficient pathway for energy substrate oxidation in hypoxia; (2) *compensatory* function focused on formation of immediate adaptive responses to hypoxia and hypoxic resistance of the body; (3) *transcriptional* function focused on activated synthesis of HIF-1 and the genes providing long-term adaptation to low pO_2; (4) *receptor* function, which reflects participation of mitochondria in the intercellular signaling system via the succinate-dependent receptor, GPR91. In all cases, the desired result is achieved by activation of the succinate-dependent oxidation pathway, which allows considering succinate as a signaling molecule.

Therefore the overall response of the body to oxygen shortage reflects a sophisticated, evolutionarily formed, multifunctional cell response, where energy metabolism performs a trigger, coordinating function of a protective signaling mechanism in the general hierarchy of intracellular processes.

Although succinate-dependent processes occur in different hypoxic tissues, in the BC these processes are observed in a greater range of low oxygen concentrations, more coordinated in time, and more intensive. This suggests, first, well-regulated endogenous, metabolic, anti-hypoxic defense, and adaptive capability of the brain and, second, a particular significance of

brain adaptive processes for the development of tolerance in the whole body.

Analysis of bioenergetics mechanisms and related succinate-dependent signaling reactions in different hypoxia regimens showed that parameters of energy metabolism can be used as predictors and prognostic criteria for severity of hypoxic disorders.

The leading regulatory role of mitochondrial respiratory chain and succinate, its energy substrate, in the development of immediate adaptation to hypoxia suggests a possibility of modulating this process to optimize it. Practical medicine successfully uses various succinate-containing drugs, which exert beneficial effects in pathologies with a hypoxic component, due to their antihypoxic and energotropic effects.

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Fatty Acid Biosynthesis Inhibition Increases Reduction Potential in Neuronal Cells under Hypoxia

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Recently, we have reported a novel neuronal specific pathway for adaptation to hypoxia through increased fatty acid (FA) biosynthesis followed by esterification into lipids. However, the biological role of this pathway under hypoxia remains to be elucidated. In the presented study, we have tested our hypothesis that activation of FA synthesis maintains reduction potential and reduces lactoacidosis in neuronal cells under hypoxia. To address this hypothesis, we measured the effect of FA synthesis inhibition on $NADH_2^+/NAD^+$ and $NADPH_2^+/NADP^+$ ratios, and lactic acid levels in neuronal SH-SY5Y cells exposed to normoxic and hypoxic conditions. FA synthesis inhibitors, TOFA (inhibits Acetyl-CoA carboxylase) and cerulenin (inhibits FA synthase), increased $NADH_2^+/NAD^+$ and $NADPH_2^+/NADP^+$ ratios under hypoxia. Further, FA synthesis inhibition increased lactic acid under both normoxic and hypoxic conditions, and caused cytotoxicity under hypoxia but not normoxia. These results indicate that FA may serve as hydrogen acceptors under hypoxia, thus supporting oxidation reactions including anaerobic glycolysis. These findings may help to identify a radically different approach to attenuate hypoxia related pathophysiology in the nervous system including stroke.

Keywords: hypoxia, reduction and oxidation potential, lactic acid, NAD, NADP, fatty acids, lipid biosynthesis

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INTRODUCTION

Despite the significant role for brain hypoxia in the development of many of pathophysiological conditions including stroke, traumatic brain injury, tumorigenesis, aging, and neurodegenerative diseases (Gallagher and Hackett, 2004; Wilson et al., 2009; Raymond et al., 2011; Clambey et al., 2012; Kirby et al., 2012; Lin et al., 2013), biochemical mechanisms for adaptation to hypoxia are still poorly understood. Recently, we have discovered a previously unknown and neuron-specific mechanism for utilizing anaerobic metabolism during hypoxia. We have found that hypoxic-stressed neurons have a unique response of dramatically increased fatty acid (FA) synthesis from glutamine and glutamate (Gln/Glu) (Brose et al., 2014). However, the biochemical significance of this pathway in neuronal adaptation to hypoxia is unknown. Previously, we have hypothesized a few mechanisms to address the importance for activated FA synthesis from Gln/Glu under hypoxia (Brose et al., 2014) including balancing Glu levels, protection against oxidative stress, and maintaining reduction potential with support of anaerobic glycolysis. In the present study,

Abbreviations: FA, fatty acids; Gln, glutamine; Glu, glutamate; LDH, lactate dehydrogenase; MS, mass spectrometry; UPLC, ultra-pressure liquid chromatography.

we have tested one of these hypotheses that FA SYNTHESIS supports anaerobic metabolism under hypoxia through accepting hydrogen from reduced cofactors ($NADH_2^+$, $NADPH_2^+$, $FADH_2$), thus maintaining reduction potential.

Under hypoxia, hydrogen accumulates on reduced cofactors ($NADH_2^+$, $NADPH_2^+$, $FADH_2$; Garofalo et al., 1988; Obi-Tabot et al., 1993; Foster et al., 2005), due to the decrease in O_2 as its final acceptor. This results in a decreased ATP production through oxidative phosphorylation, and an increased ratio of reduced/oxidized cofactors in the cell and therefore, an increased reduction potential. The altered reduction potential has several devastating effects on cells including lactate accumulation as an alternative H_2 acceptor and subsequent pH drop (Payen et al., 1996; Malisza et al., 1999; Zhang et al., 2006), increased formation of reactive oxygen species which damage lipids, proteins, and DNA (Magalhães et al., 2005), DNA modification through modulation of sirtuin Sirt1 activity (Lin et al., 2004), decreased rates of oxidation reactions such as glycolysis or Glu/Gln oxidation (McKenna, 2007), and a further decrease in ATP production (Pettit et al., 1975). Because FA synthesis consumes two hydrogens from reduced cofactors for each 2 carbons incorporated, we hypothesize that FA synthesis may have a role as a hydrogen acceptor from reduced cofactors under hypoxia, thus maintaining cellular reduction potential.

To address this hypothesis, we applied a previously validated *in-vitro* model for neuronal hypoxia using SH-SY5Y cells exposed to 19% (normoxia) or 1% (hypoxia) oxygen levels (Brose et al., 2014). FA synthesis was inhibited at two different steps in the biosynthetic pathway using tetradecyloxy-2-furoic acid (TOFA, inhibits Acetyl-CoA carboxylase Loftus et al., 2000) and cerulenin (inhibits FA synthase, Heiligtag et al., 2002; Lupu and Menendez, 2006). FA synthesis inhibition significantly increased $NADH_2^+/NAD^+$ and $NADPH_2^+/NADP^+$ ratios under hypoxia and resulted in increased lactic acid under both normoxic and hypoxic conditions. Importantly, FA synthesis inhibition caused cytotoxicity under hypoxia but not normoxia. These results indicate that FA may serve as hydrogen acceptors under hypoxia, thus supporting oxidation reaction including anaerobic glycolysis. These findings may help to identify a radically different approach to attenuate hypoxia related pathophysiology in the nervous system including stroke.

MATERIALS AND METHODS

Materials

SH-SY5Y cells were a gift from Dr. Colin Combs. All culture media and horse serum were purchased from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Serum Source International (Charlotte, NC, USA). L-[U ^{14}C] glutamaic acid (260 mCi/mmol) was purchased from PerkinElmer (Waltham, MA, USA). TOFA and cerulenin were purchased from Cayman Chemical (Ann Arbor, MI, USA). All other chemicals and solvents used were purchased from Fisher Scientific (Waltham, MA USA) and were LC-MS grade.

Cell Culture and Hypoxic Treatment

Cells were plated 3 days before the experiment on a six-well plate (Cellstar, Griner Bio-One, Monroe, NC, USA) at a density of 1.5 million cells per well. The cells were grown in Dulbecco's modified Eagle medium with nutrient mixture F-12 (DMEM/F-12) with 10% FBS and 5% horse serum at 37°C and 5% CO_2.

The hypoxic treatment was as described earlier (Brose et al., 2014). Briefly, the cells were preconditioned by replacing the growth medium with serum-free minimum essential media (MEM) and incubating in 19% O_2 (Normoxia) or 1% O_2 (Hypoxia) in 5% CO_2 at 37°C using nitrogen gas to purge the oxygen. After 24 h, the media was replaced with 2 mL of fresh serum-free MEM containing radiolabeled tracer (2 μCi [U-^{14}C Glu]) and/or fatty acid synthesis inhibitor (TOFA, 2 μg/mL; cerulenin, 1 μg/mL) and returned to their respective incubation conditions for another 18 h. A short re-oxygenation during media change did not significantly affect the FA synthesis rate as was estimated using de-oxygenated media (data not shown).

Lipid Extraction and Saponification

To measure incorporation of radiotracer into fatty acids, cellular lipids were extracted and saponified as described earlier (Brose et al., 2014). Briefly, the media was removed and the cells were washed twice with ice-cold phosphate buffered saline. After removing the final wash, 0.5 mL of methanol was added to the cells; they were scraped and transferred into a silanized with Sigmacote (Sigma Chemical Co., St. Louis, MO) screw top glass tube. Another, 0.5 mL of methanol was added, the plates were scraped again, and the solution was combined with the methanol solution. A Folch extract (Folch et al., 1957) was performed by adding an additional 1 mL of methanol and 4 mL chloroform. The mixture was soniciated using a probe sonicator (Model 150 Sonic Dismembrator, Fisher Scientific) and centrifuged at 2000 × g for 10 min. The supernatant was transferred into a new silanized screw top glass tube and was washed with 1.2 mL saline (0.9% sodium chloride). The extract was washed an additional two times with 1.2 mL chloroform:methanol:water (3:48:47). The extract was dried under nitrogen, re-dissolved in the saponification solution (180 μL methanol and 20 μl 5M potassium hydroxide in water) and heated to 60°C for 60 min to saponify. The samples were then neutralized with 20 μL 5 M hydrochloric acid in water. After neutralization, 780 μL of saline was added and the fatty acids were extracted with 2 mL hexane three times. The combined hexane extracts were evaporated and the fatty acids were re-dissolved in 1 mL of hexane. Radioactivity of an aliquot of the samples was measured in 10 mL Cytoscint (MP Biomedicals; Solon, OH, USA) using a scintillation counter (LS-6500, Beckman Coulter, Pasadena, CA, USA).

Cytotoxicity

Cytotoxicity was measured as a percent of lactate dehydrogenase (LDH) released from the cells into the media using an enzymatic kit (BioVision, Milpitas, CA, USA). Media was collected, and the cells were lysed in 1 mL of the included lysis buffer. For both media and cell lysate, 10 μL was used for LDH measurement. Absorbance was measured at 450 nm using a Flexstation III plate

reader (Molecular Devices; Sunnyvale, CA, USA). LDH released was calculated as ($LDH_{medium}/LDH_{medium+cells}$)×100%.

Lactic Acid

Lactic acid was measured using 20 μL of media with a fluorescence-based enzymatic kit from Cayman Chemical. Fluorescence was measured with a Flexstation III plate reader using an excitation wavelength of 535 nm and an emission wavelength of 590 nm with a cutoff filter of 570 nm.

$NAD^+/NADH_2$ and $NADP^+/NADPH_2$ Measurement

Nucleotides were extracted from cells under ice-cold conditions by adding ice-cold 0.5 mL methanol:water (80:20) containing 0.1 mg/mL ethylenediaminetetraacetic acid and scraping the cells. The solution containing cells was transferred to a 1.5 mL microcentrifuge tube. Another 0.5 mL of the methanol:water solution was added to each well, the wells were scraped again, and the wash was combined with the previous wash. The combined solutions were sonicated, centrifuged at 10000 × g for 10 min at 4°C. The supernatant was transferred into a 2 mL microcentrifuge tube and was washed from lipids with 1 mL hexane 2 times. The remaining solution was evaporated in a vacuum concentrator. The residue was redissolved in 20 μL water and transferred into a silanized microvial insert (Microsolv, Eatontown, NJ USA part number 9502S-02ND) and 10 μL was injected into the LC-MS.

Nucleotides were separated on the same day using a HYPERCARB column (3 μm, 250 Å, 150 × 2.1 mm; Thermo Fisher Scientific; Waltham, MA, USA) maintained at room temperature. The LC system was a Waters AQUITY UPLC pump and a well plate autosampler (Waters; Milford, MA, USA). The autosampler temperature was 8°C. Solvent A consisted of water containing 2 mM ammonium acetate at pH 10 and solvent B consisted of acetonitrile containing 2 mM ammonium acetate at pH 10. The flow rate was 0.3 mL/min and the initial conditions were 97% A and 3% B. The initial conditions were held for 0.5 min. B was increased to 10% over 8 min and held for 2 min. B was then further increased to 25% over 2 min and held for 4 min. Finally, B was increased to 98% over 4 min and held for 7.5 min. B was then returned to the initial conditions over 0.5 min and held for 2 min.

Quantification of nucleotides was performed on a Waters Synapt G2-S quadrupole time-of-flight mass spectrometer (Q-TOF). The electrospray ionization was in negative ion mode as previously described (Brose et al., 2013). The cone voltage was 20 V with a capillary voltage of 1.51 kV. The source temperature was 110°C. The desolvation temperature was 350°C. The cone gas flow was 10 L/h, the desolvation gas flow was 1000 L/h and the nebulizer gas was 6bar. The analyzer was operated in the centroid sensitivity mode with an extended dynamic range with a resolution of 10,000. Mass correction was performed using leucine enkephalin (400 pg/μL, ACN: water, 50: 50) which was infused at 10 μL/min. The acquisition rate was 10 hertz. NAD^+, $NADH_2^+$, $NADP^+$, and $NADPH_2^+$ were quantified using m/z 662.1013, 664.1161, 742.0670, and 744.0833 Da, respectively. Instrument control, acquisition, and sample analysis was performed using MassLynx V4.1 software (Waters).

Statistics

Statistical comparisons were determined using an ANOVA with Tukey's *post-hoc* test. Statistical significance was defined as <0.05. Values are expressed as mean ± SD. GraphPad Prism 6 (GraphPad; San Diego, CA) software was used for statistical analysis.

RESULTS

To address the role for increased FA synthesis under hypoxia, we use our previously validated model for neuronal cell hypoxia (Brose et al., 2014). Consistent with our previous results, FA synthesis from Glu was dramatically 6.4-fold increased in SH-SY5Y cells under 1% O_2 (**Figure 1**). Next, we inhibited FA synthesis at the Acetyl-CoA carboxylase (TOFA, Loftus et al., 2000) or FA synthase (cerulenin, Heiligtag et al., 2002; Lupu and Menendez, 2006) reactions. The inhibitors TOFA and cerulenin were used at concentrations 5- and 3-fold above their IC_{50} values, respectively (Zhu et al., 2004; Wu et al., 2011), and significantly inhibited FA synthesis from Glu under both normoxic and hypoxic conditions (**Figures 1A,B**) while they were not toxic under normoxia (**Figure 1C**). Importantly, at the concentrations used, TOFA demonstrated a higher potency to inhibit FA synthesis under both normoxia (5.1-fold FA synthesis inhibition by TOFA compared to 1.7-fold inhibition by cerulenin) and hypoxia (8.1-fold FA synthesis inhibition by TOFA compared to 2.2-fold inhibition by cerulenin) (**Figures 1A,B**).

To assay the effect of FA synthesis inhibition on cellular reduction potential, we applied a high resolution accurate mass LC-MS approach to measure $NADH_2^+/NAD^+$ and $NADPH_2^+/NADP^+$ ratios under normoxia and hypoxia (**Figure 2**). In the control (vehicle treated) cells, hypoxia resulted in an increased $NADH_2^+/NAD^+$ ratio in both TOFA and cerulenin experiments. This is consistent with hypoxic conditions when O_2 levels are insufficient to accept H_2 from reduced cofactors through the electron transport chain. Slight differences in the $NADH_2^+/NAD^+$ and $NADPH_2^+/NADP^+$ ratios between experiments may be attributed to the differences between culture age and density because TOFA and cerulenin experiments were performed at different times, and the $NADH_2^+/NAD^+$ ratio is closely linked to physiological and pathological states (Schwartz et al., 1974; Atzori et al., 1990; Zhang et al., 2006; Sun et al., 2012). Surprisingly, hypoxia decreased $NADPH_2^+/NADP^+$ ratio. This is consistent with previous reports (Tribble and Jones, 1990; Gupte and Wolin, 2006; Kathagen-Buhmann et al., 2016) and may be associated with the depression of pentose-phosphate pathway (Gupte and Wolin, 2006; Kathagen-Buhmann et al., 2016). Alternatively, decreased $NADPH_2^+$ under hypoxia may be explained through significant increased FA synthesis that utilizes $NADPH_2^+$ as a cofactor.

Consistent with our hypothesis, both inhibitors significantly increased both $NADH_2^+/NAD^+$ and $NADPH_2^+/NADP^+$ ratios under hypoxia as compared to vehicle treated hypoxic cells

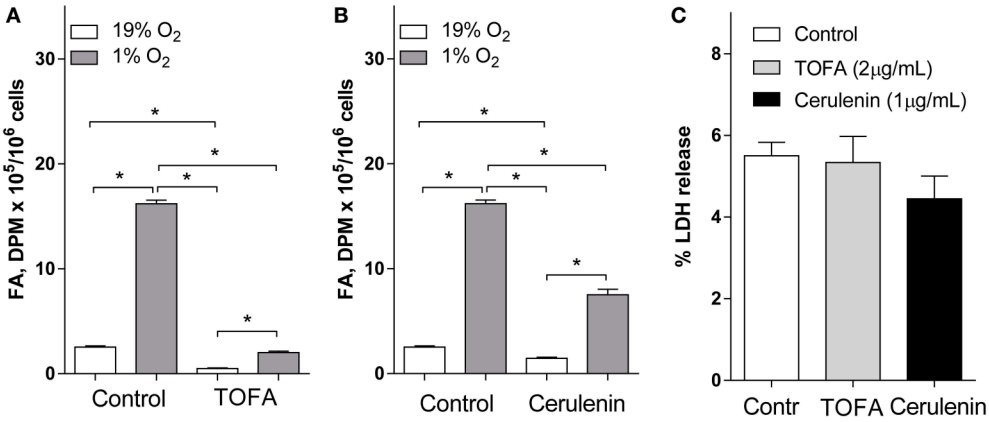

FIGURE 1 | Fatty acid synthesis from glutamate in SH-SY5Y cells was inhibited by TOFA and cerulenin under normoxia (19% O_2) and hypoxia (1% O_2) and did not cause toxicity under normoxia. (A,B): SH-SY5Y cells were preconditioned in serum-free MEM for 24 h under normoxia or hypoxia. The media was replaced with a fresh media and the cells were pretreated with vehicle (control, 1 μL/mL DMSO), **(A)** TOFA (2 μg/mL), or **(B)** cerulenin (1 μg/mL) for 30 min. [U-^{14}C] glutamate (2 μCi) was then added to the wells. The cells were incubated for another 18 h under normoxia or hypoxia. Fatty acid (FA) radioactivity was determined as described in the Materials and Methods. **(C)**: Percent of LDH released into the media was measured under normoxic (19% O_2) conditions to confirm the inhibitors TOFA (2 μg/mL) and cerulenin (1 μg/mL) were not toxic at the concentrations used. *-significantly different, $p < 0.05$. Values are mean ± SD, $n = 3$.

(**Figure 2**). Similar to the effect on FA synthesis, TOFA had a stronger effect on $NADH_2^+/NAD^+$ ratios (1.8-fold increase as compared to vehicle treated hypoxic cells) as compared to cerulenin (1.3-fold increase), while the effect on $NADPH_2^+/NADP^+$ ratios was similar for both inhibitors (∼3-fold increase as compared to vehicle treated hypoxic cells).

Because the $NADH_2^+/NAD^+$ ratio is closely related to anaerobic glycolysis and lactic acidosis which are both activated under 1% hypoxia (Zhang et al., 2006), we assayed the effect of FA synthesis inhibition with TOFA on media lactic acid (**Figure 3A**). Consistent with anaerobic glycolysis activation under hypoxia, 1% O_2 increased media lactic acid 1.9-fold. TOFA did not have an effect on lactic acid under normoxia, but significantly increased lactate under hypoxia 2.6-fold as compared to vehicle treated hypoxic cells (**Figure 3A**). Consistent with increased $NADH_2^+/NAD^+$ and $NADPH_2^+/NADP^+$ ratios, and increased lactoacidosis under hypoxia, TOFA dramatically increased cytotoxicity under hypoxia but not normoxia as measured by cellular LDH release (**Figure 3B**).

DISCUSSION

Despite the significant contribution of brain hypoxia in the development of many of pathophysiological conditions, biochemical mechanisms for neuronal adaptation to hypoxia are still not completely understood. Previously, using both primary neurons and neuronal cell lines, we have reported a novel response of neuronal cells to hypoxia through a dramatic increase in FA synthesis from Gln/Glu (Brose et al., 2014). However, the biological importance for this pathway has not been addressed.

To explain the role for increased FA synthesis under neuronal hypoxia, we have previously hypothesized few mechanisms that may have a complimentary protective role, including balancing Glu levels, protection against oxidative stress, and maintaining reduction potential with support of anaerobic glycolysis (Brose et al., 2014). In the current study, we have addressed the role for FA synthesis in supporting reduction potential as a potent acceptor of hydrogen under hypoxia.

The mechanism for alterations in reduction potential under hypoxia is well understood. Under hypoxia, hydrogen transfer from substrates to oxygen in the electron transport chain in mitochondria is decreased. This leads to accumulation of hydrogen on intermediate cofactors ($NADH_2^+$, $NADPH_2^+$, $FADH_2$; Garofalo et al., 1988; Obi-Tabot et al., 1993; Foster et al., 2005). Because the total pool of reduced and oxidized co-factors is unchanged, the level of oxidized cofactors is decreased, thus the ratio between reduced and oxidized cofactors is increased. As a result, ATP levels are decreased, while the reduced cofactors ($NADH_2^+$, $NADPH_2^+$, $FADH_2$) are significantly increased, limiting energy production and increasing reduced/oxidized cofactor ratio in cells. In addition, cells are unable to completely oxidize pyruvate and acetyl-CoA produced in glycolysis and glutaminolysis (McKenna, 2007). In line with this mechanism, and consistent with previous studies when 1% O_2 was used to model hypoxia (Zhang et al., 2006), we observed a significant increase in the $NADH_2^+/NAD^+$ ratio and lactic acid accumulation in control hypoxic cells (**Figures 2**, **3**). However, similar to previous reports (Tribble and Jones, 1990; Gupte and Wolin, 2006; Kathagen-Buhmann et al., 2016), hypoxia decreased the $NADPH_2^+/NADP^+$ ratio (**Figure 2**) which may be explained through the depression of the pentose-phosphate pathway(Gupte and Wolin, 2006; Kathagen-Buhmann et al., 2016). Alternatively, decreased $NADPH_2^+$ under hypoxia may be explained through significantly increased FA synthesis under hypoxia since FA synthesis utilizes $NADPH_2^+$ as a cofactor. Astrocytes and, to a lesser extent, neurons adapt to hypoxic conditions through switching to anaerobic glycolysis. However,

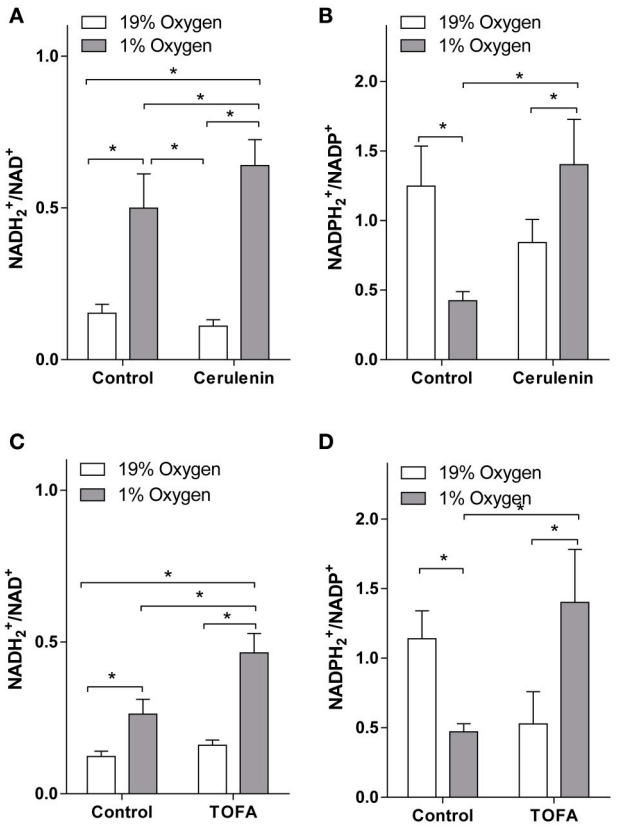

FIGURE 2 | $NADH_2^+/NAD^+$ and $NADPH_2^+/NADP^+$ levels under hypoxia are increased with fatty acid synthesis inhibition. Inhibition of fatty acid synthesis in hypoxic SH-SY5Y cells increases the amounts of $NADH_2^+$ **(A,C)** and $NADPH_2^+$ **(B,D)** relative to their oxidized forms. SH-SY5Y cells were preconditioned in serum-free MEM for 24 h under normoxia or hypoxia. The media was switched to a fresh media and the cells were treated with vehicle (control, 1 μL/mL DMSO), cerulenin (1 μg/mL) **(A,B)**, or TOFA (2 μg/mL) **(C,D)** and were incubated for another 18 h under normoxia or hypoxia. Nucleotides were extracted with 80:20 methanol: water containing EDTA and were analyzed with LC-MS as described in the Materials and Methods. *-significantly different, $p < 0.05$. Values are mean ± SD, $n = 3$.

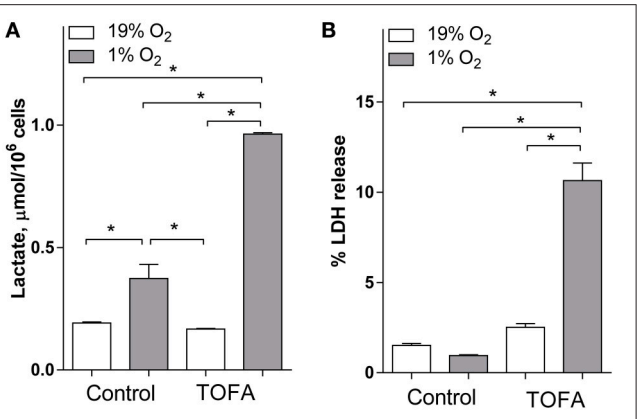

FIGURE 3 | Inhibition of fatty acid synthesis with TOFA in SH-SY5Y cells increases lactic acid levels and toxicity under hypoxia. SH-SY5Y cells were preconditioned in serum-free MEM for 24 h under normoxia or hypoxia. The media was switched to a fresh media and the cells were treated with vehicle (control, 1 μL/mL DMSO) or TOFA (2 μg/mL). The cells were incubated for another 18 h under normoxia or hypoxia. **(A)**: Lactic acid was measured using an enzymatic fluorescent kit. **(B)**: Toxicity was determined through percent of LDH release which was measured using a colorimetric enzymatic kit. *significantly different, $p < 0.05$. Values are mean ± SD, $n = 3$.

under hypoxia anaerobic glycolysis leads to further accumulation of both reduced cofactors and lactic acid (**Figure 3**). This is consistent with attenuation of $NADH_2^+$ accumulation under hypoxia when glucose levels are decreased (Garofalo et al., 1988).

The altered reduction potential has several devastating effects on cells. It results in: **1.** Further lactate accumulation (as an alternative acceptor of hydrogen), and pH drop (Payen et al., 1996; Malisza et al., 1999; Zhang et al., 2006). Importantly, even under normoxia, astrocytic lactate production is very high to provide this key metabolite to neurons for energy metabolism (Hu and Wilson, 1997; Galeffi et al., 2007). Under hypoxia, neuronal oxidative potential is limited, decreasing lactate utilization; **2**. Reactive oxygen species formation with consequential damage to lipids, proteins, and DNA (Magalhães et al., 2005). This paradoxical phenomenon of hypoxia-induced oxidative stress is explained through increased mitochondrial reductive stress (Turrens et al., 1985; Duranteau et al., 1998).

One of the additional mechanisms for increased oxidative damage under accumulation of $NADH_2^+$ is the formation of H_2O_2 that promotes reactive oxygen species formation (Circu and Aw, 2010). Intriguingly, the $NADPH_2^+$ to $NADP^+$ ratio is decreased under hypoxia as we discussed above (**Figure 2**). This may cause additional oxidative damage due to involvement of $NADPH_2^+$ in enzymatic detoxification of reactive oxygen species. However, because an active mitochondrial FA synthase may use $NADH_2^+$ as a cofactor (Podack and Seubert, 1972; Seubert and Podack, 1973; Hinsch and Seubert, 1975; Whereat and Rabinowitz, 1975; Hinsch et al., 1976; Hiltunen et al., 2010; Smith et al., 2012) and H_2 is readily transferred from $NADH_2^+$ to $NADP^+$ by transhydrogenases (Bizouarn et al., 2000; Jackson et al., 2002), increased FA synthesis may not cause but rather protect against oxidative damage; **3**. Altered gene expression and protein modifications through increased protein acetylation. The $NADH_2^+/NAD^+$ ratio modulates the activity of the NAD-dependent deacetylase sirtuin Sirt1 (Lin et al., 2004) that plays a central role in the regulation of thousands of metabolic enzymes and transcription factors in the cytosol and mitochondria (Hallows et al., 2006; Herranz and Serrano, 2010; Wang et al., 2010; Zhao et al., 2010; Hirschey et al., 2011); **4**. Decreased rates of oxidation metabolic reactions including the glycolytic pathway. Because over 700 oxidoreduction enzymes use NAD^+ or $NADP^+$ as cofactors (Sun et al., 2012), the reduced availability of oxidized NAD^+ and $NADP^+$ globally effects cellular biochemical processes; **5**. In addition, the altered reduction potential further reduces ATP production (Pettit et al., 1975), causing neuronal damage. Few mechanisms are involved in the reduction of ATP production including allosteric regulation and oxidized cofactor availability.

Because FA synthesis consumes H_2 from two $NADPH_2^+$ per each acetyl-CoA incorporated into FA chain, H_2 is readily

transferred from $NADH_2^+$ to $NADP^+$ by transhydrogenases (Bizouarn et al., 2000; Jackson et al., 2002), and an active mitochondrial FA synthase may use $NADH_2^+$ as a cofactor (Podack and Seubert, 1972; Seubert and Podack, 1973; Hinsch and Seubert, 1975; Whereat and Rabinowitz, 1975; Hinsch et al., 1976; Hiltunen et al., 2010; Smith et al., 2012), we hypothesized that increased FA synthesis under hypoxia has a role in maintaining cellular reduction potential. Importantly, under hypoxia each acetyl-CoA produced from glucose also produces two $NADH_2^+$. One is produced during anaerobic glycolysis, and another one during pyruvate oxidative decarboxylation. Thus, activation of FA synthesis will stoichiometrically use all $NADH_2^+$ produced in the anaerobic glycolysis and will prevent hydrogen accumulation in the form of lactic acid. In addition, it will support Glu oxidation also associated with $NAD(P)H_2^+$ formation (McKenna, 2007). Using a loss of function approach through inhibition of FA synthesis at two different metabolic reactions at not-cytotoxic levels, we demonstrated a dramatic increase in $NADH_2^+/NAD^+$ and $NADPH_2^+/NADP^+$ ratios under hypoxia compared to vehicle-treated hypoxic neuronal cells (**Figure 2**).

FA synthesis inhibition also resulted in the increased lactic acid levels, and caused a significant toxicity under hypoxia but not normoxia (**Figure 3**). Because TOFA may alter a number of different pathways, it is difficult to provide a conclusive interpretation of the toxicity mechanism of fatty acid inhibition under hypoxia. However, because reductive potential was increased with both inhibitors under hypoxia, and increased reductive potential is the cause but not a direct result of apoptosis (Circu and Aw, 2010; Redza-Dutordoir and Averill-Bates, 2016), we speculate that decreased consumption of reduced cofactors in fatty acid synthesis pathway through inhibition may lead to cellular death under hypoxia.

Together, these data strongly indicate that FA synthesis is important for maintaining reduction potential and decreasing lactic acid, in order to support cell survival under hypoxia. These findings may help to identify a radically different approach to attenuate hypoxia related pathophysiology in the nervous system including stroke.

AUTHOR CONTRIBUTIONS

SB: Conducted experiments, participated in designing the study and method development, writing and editing the manuscript, analyzed and interpreted the data. SG: Conducted MS experiments, participated in LC-MS method development and MS data analysis and interpretation. MG: Supervised and designed the study and method development, wrote and edited the manuscript, analyzed and interpreted the data.

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REVIEW
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Role of Homocysteine in the Ischemic Stroke and Development of Ischemic Tolerance

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Homocysteine (Hcy) is a toxic, sulfur-containing intermediate of methionine metabolism. Hyperhomocysteinemia (hHcy), as a consequence of impaired Hcy metabolism or defects in crucial co-factors that participate in its recycling, is assumed as an independent human stroke risk factor. Neural cells are sensitive to prolonged hHcy treatment, because Hcy cannot be metabolized either by the transsulfuration pathway or by the folate/vitamin B12 independent remethylation pathway. Its detrimental effect after ischemia-induced damage includes accumulation of reactive oxygen species (ROS) and posttranslational modifications of proteins via homocysteinylation and thiolation. Ischemic preconditioning (IPC) is an adaptive response of the CNS to sub-lethal ischemia, which elevates tissues tolerance to subsequent ischemia. The main focus of this review is on the recent data on homocysteine metabolism and mechanisms of its neurotoxicity. In this context, the review documents an increased oxidative stress and functional modification of enzymes involved in redox balance in experimentally induced hyperhomocysteinemia. It also gives an interpretation whether hyperhomocysteinemia alone or in combination with IPC affects the ischemia-induced neurodegenerative changes as well as intracellular signaling. Studies document that hHcy alone significantly increased Fluoro-Jade C- and TUNEL-positive cell neurodegeneration in the rat hippocampus as well as in the cortex. IPC, even if combined with hHcy, could still preserve the neuronal tissue from the lethal ischemic effects. This review also describes the changes in the mitogen-activated protein kinase (MAPK) protein pathways following ischemic injury and IPC. These studies provide evidence for the interplay and tight integration between ERK and p38 MAPK signaling mechanisms in response to the hHcy and also in association of hHcy with ischemia/IPC challenge in the rat brain. Further investigations of the protective factors leading to ischemic tolerance and recognition of the co-morbid risk factors would result in development of new avenues for exploration of novel therapeutics against ischemia and stroke.

Keywords: hyperhomocysteinemia, ischemic preconditioning, intracellular signaling, MAP kinases, neurodegeneration, brain

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INTRODUCTION

Many experimental and clinical studies provide evidence that co-morbid disorders are potential risk factors for development of vascular disorders in humans including stroke (Lehotský et al., 2009a; Kwon et al., 2014). At present, there are several known factors elevating the risk of ischemic stroke which include transient ischemic attack (TIA), arterial diseases, atrial fibrillation, improper diet and/or obesity and physical inactivity (Dirnagl et al., 2009). As it has been verified by many studies, even mild hyperhomocysteinemia (hHcy) may increase the risk for clinical manifestations of stroke, probably due to the pleiotropic biochemical properties of homocysteine (Hcy) and its impact on venous and arterial atherosclerotic modifications (Refsum et al., 1998; Steele et al., 2013; Kwon et al., 2014; Petras et al., 2014; Williams et al., 2014). In fact, Hcy suppresses NO production by endothelial cells and platelets and increases generation of reactive oxygen species (ROS) by the release of arachidonic acid from the platelets. It also inhibits glutathione peroxidase and thus stimulates proliferation of endothelial cells (see Petras et al., 2014, for review). In addition, Hcy has been shown to inhibit methyltransferases, to suppress DNA repair and to facilitate apoptosis when accumulated inside the cells. Autooxidation of Hcy metabolites results in H_20_2 accumulation (Boldyrev et al., 2013) and challlenging neurons to Hcy metabolites for longer period leads to necrotic cell death (Ziemińska et al., 2003). Clinical studies suggest that elevated homocysteine level frequently parallels progressive aging as well as neurodegenerative and acute disorders of the CNS, e.g., Alzheimer's disease or Parkinson's disease (Dionisio et al., 2010). Designing appropriate animal models relevant to the clinical conditions of human stroke is an important step for studying the disease ethiology. Until now, only sparse studies have been developed to explore the mutual effect of HCy and ischemic preconditioning (IPC) in animal models of ischemic stroke.

In this paper we summarize current overview on homocysteine conversion steps in the organism and present the genetic and metabolic causes of hyperhomocysteinemia-related neurotoxicity. Based on the results from our laboratory, we also document, in this context, that mutual effect of experimental hyperhomocysteinemia (hHCy) and ischemic insult with or without pre-ischemic challenge can have different outcomes on the extent of neuronal degeneration as well as on the intracellular signaling pathways leading to the preconditioning phenomenon.

Evolution of Ischemic Tolerance

The brain is the most sensitive organ to hypoxia or ischemia and many endogenous protective mechanisms have been evolved by nature to protect it against the failure caused by the lack of oxygen and energy substrate supply. These mechanisms can also be artificially induced by various approaches resulting in the protective state known as ischemic tolerance (Dirnagl et al., 2009). Many stressors in acute or chronic paradigms are efficient to suppress subsequent injurious/lethal events in the brain caused by hypoxia/ischemia. Hypoxic or IPC is a widely recognized strategy which eventually leads to the state of ischemic tolerance (for review see Rybnikova and Samoilov, 2015; Wang et al., 2015). It could potentially be used as a preventative measure in high risk individuals or as a precaution against secondary stroke following medical procedures such as aneurysm repair or cardiac surgery (Dirnagl et al., 2009; Lehotský et al., 2009a; Thompson et al., 2013). Initial pre-clinical studies of this preconditioning phenomenon (IPC) relied primarily on brief periods of ischemia or hypoxia known as the IPC stimulus but it was later realized that many other stressors, including pharmacological agents, are also effective. Definitive validation of the protective efficacy of preconditioning agents is still missing. On the other hand, some of these strategies/agents are already adapted in clinical practice and thus the future translational approach seems to be promising. Unfortunately, human stroke is not predictable, but the maneuver of postconditioning has a higher potency to elevate protection/adaptation mechanisms, or as a precaution against stroke recurrence (Danielisova et al., 2014). As in many cases, several evidences suggest that preconditioning is beneficial in the short period after stroke since the brain parenchyma in a longer term manifests alterations or tissue damage which are only postponed (Lehotský et al., 2009a; Thompson et al., 2013). More animal and clinical experiments are required to prove and validate the safety and efficacy of these strategies (Dirnagl et al., 2009). Molecular ethiology of IPC is complex and not yet well understood. However, it was shown to affect many pathways in intracellular signaling including receptor modifications, induction of various kinases, e.g., mitogen activating protein (MAP) and switching on apoptotic mechanisms. Cellular factors included in more clinically relevant postconditioning maneuvers represent inhibition of metalloproteinase 9 (MMP-9) expression, which suppresses the extracellular matrix degradation (Chaturvedi and Kaczmarek, 2014; Turner and Sharp, 2016). Remarkably, ischemic tolerance in the brain can also be activated remotely by application of a tourniquet to one of the limbs (Hu et al., 2012; Liu et al., 2016). Remote preconditioning has even been shown to have beneficial effects in human patients with subarachnoid hemorrhage but further studies are still needed (Dirnagl et al., 2009; Lehotský et al., 2009a; Thompson et al., 2013; Cox-Limpens et al., 2014).

In spite of the high clinical relevance, only a limited number of experimental approaches can be found in the literature to describe the influence of co-morbid hyperhomocysteinemia to ischemic damage in animal models of human stroke. Early experimental data which deal with the effect/attenuation of hyperhomocysteinemia on IPC come from cardiovascular studies (Balakumar et al., 2009; Rohilla et al., 2010; Rana et al., 2015). Experiments documented that probably the high degree of oxidative stress occurred in the hyperhomocysteinemic conditions may be responsible for abolishing/attenuation of the cardioprotective potential of IPC (Balakumar et al., 2009). Although IPC-induced cardioprotection has been documented, the experimental data clearly suggest critical abrogation of the beneficial effects of IPC in metabolic dysregulation caused by hyperhomocysteinemia, probably due to reduced release of calcitonin gene-related peptide (CGRP), the over-expression of glycogen synthase kinase-3β (GSK-3β) and phosphatase and tensin homolog (PTEN), impairment of mito-KATP channels, the consequent opening of mitochondrial permeability transition

pore (MPTP) protein kinase C delta, and other mechanisms (Rana et al., 2015).

Cellular Toxicity of Homocysteine Resulting from Its Metabolic Conversions

Homocysteine (Hcy) is an intermediate sulfhydryl- containing amino acid derived from methionine. It has been proved to be toxic for neuronal and vascular endothelial cells (**Figure 1**). In humans it comes from the dietary protein rich in sulfur aminoacids through S-adenosyl methionine conversion (Medina et al., 2001). The causal link between hyperhomocysteinemia and vascular diseases has been described already in the early 1960s (for review see Durand et al., 2001). Remarkably, patients suffering with severe hyperhomocysteinemia (hHcy) manifest typical clinical cardiovascular symptoms as well as neurological disorders, such as cerebral atrophy, dementia and seizures (Obeid and Herrmann, 2006). As shown by many epidemiological observations the association of folate deficiency and hyperhomocysteinemia is frequently correlated with the incidence of vascular diseases and in recent years, also with the incidence of ischemic stroke (Herrmann and Obeid, 2011; Petras et al., 2014).

Homocysteine is metabolized from methionine by three independent alternative pathways:

(i) re-methylation,
(ii) transmethylation to methionine,
(iii) trans-sulfuration to cysteine.

Though mutations or polymorphisms in the key genes encoding enzymes of Hcy metabolic pathways have been well elucidated in cardiovascular disorders and also in stroke, the epigenetic mechanisms, such as DNA methylation, chromatin remodeling, RNA editing, noncoding RNAs (ncRNAs) and microRNAs (miRNAs) are now involved in the ethiology of stroke (Dirnagl

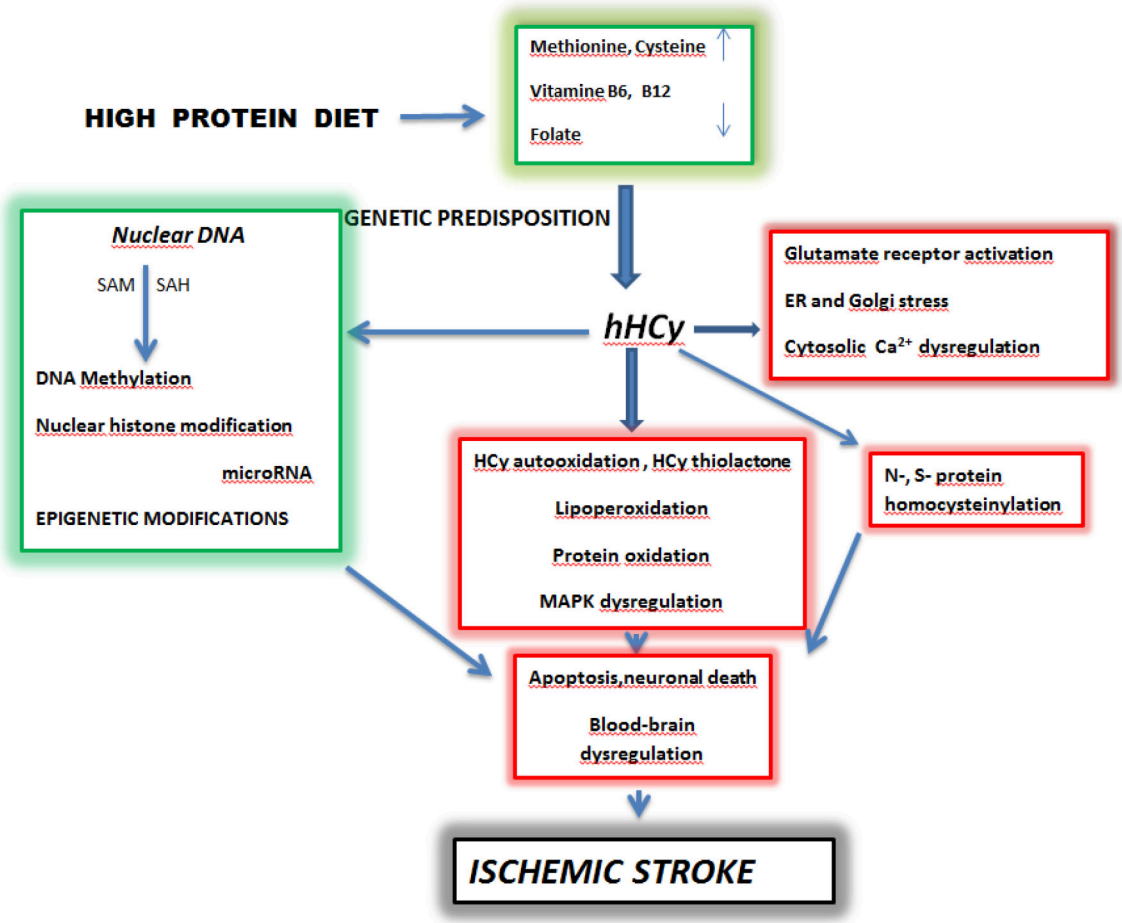

FIGURE 1 | Schematic pathways of homocysteine toxicity leading to brain ischemic stroke. SAM-S—Adenosyl Methionine, SAH-S—S -Adenosyl Homocysteine, hHcy—hyperhomocysteinemia. High dietary intake of diet rich in methionine and deficiency of vitamine B_6, B_{12} and folate lead to hyperhomocysteinemia in predisposed individuals. A prolonged elevated level of homocysteine initiates complex processes which include oxidative stress, protein homocysteinylation and Ca^{2+} dysregulation. These events in parallel with epigenetic changes can culminate in apoptosis, neuronal death and blood-brain barrier dysregulation manifested as ischemic stroke (Kalani et al., 2013; Petras et al., 2014; Kovalska et al., 2015; Lehotsky et al., 2015; Škovierová et al., 2015). Adapted from Lehotsky et al. (2015).

et al., 2009; Kalani et al., 2014). Clinico-genetic observations prove that genetic polymorphisms of the metabolic genes, such as methylentetrahydrofolate reductase (MTHFR), cystathionine β–synthase (CBS), DNA methyltransferase (DNMT) and nicotinamide N-methyl-transferase (NNMT) might be involved in the propensity for stroke due to elevated level of Hcy (Hozyasz et al., 2012; Balcerzyk et al., 2015). Nutritional supplements, e.g., folic acid (a co-factor in one-carbon metabolism), can prospectively interfere with the epigenetic regulations of neuronal cells and may also be involved in the sustainability of neuronal functions and integrity (Obeid and Herrmann, 2006; Kalani et al., 2013).

HOMOCYSTEINE METABOLIC CONVERSIONS

In humans Hcy is metabolized mainly by methyl group transfer and re-methylation and requires presence of dietary vitamin B_{12} and folic acid for N-5-methyltetrahydrofolate-homocysteine methyltransferase activity. Additionally, the trans-sulfuration reaction of Hcy depends on the presence of dietary vitamin B_6 (**Figure 2**). Hcy metabolism in brain parenchyma has some peculiarities in comparison to other organs. The trans-sulfuration pathway is entirely restricted since the remethylation pathway is using betaine (an intermediatory product) (Smulders et al., 2006; Petras et al., 2014) and the possibilities to metabolize/convert HCy mostly depend on the external supplies of folate and cobalamin as dietary vitamins. The glial cells possess very low stores of vitamin B_{12} that are quickly depleted during its negative balance and as such cannot sufficiently support neuronal survival. The toxicity of HCy to CNS neurons is widely recognized affecting both the neuronal survival rate and the ability of neurons to transmit signal and thus to form functional neural networks. This demonstrates that Hcy effects go far beyond the neuronal survival. In our experiments we have evaluated the neurotoxic properties of Hcy on glial cells, using a glioblastoma cell line as a model system. The viability of cells was assayed both biochemically and cytologically. At Hcy concentration around 50 μmol/l in the culture medium (clinically comparable to intermediate hHcy) we observed significant cell death which allowed us to suggest that Hcy-induced impairment of neuronal functions with damage to the glial cells might contribute to the etiopathogenesis of neurological disorders associated with hHcy (Škovierová et al., 2015). Interestingly, the increased Hcy level in humans has been detected both in acute CNS disorders, such as stroke, and chronic diseases such as epilepsy, Alzheimer's disease, dementia, as well as in clinically manifested classical homocystinuria (Seshadri et al., 2002; Kwon et al., 2014; Petras et al., 2014). Very recently it has been reported that the ratio between S-Adenosylmethionine (SAM) and S-Adenosylhomocysteine (SAH) should be used as a biomarker and may provide a sensitive indicator for the clinical diagnosis of atherosclerosis (Zhang et al., 2016). Another scientific group investigating the effect of Hcy on fatty acid binding protein 4 (FABP4) suggested that FABP4 plays a key role

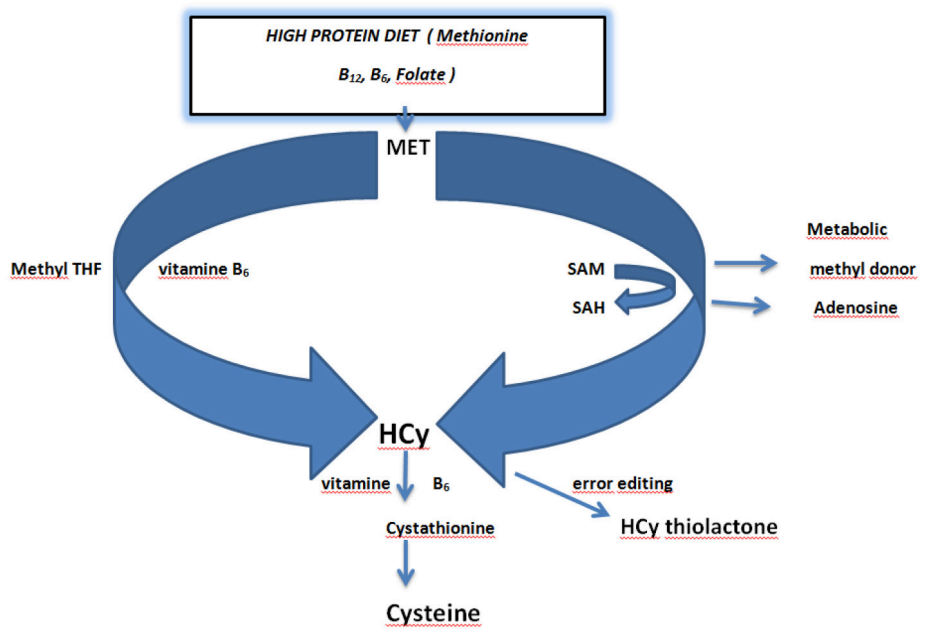

FIGURE 2 | Schematic overview of homocysteine metabolism and the role of dietary vitamins folate and vitamin B_6. Dietary methionine acts as a methyl donor via conversion of S-Adenosyl Methionine (SAM) to S-Adenosyl Homocysteine (SAH). SAH is converted to homocysteine by releasing adenosine. Methionine is directly converted to homocysteine in the presence of Methyl tetrahydrofolic acid (THF) and vitamin B_6. Homocysteine is converted in error editing reaction to homocysteine thiolactone. Conversion of homocysteine to cysteine requires vitamin B_6 (Kalani et al., 2013; Petras et al., 2014; Lehotsky et al., 2015). Adapted from Lehotsky et al. (2015).

in Hcy mediated disturbance of lipid metabolism and that DNA methyltransferase 1 (DNMT1) may be a novel therapeutic target in Hcy-related atherosclerosis (Yang et al., 2015).

HOMOCYSTEINE TRANSPORT IN THE BRAIN

Little is known about Hcy transport in the brain. Early animal studies indicated that Hcy can be transported through the blood brain barrier (BBB) epithelial cells via a specific saturable receptor in addition to simple diffusion (Grieve et al., 1992). It was demonstrated that in the rat capillary endothelium L-homocysteine shares both the sodium-dependent and independent cysteine transporters (XAG, L, ASC, A) with a varying degree of affinity (Büdy et al., 2006). However, in subjects with moderate hHcy, the majority of Hcy is disulphide-linked to plasma protein cysteine residues, and is, therefore, unavailable for transport via the cysteine transport systems (Sengupta et al., 2001; Lim et al., 2003). Ho et al. (2003) showed an increased production of Hcy in neuronal cells incubated in the folate-deficient media demonstrating that Hcy can be produced within the brain parenchyma. Moreover, accumulation of Hcy in the brain has been shown to be associated with the increased concentration of total plasma Hcy and S-adenosylmethionine (SAM) in the cerebrospinal fluid (CSF) (Herrmann and Obeid, 2011). Kamath et al. (2006) found a significant toxic effect of elevated Hcy on brain microvessels and implicated a role for Hcy in the disruption of the BBB, particularly affecting astrocytes. Thus, Hcy-induced endothelial and astrocytic dysfunction could also alter neuronal function.

CAUSES OF CLINICALLY RECOGNIZED HYPERHOMOCYSTEINEMIA IN HUMANS

As in the case of other metabolites, the clinically accepted reference total plasma Hcy concentration varies between 5 and 10 μmol/l. According to the laboratory analyses, classification of hHcy severity depends on its concentration in the plasma: mild (for concentration slightly above 10 μmol/l, moderate (for concentrations between 16 and 30 μmol/l), intermediate (for concentrations of 31–100 μmol/l) and severe (for concentrations higher than 100 μmol/l) which is also manifested with clinical symptoms of homocystinuria (Herrmann and Obeid, 2011). hHcy develops as a result of impaired Hcy metabolism under conditions of severe dietary deficiencies in folic acid, vitamin B_6 and/or vitamin B_{12} (**Figure 1**). The hHCy can also be a result of genetic predispositions due to perturbations of genes in methionine and homocysteine metabolism, most likely by methylenetetrahydrofolate reductase (MTHFR) deficiencies. Although Hcy is produced in all tissues, its conversion takes part only in the liver/kidney, mainly through the trans-sulfuration pathway. That is why such tissues as the blood vessels and the brain utilize remethylation as the only alternative. With significant reduction in MTHFR activity in these organs Hcy cannot be remethylated to methionine, hence accumulates in the blood as well as within the nervous system. Interestingly, the role of the MTHFR C677T polymorphism, as a risk factor for ischemic stroke, has recently been established in different laboratories and also in different genetic cohorts (Kim et al., 2013; Song et al., 2016). Remarkably, in orthotopic heart transplantation patients the risk of consequent brain ischemic stroke and transient ischemic attack (TIA) well correlates with plasma Hcy levels. Thus, hHcy could be involved in the pathogenesis of these stroke types mainly by increasing the risk of atrial myocardial fibrillation (Acampa et al., 2016).

TOXICITY OF HOMOCYSTEINE TO NEURAL CELLS AS A STROKE RISK FACTOR

Over the years, several theories concerning the toxicity of Hcy have been elaborated. But despite the efforts, none does clearly explain its toxicity. The toxic effect of Hcy on brain tissue is influenced by the absence of two major metabolic routes for Hcy elimination: betaine-mediated conversion of Hcy to Met and transsulfuration of Hcy to Cys. In addition, Hcy acts as an agonist for both groups of glutamate receptors, metabotropic (group I and III) and ionotropic (AMPA) receptors, as well as for N-methyl-D-aspartate receptor (NMDA) (Boldyrev et al., 2013). Overstimulation of these receptors results in an increased level of cytoplasmic calcium, higher production of free radicals and activation of caspases leading to apoptosis (Mattson and Shea, 2003). Not only neuronal cells are exposed to toxic effects of Hcy, but glial cells too (Verkhratsky and Toescu, 2006; Škovierová et al., 2015). The importance of astrocytes for brain homeostasis assisting neurogenesis, determining the micro-architecture of the gray matter and also energy metabolism has been well documented (Verkhratsky and Toescu, 2006). Hcy mediated NMDA receptor induction of neuronal cells could lead to their death due to the transient activation of extracellular signal-regulated kinases, ERK, MAPK, and p38 MAPK (Poddar and Paul, 2013) that is different from the downstream signaling pathways triggered by other NMDA receptor agonists. The Hcy induced glutamate receptor activated neurotoxicity is widely recognized (da Cunha et al., 2012; Kwon et al., 2014). Interestingly, ischemic insults also activate glutamatergic excitotoxicity with the promotion of neuronal death. Additionally, Hcy acts as an inducer of caspase-dependent neuronal apoptosis via several detrimental pathways, such as DNA damage, poly-ADP-ribose polymerase (PARP) dysbalance, and mitochondrial dysregulation by caspase-3 activation (Kruman et al., 2000; Fang et al., 2014; Kamat et al., 2015). Notably, glial-vascular interface communication as a part of BBB is also affected by Hcy (Mattson and Shea, 2003; Verkhratsky and Toescu, 2006; Loureiro et al., 2010; Kalani et al., 2014). As a consequence, elevated levels of Hcy lead to an enhanced excitatory glutamatergic neurotransmission in different brain regions and, as a result, to neuronal damage induced by glutamatergic derived excessive Ca^{2+} influx and generation of ROS.

In humans, the increased level of Ca^{2+} damages mitochondria by collapsing the mitochondrial membrane potential and

suppresses ATP production. Furthermore, the consecutive leakage of cytochrome c from mitochondria as well as ROS activate the caspase 3 pathway which leads to DNA fragmentation (Huang et al., 2001; Kwon et al., 2013).

It was proved that Hcy itself is able to induce BBB disruption (Kamath et al., 2006). This disruption can be due to several different processes. Firstly, Hcy induces an imbalance between the activity of MMP-9 and the tissue inhibitor of metalloproteinase 4 (TIMP-4), in the way of increasing MMP-9 and decreasing TIMP-4 activity (Tyagi et al., 2009). Subsequently, MMP-9 interacts with different components of the BBB and leads to disruption of this structure. Secondly, Hcy acts as an excitatory neurotransmitter for (i) γ-aminobutyric acid (GABA) receptors A, which leads to increased vascular permeability and (ii) NMDA receptor (Betzen et al., 2009). The expression of NMDA receptor is not confined to neurons only. Other cells, including endothelial cells from the cerebral tissue, can also express this type of receptor. Free radicals, inducing up-regulation of the NR1 subunit of the NMDA receptor, increase the susceptibility of cerebral endothelial cells to excitatory amino acids, favoring BBB disruption (Betzen et al., 2009; Tyagi et al., 2009).

One of the first hypotheses suggested that ROS and hydrogen peroxide (H_2O_2) formed in redox reactions involving the thiol group of Hcy was responsible for the toxicity of this compound. The major drawback of this hypothesis was that Cys (which is a common amino acid) is not a risk factor for vascular diseases, despite its up to 30-fold higher concentration than Hcy (Jakubowski et al., 2009). However, different studies showed that redox reactions may be a key factor in the development of atherosclerosis, vascular hypertrophy and possibly neurotoxicity in animals with hHcy. Thus, a dysbalance in the redox state and oxidative stress can be a primary mechanism responsible for hHcy-related pathogenesis, such as vascular hypertrophy, thrombosis and atherosclerosis (Dayal et al., 2004; Herrmann and Obeid, 2011; Marković et al., 2011; Petras et al., 2014). ROS are generated during oxidation of the free thiol group of Hcy, when Hcy binds via a disulphide bridge with plasma proteins—mainly albumin—or with other low-molecular plasma thiols, or secondarily with another Hcy molecule. The proposed mechanisms for Hcy-induced oxidative stress, therefore, can be classified as follows based on the existing knowledge (Jones et al., 1994; Streck et al., 2002; Tasatargil et al., 2004; Topal et al., 2004; Jiang et al., 2005; Lubos et al., 2007; Loureiro et al., 2010; Jakubowski, 2011; Boldyrev et al., 2013; Petras et al., 2014):

(i) Restriction of the activity of cellular antioxidant enzymes,
(ii) Hcy auto-oxidation,
(iii) nitric oxide synthase (NOS)-dependent generation of the superoxide anion via uncoupling of endothelial NOS (eNOS),
(iv) disruption of extracellular superoxide dismutase from endothelial surfaces, and
(v) activation of NADPH oxidases.

Moreover production of a strong oxidant peroxynitrite activates tyrosine nitration and results in modifications of protein function and cellular dysfunction (Postea et al., 2006).

Hcy can also be converted to its thioester forming the Hcy-thiolactone in an error-editing reaction in proteosynthesis (Gurda et al., 2015). Human and experimental animal studies have documented that formation of Hcy-thiolactone, indeed, is involved in the Hcy patho-biology. Its toxicity is based on a reaction which leads to protein N-homocysteinylation through the formation of amine bonds with protein lysine residues (Jakubowski, 2011), which impairs or alters the structural and functional properties of a particular protein.

Several clinical studies have reported that increased plasma Hcy levels may provoke seizures. In agreement with this finding it was suggested that systemic administration of Hcy at high doses provokes convulsive attacks in mice and it was proposed that similar detrimental effects can be manifested in patients suffering from temporal lobe epilepsy (Baldelli et al., 2010). Consequently, the Hcy-derived chemical reactive metabolites are thought to have an important role in Hcy-induced seizures.

High level of plasma Hcy is a recognized risk factor for developing Alzheimer's disease. Li J. G. et al. (2014) have documented that Hcy exacerbates β-amyloid and tau pathology with plaques and tangles, as well as cognitive deficit, in a mouse model of Alzheimer's disease which supports the concept that dietary lifestyle which leads to hHcy can act as a risk factor and actively contribute to the development of the disease.

Many experimental studies from our and other laboratories (Dirnagl et al., 2009; Urban et al., 2009; Łehotsky et al., 2009b; Pavlíková et al., 2009; Danielisova et al., 2014; Stetler et al., 2014) have documented that ischemia/reperfusion injury (IRI) in rats is followed by time-dependent dysbalance in the redox state in the cortex and hippocampus. The ischemic attack also activates alterations at the genetic level resulting in different expression of mRNA and proteins. Results from other laboratories also demonstrate a beneficial effect of preconditioning to the redox balance and gene expression (Stetler et al., 2014). In spite of high clinical relevance, the published data which described complex effect of IRI and endogenously formed Hcy as a recognized risk factor to the ischemic insult are sparse (Kwon et al., 2014).

An experimental approach (Streck et al., 2002; Loureiro et al., 2010; Lehotský et al., 2011; Pavlikova et al., 2011; Kovalska et al., 2012; Kolling et al., 2016) which is modeling development of hHcy by alimentary fortified or intraperitonealy injected Hcy initiated elevation of lipoperoxidative and protein oxidative products in rat hippocampus (Petras et al., 2014). It is well know that Hcy crosses BBB and reaches a peak of its concentration in the cerebrum and parietal cortex between 15 and 60 min after subcutaneous injection. Plasma Hcy concentration in rats treated via this way achieved the levels similar to those found in homocystinuric patients (moderate hHcy). An increased Hcy level is potent enough to induce and to accumulate hydroxyl radicals as the most powerful free radicals with the ability to remove electrons from other molecules including lipids, proteins, carbohydrates and DNA practically in all cellular components (Kolling et al., 2011; da Cunha et al., 2012; Petras et al., 2014). Thus, as seen from literature data (Streck et al., 2002; Loureiro et al., 2010) and also from our results (Lehotský et al., 2011; Pavlikova et al., 2011; Petras et al., 2014; Kovalska et al., 2015; Lehotsky et al., 2015), chronic elevated levels of Hcy manifest

high cellular toxicity. The biological consequence of this toxic effect in the form of neuronal degeneration (measured by the number of Fluro Jade C positive- and TUNEL positive cells) (Lehotský et al., 2011; Pavlikova et al., 2011; Kovalska et al., 2012; Lehotsky et al., 2015) and expressed as the ratio of degenerated cells over intact neurons is elevated in the hippocampus of hHcy animals. This index reaches almost the levels which is obtained after ischemic/reperfusion insult (Kovalska et al., 2014).

Earlier studies have also demonstrated that auto-oxidation of Hcy metabolites results in H_2O_2 production with consequent induction of necrotic cell death (Ziemińska et al., 2003; Boldyrev et al., 2013). In the study of Ataie et al. (2013), it was shown that direct intracerebroventricular injection of Hcy activates apoptotic cell death in the *substantia nigra* which is consequently followed by typical Parkinson's disease-like behavior in rats. In the clinical conditions, in Alzheimer's patients and patients with mild cognitive impairment, the plasma levels of Hcy correlates with the alterations in the hippocampal volume and disease progression. Remarkably, this effect is not mediated by cerebral amyloid β peptide deposition, or vascular burden, and as such Hcy-induced oxidative dysbalance is the most likely explanation (Kwon et al., 2013; Choe et al., 2014).

In another study Pavlikova et al. (2011), using the hHcy model in rats, have observed significant variations both in the level of mRNA and protein expression for the calcium pump in the secretory pathways (SPCA1. The important role of this protein in normal neural development and migration has been documented in previous studies (Sepúlveda et al., 2008) and SPCA1 reduction was shown to initiate stress of the Golgi apparatus manifested by the changes in membrane structure and redox dysbalance in neurons. Untill now, no literature papers can be found to describe Hcy effect on the expression profile of the Ca^{2+}-transport proteins in neuronal cells and the character of transcriptional regulation of the SPCA1 gene is not yet clarified. As shown by Kawada et al. (2005), the transcription factors Sp1 and YY1 may play role in its gene regulation by the cis-enhancing elements in the 5'-untranslated regions.

Another aspect of hHcy is an intracellular Ca^{2+} mobilization and endoplasmic reticulum (ER) stress (Kalani et al., 2013; Petras et al., 2014), which results in development of apoptotic events, endothelial dysfunction and remodeling of the extracellular matrix in brain parenchyma (Li M. H. et al., 2014). Interestingly, Hcy itself, by interfering with the level of S-adenosylmethionine (as a donor of the methyl group), has also been reported to induce modulation of gene expression through epigenetic alteration of the gene methylation status (Dionisio et al., 2010).

Notably, another etiopathogenic processes related to Hcy-induced neurotoxity might involve modifications of protein structure. Protein homocysteinylation includes:

(i) S-homocysteinylation and
(ii) N-homocysteinylation,

both of which are considered as posttranslational protein modifications. The degree of protein homocysteinylation correlates with the plasma Hcy level (Kolling et al., 2011) and conversion of Hcy to Hcy-thiolactone (Hcy-TL) results in increased protein N- homocysteinylation. As a consequence, homocysteinylation modifies functions of the proteins and elevates the rate of their proteolysis leading to cell damage (Jakubowski, 2004). *In vivo*, Hcy-TL targets and modifies blood albumin, hemoglobin, immunoglobulins, LDL, HDL, transferrin, antitrypsin, and fibrinogen (Jakubowski et al., 2009). Also, Hcy-TL acts as an inhibitor of Na^+/K^+-ATP-ase from the cortex, hippocampus, and brain cells of rats, affecting the membrane potential with deleterious effects for neurons (Rasić-Marković et al., 2009). Elevated plasma levels of Hcy-TL and N-homocysteinylated proteins can result either from the genetic defects in Hcy metabolism or a methionine-rich diet (Jakubowski et al., 2009). Gurda et al. (2015) analyzing the changes in gene expression profiles induced by Hcy and its products using microarray technology, real-time quantitative PCR and bioinformatic analysis have identified 47, 113, and 30 different mRNA regulated by N-homocysteinylated proteins, Hcy-TL and Hcy, respectively, and found that each metabolite induced a unique pattern of gene expression. Major molecular pathways affected by Hcy-TL were chromatin organization, "one carbon" metabolism and lipid-related processes while the major pathways of N-homocysteinylated protein and Hcy were blood coagulation, sulfur amino acid metabolism and lipid metabolism. It all indicated that the main diseases related to all three inductors are atherosclerosis, coronary heart disease and stroke.

In addition to this, it was proved that proteins modified by N-homocysteinylation could act as neoantigens, triggering activation of the inflammatory response which is a key component of atherogenesis, atherotrombosis and stroke etiology. Moreover, these neoantigens induce an autoimmune response and the concentration of autoantibodies is higher in some human pathologies (i.e., cerebrovascular disease, renal failure) comparing to normal individuals (Jakubowski, 2011). N-homocysteinylated proteins in the luminal face of vascular endothelial cells are recognized by specific antibodies and this neoantigen-autoantibody interaction leads to the activation of circulating macrophages, which become responsible for repeated vascular endothelium damage. Furthermore, Hcy-TL impairs the ability of the vascular endothelium to regenerate itself by direct inhibition of lysyl oxidase which is responsible for the correct cross-linking of collagen and elastin in the arterial wall (Raposo et al., 2004).

More recently Petras et al. (2014) documented a 57.9% decrease of $Mn^{2\pm}$ activated superoxide dismutase (Mn-SOD) activity in cortical mitochondria in the hHcy model in rats. This enzyme belongs to the first line of cellular defense against oxidative injury and has been observed to be diminished in the hHcy group compared to the control group. These results are consonant with very recent experiments of Longoni et al. (2016), which also emphasize a high potency of hHcy conditions for putative posttranslational Hcy induced protein modifications. This likely could lead to enzyme(s) homocysteinylation and thiolation which might contribute to the partial enzyme inactivation. Petras et al. (2014), also reported a small increase of catalase (CAT) activity in the hHcy group compared to the

control group which is likely to be a response to the increased level of ROS.

Very little is known about the effect of hHcy on the mitochondrial energy transduction system. The results indicate that hHcy reduces mitochondrial respiration, increases activity of the electron transport chain (ETC) complex II, and inhibits the ETC complex IV activity (Chang et al., 2008). In our laboratory we have investigated the effect of hHcy (on rat heart function, activities of the ETC complexes, mitochondrial protein expression and protein oxidative damage (Timkova et al., 2016). Left ventricular developed pressure, as well as maximal rates of contraction and relaxation, were significantly depressed in hHcy rats. Interestingly, hHcy was accompanied by a significant inhibition of ETC complexesII–IV, whereas activity of the complex I was unchanged. The decline in ETC activities was not associated with elevated protein oxidative damage, as indicated by unchanged protein carbonyl, thiol, and dityrosine contents. Moreover, the level of protein adducts with 4-hydroxynonenal was decreased in hHcy rats. Additionally, 2D-gel electrophoresis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry did not show alterations in the content of inhibited ETC complexes. However, mass spectrometry analyses identified 8 proteins whose expression was significantly increased by hHcy. These proteins are known to play important roles in the cellular stress response, bioenergetics, and redox balance. Altogether, the results of this study (Timkova et al., 2016) suggest that hHCy induced ETC dysfunction is not causally linked with the altered protein expression. Additionally, an altered expression of other mitochondrial proteins suggests an adaptation response to Hcy-induced myocardial injury.

In this context, Kolling et al. (2016) examined the effects of severe hHcy on brain metabolism, and evaluated a possible neuroprotective role of creatine (50 mg/kg body weight) in young rats (6-day-old). In the *amygdala* region Hcy treatment decreased the activities of succinate dehydrogenase and cytochrome c oxidase but did not alter complex II activity. Hcy treatment also increased the number of cells with high mitochondrial mass, high mitochondrial membrane potential and undergoing late apoptosis. Importantly, creatine administration prevented some of the key effects of Hcy in the amygdala. These authors also observed a decrease in the activity and immuno- content of the α1 subunit of the Na^+,K^+-ATPase in the amygdala after Hcy-treatment. These findings support the notion that Hcy modulates mitochondrial function and bioenergetics in the brain, as well as Na^+,K^+-ATPase activity and suggest that creatine might represent an effective adjuvant to protect against the effects of high Hcy plasma levels.

Similarly, results from hHcy experiments (Longoni et al., 2016) pointed out the neuroprotective effect of vitamin D (calcitriol) against hHCy as an important factor of brain development, brain metabolism and neuroprotection. The authors showed that pre-treatment with calcitriol up to 250 nmol/l has a remarkable protective effect on cortical slices. Hcy caused changes in bioenergetics parameters (e.g., respiratory chain enzymes) and mitochondrial functions by inducing changes in mitochondrial mass and swelling. Additionally, Hcy induced an increase in $NeuN^{(+)}$/PI cells but did not induce $GFAP^{(+)}$/PI cells. Calcitriol prevented these alterations likely by increasing the level of the vitamin D receptor. These findings suggest that calcitriol treatment may be one of the therapeutic strategies against cerebral complications caused by Hcy.

EFFECT OF HYPERHOMOCYSTEINEMIA ON ISCHEMIC/REPERFUSION INJURY AND ISCHEMIC TOLERANCE INDUCED BY ISCHEMIC PRECONDITIONING

Effect of Hyperhomocysteinemia on Ischemic Reperfusion Injury (IRI)

Clinical relevance of elevated total HCy level in plasma in the development of human stroke (Shi et al., 2015), stroke reoccurrence and in the prediction of mortality, especially in stroke patients with the large-vessel atherosclerosis subtype (Kumral et al., 2016) has been proved by many studies. Moreover, hHcy contributes in humans to vascular dysregulation, cognitive impairment and dementia (Hainsworth et al., 2016). Increased level of Hcy also predicts the risk of incident dementia which is independent of cerebral small-vessel diseases and vascular risk factors (Miwa et al., 2015).

Interestingly, in spite of the high clinical significance, only a limited number of experimental approaches can be found in the literature to describe the mutual influence of co-morbid hHcy to ischemic damage in animal models of human stroke (Sato et al., 1998; Thompson et al., 2013; Stetler et al., 2014). As shown by several studies, the ischemic/reperfusion insult induces degeneration of the majority (more than 64%) of hippocampal neurons (Dirnagl et al., 2009; Kovalska et al., 2012). An experimental approach (Streck et al., 2002; Loureiro et al., 2010; Lehotský et al., 2011; Pavlikova et al., 2011; Kovalska et al., 2012; Kolling et al., 2016) which is modeling development of hHcy by alimentary fortified or intraperitonealy injected Hcy has proved that in the rat cortical and limbic areas neurons demonstrate functional and morphological changes. The changes lead to the impairment of ion transport mechanisms, mitochondrial alterations and cytoskeltal remodeling. However, by combination of this metabolic stressor with 15 min forebrain ischemia/reperfusion (Kovalska et al., 2012, 2015), morphological changes in the neurons and disturbances of glial cells are aggravated and the extent of intact –like cells is altered in comparison to the naive ischemic/reperfusion group.

The effect of hHcy on cellular degeneration and morphological changes was manifested in rat hippocampal and cortical regions (Kovalska et al., 2012, 2015). The increased number of Fluoro-Jade C+ and TUNEL+ neuronal and glial elements supports toxic effect of hHcy. The thickened and collapsed processes that poorly extend to the area of pyramidal neurons in the CA1 region and M1 cortex are presumably due to the morphological alterations of astrocytes and cytoskeletal remodeling. This might be an indicator of severity of neuronal injury induced by hHCy (da Cunha et al., 2012; Kwon et al., 2014). Astrocytes are highly plastic cells and their dynamic morphological changes could affect the intercellular communication with surrounding synapses that

are important in the development of brain lesions (Buffo et al., 2010). Maler et al. (2003) reported that Hcy level of 2 mmol/l and above induced a dose-dependent cytotoxic effect on cortical astrocytes. Astrocytes regulate expression of the NMDA receptor subtypes, which increase neuronal sensitivity to glutamate toxicity and thus accelerate the initial step in the program of reactive astrogliosis and dynamics of astrocyte response to the damage (Škovierová et al., 2015). On the other hand, in response to the injury, astrocytes synthesize a number of factors that may play either neuroprotective or neurotoxic roles.

Increased neuronal damage by Hcy was also detected in *in vitro* ischemic model of hippocampal slices with oxygen and glucose deprivation. Injury of the neural cells was analyzed by quantification of lactate dehydrogenase (LDH) release into the extracellular fluid. Hcy increased the LDH release suggesting an aggravated tissue injury caused by hypoxic/ischemic conditions (Tagliari et al., 2006).

Remarkably, combination of both stressors (ischemia + Hcy) initiated morphological degenerative alteration and also affected considerably the expression of the MAPKs pathways by massive activation of MAPK/p-p38 with a maximum at 24 h after reperfusion (Kovalska et al., 2012, 2015). This dynamic of MAPK/p38 activation could contribute to a more extensive progression of tissue injury (Poddar and Paul, 2013; Zhou et al., 2015). MAPK/ERK are versatile protein kinases that are ubiquitously expressed in the CNS. The studies document the robust expression changes in the hippocampus and modest posttranslational changes in the MAPK/ERK pathway in less sensitive vulnerable neurons of the cortical layers III and V (Zhang et al., 2009; Kovalska et al., 2012, 2015). Extensive experiments have shown an interplay and tight integration of MAPK/ERK signaling in promoting neuronal cell death both in development and in neurodegenerative disorders (Zhang et al., 2009; Poddar and Paul, 2013). It has been proposed that transient activation of MAPK/ERK kinase has different consequences as compared with sustained activation. Transient activation of MAPK/ERK plays a pivotal role in neuronal maturation, survival, and long-term potentiation (Zhou et al., 2015). On the other hand, sustained activation of MAPK/ERK may play a critical role in triggering pro-apoptotic signals and neuronal cell death (Zhang et al., 2009; Poddar and Paul, 2013). It is well known that hHcy mediates glutamate-mediated NMDA receptor stimulation, which eventually leads to the activation of both stimulatory and inhibitory pathways involved in the modulation of MAPK/ERK signaling (Zhang et al., 2009). In fact, the dual role of MAKP/ERK kinases in cell survival and death suggests that a unique profile of gene expression may be elicited depending on the duration and/or magnitude of MAPK/ERK kinase activation (Zhou et al., 2015). Thus, the duration of MAPK/ERK kinase activation following MAPK/p38 stimulation depends on the nature of the extracellular stimuli (in our experimental conditions hHCy and or ischemia or combination of both, Kovalska et al., 2015) and may have different consequences on intracellular signaling pathways eventually leading to different cellular responses. This is in line with previous experiments which documented, that Hcy promotes p38-dependent chemotaxis in bovine aortic smooth muscle cells and this mechanism is important for Hcy-induced atherogenesis with the potential therapeutic implications (Akasaka et al., 2005).

In the context of verification of HCy toxicity, study of Sato et al. (1998) documents that S-adenosyl-L-methionine (SAM) manifested a dose-dependent protection of hippocampal CA1 neurons after transient forebrain ischemia in rats. As was expected, this protective effect was suppressed by S-adenosyl-L-homocysteine challenge, which acts as a potent inhibitor of transmethylation. From these results, authors concluded that the enhancement of cerebral SAM level and activation of transmethylation using SAM as a methyl donor in postischemic brain is vital for protecting neurons against delayed neuronal death. Sharma et al. (2015) proposed that pathological consequences of N-homocysteinylation due to the elevated level of Hcy result in cytotoxicity and even amylod formation. In fact, high levels of Hcy was shown to aggravates cortical neuronal cell injury after cerebral ischemia through neuronal autophagy over-activation detected by significantly increased formation of autophagosomes and the expression of LC3B and Beclin-1and causes increased cerebrovascular permeability (Tyagi et al., 2012; Zhao et al., 2016). As was proposed by authors, the oxidative damage-mediated autophagy may be a molecular mechanism underlying neuronal cell toxicity of elevated Hcy level during ischemic insult.

Interestingly, in another study by Veeranki et al. (2015a) in CBS-/+ mice under the hHcy condition, the satellite cells from the skeletal muscle not only expressed lower *in vitro* proliferative activity, but also manifested increased oxidative stress. In addition, they shown elevation of p38 MAPK as well as p16 and p21 expression after hHcy treatment suggested that hHcy-induced suppression of satellite cell proliferation involves p38 MAPK signaling.

In fact, Tyagi et al. (2012) found homocysteinylated cytochrome-c mediated autophagy in hHcy mice after cerebral ischemia. Cytochrome-c transports electrons and facilitates bioenergetics in the system and authors found that tetrahydrocurcumine ameliorates autophagy during this condition by reducing homocysteinylation of cytochrome-c in-part by MMP-9 activation.

In a clinical settings, Hcy reduces number of endothelial progenitor cells (EPCs) in patients with cerebral stroke through apoptosis. Ischemic insults activates migration of EPCs from bone marrow to repair damaged sites either through direct incorporation of EPCs or by repopulating mature endothelial cells. Increased level of Hcy mediates toxicity to EPC due to apoptosis involving caspase-8, cytochrome c release, and caspase-3 activation and thus making reduction of EPC numbers (Alam et al., 2009). Yang et al. (2016) presented data which indicates that co- morbid Hcy in cerebral stroke stimulates hypermethylation of the trombomodulin gene which leads to gene silencing. Since thrombomodulin may be protective against cerebral ischemia by downregulating coagulation, increased Hcy may play an important role in the occurrence and development of cerebral infarction after ischemic stroke. In a recent paper Caldeira et al. (2014) discusses the role of the ubiquitin-proteasome system in brain ischemia which emphasizes the deletarious role of increased Hcy in the control of ubiquitin-containing

proteinaceous deposits accumulation and modulation within the ischemic injury. Furthermore, Toda and Okamura (2016) attributed the idea that hHcy due to the impaired synthesis of NO in the endothelium and by the increased production of asymmetric dimethylarginine is responsible for the impaired circulation in the brain and hypoperfusion/transient ischemia may act as a triggering factor for dementia and Alzheimer's disease. Reduced actions of NO and brain hypoperfusion trigger increased production of amyloid-β that inhibits endothelial function, thus establishing a vicious cycle for impairing brain circulation.

Combination of Hyperhomocysteinemia with Preischemia/Preconditioning Followed by Ischemic Insult

The pre-ischemic maneuver as a form of evolved tolerance to consequent ischemia is known to rescue the majority of hippocampal neurons (Lehotský et al., 2009a; Kovalska et al., 2012; Rybnikova and Samoilov, 2015; Wang et al., 2015). It has been previously shown that ischemic/reperfusion injury (IRI)t leads to neurodegeneration of neurons in the CA1 region of hippocampus as detected by 75–80% Fluoro-Jade C+ and 90 times (9 ± 1.2 cells/mm2) higher TUNEL+ cells in comparison to the control. On the other side, ischemic precondtioning (IPC), remarkably, leads to suppression of the number of positive cells to more than 70% and conferred neuroprotection (Kovalska et al., 2012, 2014, 2015). Combination of hHcy with ischemic injury increases the extent of neurodegeneration, however, if hHcy is combined with preconditioning (Lehotský et al., 2011; Pavlikova et al., 2011; Kovalska et al., 2012, 2015; Petras et al., 2014; Lehotsky et al., 2015), this maneuver leads to the massive suppression of cell degeneration (as compared to IPC alone) not exceeding 5% of the total number of neurons. It apparently seems that, at least in this hHcy model in rats (Kovalska et al., 2015), IPC significantly corroborates the protective mechanisms.

Recently, we have shown that IPC prior to the lethal ischemia affects MAPK/ERK and MAPK/p38 pathways in the cerebral cortex (Kovalska et al., 2012) as well as in the hippocampus (Kovalska et al., 2014). There is only sparse literature data focusing on the effect of Hcy on the protein expression of MAPKs in neuronal cells (Poddar and Paul, 2013). Poddar and Paul (2013) showed a biphasic response of MAPK/p38 activation in the Hcy-NMDA induced neuronal damage *in vitro*, characterized by an initial rapid elevation followed by a delayed and more prolonged secondary increase, where the later peak was primarily involved in mediating the Hcy-induced cell death. They also showed that this secondary activation of MAPK/p38 correlates with upstream MAPK/ERK activation, which plays a role in facilitating the Hcy-induced cell death. These results are consistent with the conclusions of the study of Kovalska et al. (2014, 2015). In the previous reports from this group (Kovalska et al., 2012, 2014; Lehotsky et al., 2015) it has been shown that IRI induces only a slight increase of MAPK/p38 expression. However, the combination of both stressors (ischemia+ hHcy) leads to the massive activation of MAPK/p-p38 with a maximum at 24 h after reperfusion (Kovalska et al., 2014). This dynamic MAPK/p38 activation could contribute to a more extensive progression of tissue injury (Poddar and Paul, 2013; Zhou et al., 2015). Remarkably, the protective effect of the IPC maneuver detected by the decreased numbers of degenerated neurons is also followed by the decreasing of MAPK/p-p38 expression and elevated immun-osignal for MAPK/p-ERK in ongoing reperfusion times in the CA1 hippocampal region and M1 cortical area.

Significant reductions of oxidative changes and suppression of cell degeneration in the hippocampus as well as in cortex of rat brain is generally an indicative consequence of preischemic treatment (Dirnagl et al., 2009; Lehotsky et al., 2015). Remarkably, IPC in the both paradigms (without and in hHCy condition) preserved the majority of neurons, as seen by decreasing the number of Fluoro-Jade C+ and TUNEL+ neuronal cells. Additionally, preischemia/preconditioning had a protective/stimulatory influence on the MAPK/ERK protein activation under both stress conditions with the opposing effect of MAPK/ERK and MAPK/p38 on cell survival and cell death. Conclusively, preconditioning even if combined with hHcy could still preserve the neuronal tissue from the lethal ischemic effect.

In another set of experiments, we have shown earlier that pre-ischemic treatment prior IRI retains the integrity of the majority of hippocampal neurons and also stimulates recovery of the expression rate of both the SPCA1 mRNA and protein in rats (Pavlíková et al., 2009; Stetler et al., 2014). Combination of hHcy with IRI leads to the slensing of the SPCA mRNA expression and IPC re- stimulates its rate to 2.5-fold.

In conclusion, results from our and other laboratories show that hHcy is associated with a selective degeneration of cortical and limbic structures including the hippocampal area. This degeneration involves the loss of neurons, glial activation, hypertrophy of astrocytes and probably sprouting of new connections. The morphological findings indicate that astrocytes are the first neural cells participating in the deleterious actions of Hcy on the CNS. Apparently, astrocytes are able to respond to mild hHcy by reorganizing their cytoskeleton, surviving and protecting neurons from the damage (Loureiro et al., 2010).

The biological effect of various preconditioning agents is well documented (Dirnagl et al., 2009; Lehotský et al., 2009a). Various types of preconditiong is able to attenuate adverse effect of injurious agents, such as Hcy. As it is shown by the study of Blaise et al. (2009), short hypoxia could suppress the deleterious effects of hHcy on developing rat brain by inducing neurogenesis. Brief neonatal hypoxia, as form of preconditioning, markedly stimulated migration of new neurons to the permissive areas such as the subventricular zone and the hippocampus, increased locomotor coordination and learning and memory and attenuated the long-term effects of hHcy. Similarly, physical exercise (Hrncic et al., 2014) as a type of preconditioned impulse has a beneficial effect in the hHcy induced seizures. It decreases susceptibility to seizures which, according to the authors, is, at least in part, a consequence of improved antioxidant enzymes activity.

Moreover, physical exercise reverses glutamate uptake and oxidative stress effects of hronic Hcy administration in the rats (da Cunha et al., 2012). Wei et al. (2014) documented that

application of a chemical metabolic preconditioner hydrogen sulfide inhibits Hcy-induced endoplasmic reticulum stress and neuronal apoptosis in rat hippocampus probably due to the upregulation of the BDNF-TrkB pathway.

In parallel to the above described hHcy induced alterations in intracellular signaling, deleterious effect of Hcy can include also newly described epigenetic dysregulation. The experimental evidence suggests that epigenetic mechanisms are also involved in the etiopathogenesis of stroke and the phenomenon of ischemic tolerance. In particular, it was shown that several important enzymes regulating DNA methylation (e.g., DNA-N-methyltransferas) are implicated in these processes. As a result, its might lead to hypermethylation of the genomic DNA and silencing of functional genes (Kalani et al., 2013).

However, in the context of methylation processes, the one carbon unit metabolism pathway as a part of regular Hcy metabolism correlates with amino acids methylation of the functional proteins and histones as well as of the nucleotides within the RNA and DNA. Remarkably, demethylation of S-adenosyl methionine which gives rise to S-adenosyl-homocysteine, is the sole source of de novo methyl groups for the cell. One can only speculate, that in the hHCy conditions, dysregulation of this step might have a broader implication for many cellular processes including modulation of diverse genes expression via epigenetic regulation (Dionisio et al., 2010; Kalani et al., 2013, 2014). In line with these findings it seems reasonable to consider the association of hHCy and BBB integrity in brain ischemic stroke. As was shown by the recent paper of Kalani et al. (2014), this process could be regulated by epigenetic mechanisms including expression of the specific miRNA29b. This regulates activity of methyl transferase DNMT3b which finally leads to MMP9 activated digestion of the extracellular matrix and junction proteins leading to a leaky vasculature. Moreover, Hcy itself directly affects the BBB permeability and the miR29b regulated activity of MMP9 seems a novel epigenetic mechanism. Similarly, an epigenetic regulation stimulated by hydrogen sulfide is included also in the attenuation process of Hcy-induced mitochondrial toxicity in mouse brain endothelial (bEnd3) cells (Kamat et al., 2015). In this context, the effect of hHcy was investigated in the model of skeletal muscle weakness and fatigability (Veeranki et al., 2015b). Mice with a haplotype of metabolic enzyme CBS+/- exhibited more fatigability and generated less contraction force due to reduced ATP levels. In parallel an increase in the levels of miR-31, and miR-494 that were implicated in dystrophin and mitochondrial regulation and an increase in DNMT3a and DNMT3b proteins and global DNA methylation levels suggest that hHcy plays a causal role in enhanced fatigability through mitochondrial dysfunction which involves epigenetic changes.

Recently has been shown that elimination of the toxic Hcy metabolite, Hcy-thiolactone is performed by the high density lipoprotein (HDL)—associated enzyme, Hcy – thiolactonase/paraoxonase 1 (PON1) (Domagała et al., 2006). This brings a novel pathobiochemical relevance, because it has been suggested that PON1 can protect mice against Hcy-thiolactone neurotoxicity by its hydrolyzing activity also in the brain (Borowczyk et al., 2012).

Taken together, documented responses of neuronal cells to hHcy, IRI and preischemic challenge in the hHcy model in rats (**Figure 3**) might suggest a correlation of several ethiological factors such as antioxidant defense (Borowczyk et al., 2012; Petras et al., 2014), alterations in the mechanisms of Ca^{2+} transport (Pavlíková et al., 2009) and likely newly explored epigenetic mechanisms, such as DNA methylation and chromatin remodeling in the phenomenon of ischemic damage and ischemic tolerance (Dirnagl et al., 2009; Lehotský et al., 2009a; Kalani et al., 2013; Thompson et al., 2013; Stetler et al., 2014).

CONLUSION AND CHALLENGES

hHcy manifested as an elevated plasma level of Hcy is now a widely recognized risk factor for human vascular disorders and ischemic and tromboembolic stroke. It remains unclear whether excessive Hcy concentration directly contributes to the pathogenesis of the diseases or it represents a biomarker of metabolic aberrations, such as aberrant methyl group metabolism. Different strategies to reduce plasma Hcy concentrations have reached nonconclusive results, not just in the case of vascular disorders, but also with respect to neurodegenerative disorders, or cancer. Chronic elevated plasma hHcy alters functions of the vascular endothelial cells and, by the important pathobiochemical modifications, activates thiolation and homocysteinylation of plasma proteins and enzymes with the deleterious impact on the cerebrovascular permeability and eventually on brain parenchyma. Neural cells are sensitive to prolonged hHcy treatment, because Hcy cannot be metabolized by transsulfuration pathway either by folate or by vitamine

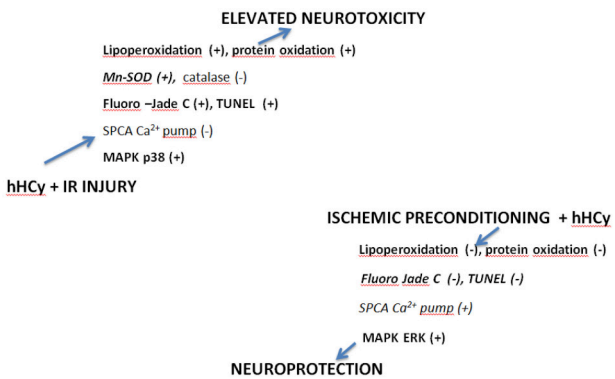

FIGURE 3 | Proposed mechanisms leading to homocysteine neurotoxicity and the protection induced by ischemic preconditioning (IPC) in hyperhomocysteinemic conditions (hHCy). (+): increased number of cells and/or activity, (–): decreased number of cells and/or activity. Homocysteine induced neurotoxicity includes dysregulation in redox balance, lipoperoxidation and protein oxidation, Ca^{2+} pump dysfunction and activation of MAPKp38 which is detected in vulnerable cells by increased Fluoro-Jade C staining (+) and TUNEL positive cells (+). Ischemic preconditioning suppresses oxidative dysregulation and activates MAPK-ERK which leads to reduced Fluoro Jade C and TUNEL positivity in sensitive cells (Pavlikova et al., 2011; Petras et al., 2014; Kovalska et al., 2015; Lehotsky et al., 2015; Škovierová et al., 2015). Adapted from Lehotsky et al. (2015).

B_{12} independent remethylation pathway. Therefore, Hcy could be used as an additional valuable prognostic and predictive biomarker in neurodegenerative diseases. Chronic challenge to hHCy induces posttranslational protein modifications which modifies the function and activity of the regulatory enzymes involved in free radical protection. Hcy as a toxic metabolic intermediate interferes with the proper redox balance and increases oxidative stress and production of free radicals in many cells including endothelial, glial and neuronal cells. Chronic elevated level of Hcy contributes to the increase of pathological neuronal lipoperoxidation and cellular protein oxidation, all the products being clearly recognized in the neurotoxicity and processes of brain damage. Moreover, Hcy treatment of cell cultures doubles the rate of telomere shortening (Zhang et al., 2016). Therefore, it would be very important to find the strategies to decrease Hcy levels. Genetic abnormalities and nutritional deficiencies explain only a part of hHcy pathologies. Hormonal and metabolic factors and also therapy with multiple vitamins and folate might be as a one clue to correct Hcy level in patients, however, clinical trials are needed to determine the optimal doses of vitamins.

The epigenetic mechanisms play an important role in elevated Hcy production. Since SAM is a universal donor of methyl group, SAH followed by Hcy are produced during these processes. It becomes more and more evident, that DNA methylation impairment might be a consequence of hHcy caused by endogenous (polymorphism of Hcy and folate pathways genes) and/or exogenous factors (dietary deficiency of folate and vitamins) and may be involved in hHcy pathogenesis. Thus, revealing many factors that may affect the methyl balance and understanding the pathophysiology of diseases from "methylation point of view" still remain a great challenge. Advanced studies are needed to understand whether and how changes in DNA methylation patterns, global and gene specific are associated with elevated levels of Hcy in the context to diseases and risk factors, such as oxidative stress, aging and exposure to drugs.

In this review we made an attempt to summarize and also emphasizes the results of novel experimental paradigm which combines only hHcy conditions (as a clinically recognized risk factor of ischemic stroke) and together with the ischemic insult and preconditioning maneuvers. Induction of hHCy alone leads to progressive neuronal cell death and morphological changes in the hippocampus and cerebral cortex in the rats. Ischemic reperfusion injury activates degeneration processes and de-regulates intracellular signaling which are aggravated under hHcy conditions.

The preischemic maneuver (preconditioning) evolves the state of ischemic tolerance manifested by the attenuated extent of neuronal degeneration as well as the intracellular signaling involved in the tolerance phenomenon. Combination of preconditioning with the hHcy retains the integrity of the majority of hippocampal neurons and also stimulates recovery of the expression rate of both the SPCA1 mRNA and the protein. Preconditioning even if combined with hHCy could still preserve the neuronal tissue from the lethal ischemic effect. The studies also emphasize the opposing effects of MAPK/ERK and MAPK/p38 signaling pathway on cell survival and cell death in the condition of preconditioned hHcy ischemia in the rat model of human stroke. The increased prevalence of hHcy in the population and crucial role of elevated Hcy levels in pathogenesis of different diseases, make this amino acid as an interesting target for future research.

In the human brain pathology, IPC is hardly suitable as a preventative measure in the predispose cerebrovascular patients. However, it could be used as a precaution against secondary stroke following medical procedures such as aneurysm repair or cardiac surgery. From the clinical point of view, use of preischemia/preconditioning challenge may bring some complications (Dirnagl et al., 2009; Lehotský et al., 2009a). Initial pre-clinical studies of this phenomenon revealed that pharmacological agents are also effective as preconditioners and there are also some other agents already clinically used. In the case of naturally occurring human strokes which cannot be predicted, maneuver of postconditioning may be a therapeutic strategy that could be used afterwards to elevate or stimulate repair mechanisms or as a precaution against stroke recurrence. It is also reasonable to summarize that preconditioning has a beneficial effect in the short term, however, the analysis of structural alterations in brain parenchyma documents that the damaging effect is only postponed. More clinically relevant experiments and clinical studies are urgently required to validate the efficacy of these paradigms in humans (Dirnagl et al., 2009).

Further investigations of the protective factors leading to ischemic tolerance and recognition of the co-morbid risk factors would result in development avenues for exploring novel therapeutics against ischemia and stroke. Usefulness and validity of Hcy as a biomarker of some diseases is another research challenge.

AUTHOR CONTRIBUTIONS

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

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frontiers in Neuroscience

REVIEW
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Current insights into the molecular mechanisms of hypoxic pre- and postconditioning using hypobaric hypoxia

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Exposure of organisms to repetitive mild hypoxia results in development of brain hypoxic/ischemic tolerance and cross-tolerance to injurious factors of a psycho-emotional nature. Such preconditioning by mild hypobaric hypoxia functions as a "warning" signal which prepares an organism, and in particular the brain, to subsequent more harmful conditions. The endogenous defense processes which are mobilized by hypoxic preconditioning and result in development of brain tolerance are based on evolutionarily acquired gene-determined mechanisms of adaptation and neuroprotection. They involve an activation of intracellular cascades including kinases, transcription factors and changes in expression of multiple regulatory proteins in susceptible areas of the brain. On the other hand they lead to multilevel modifications of the hypothalamic-pituitary-adrenal endocrine axis regulating various functions in the organism. All these components are engaged sequentially in the initiation, induction and expression of hypoxia-induced tolerance. A special role belongs to the epigenetic regulation of gene expression, in particular of histone acetylation leading to changes in chromatin structure which ensure access of pro-adaptive transcription factors activated by preconditioning to the promoters of target genes. Mechanisms of another, relatively novel, neuroprotective phenomenon termed hypoxic postconditioning (an application of mild hypoxic episodes after severe insults) are still largely unknown but according to recent data they involve apoptosis-related proteins, hypoxia-inducible factor and neurotrophins. The fundamental data accumulated to date and discussed in this review open new avenues for elaboration of the effective therapeutic applications of hypoxic pre- and postconditioning.

Keywords: hypoxic preconditioning, hypoxic postconditioning, neuroprotection, hypoxic tolerance of the brain, molecular mechanisms

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Identification of the molecular and physiological mechanisms underlying the response of organisms to environmental factors, in particular, to harmful injurious exposures represents one of the major research problems in biology and medicine. To solve this problem is fundamentally important for further elucidation of the endogenous mechanisms responsible for adaptation to the environment, as well as for elaboration of the effective tools to increase the resistance of the

organism and its most vulnerable organs, the heart and brain, to detrimental external and internal factors.

The central place in the formation of various types of adaptive reactions of the organism to environmental factors belongs to the neural process of perception and transduction of the adaptogenic signal which the famous Russian physiologist and Nobel prize laureate Ivan Pavlov designated as a "warning" signal. One of Pavlov's disciples P. K. Anokhin further developed the concept of "alarm" or "warning" activity as the basis for the *phenomenon of anticipatory reflection of reality* which is a universal phenomenon of life allowing to "anticipate the course of future events in order to better adapt to the environment" (Anokhin, 1974). According to modern concepts, in the nervous system the process of adaptation to possible damaging effects can manifest itself by the following cascade: an adaptogenic stimulus ("warning" signal, specifically injurious or stress factors of mild intensity) induces reorganization of the plasticity of the elements of the nervous system (neurons, synapses, glia). At the root of this reorganization lies the processing of anticipatory reflection of reality which prepares brain cells to the expected deleterious exposures and is associated with an induction of evolutionarily-acquired gene-determined protective mechanisms. As a result, reprogramming of death/survival mechanisms in the brain cells is activated leading to neuroprotection and compensation of the deleterious effects. By means of such cascade events, the "warning" signal results in environmentally-determined adaptive change in the phenotype, and the process represents an illustrative example of hormesis or neurohormesis. Hormesis is a basic principle of physiology which is generally defined as responses of cells or organisms to a factor which induces stimulatory or beneficial effects at low doses and inhibitory or adverse effects at high doses (Calabrese et al., 2010; Calabrese and Mattson, 2011). A preconditioning phenomenon aimed at adaptation to extreme factors is one of the typical examples of the adaptogenic "warning" signaling and, therefore, of hormesis. Thus, it is likely that various types of the preconditioning, as well as other hormetic stimuli, share some common protective mechanisms. From this point of view, elucidation of the mechanisms launched by the hypoxic preconditioning can be important in a broader context, shedding more light on the basic phenomenon of hormesis.

THE PHENOMENON OF PRECONDITIONING

The terms "preconditioning" and "tolerance" were introduced into experimental practice in 1964 (Janoff, 1964). At the end of the last century Murry and colleagues demonstrated that multiple brief ischemic episodes might actually protect the myocardium from a subsequent sustained ischemic insult, and this phenomenon has been termed "ischemic preconditioning" (Murry et al., 1986). In the 1990s a hypoxic/ischemic tolerance induced by pre-exposure to brief (sublethal) *preconditioning* ischemic episodes has been described and partially studied in the brain (Kitagawa et al., 1990; Kirino et al., 1991). In the next 25 years the mechanisms of cerebral ischemic preconditioning have been uncovered in various *in vivo* and *in vitro* models including global and focal brain ischemia, surviving brain slices, cultured primary neurons and transient ischemic attacks in humans (for review see Kirino, 2002; Steiger and Hänggi, 2007; Obrenovitch, 2008; Shpargel et al., 2008; Gidday et al., 2013). According to the current concept, formation of brain ischemic tolerance induced by ischemic preconditioning consists of two sequential phases ("windows"): the early (an induction of tolerance) and the late (an expression of tolerance) phase (Kirino, 2002; Steiger and Hänggi, 2007; Stenzel-Poore et al., 2007; Shpargel et al., 2008). The first window involves rapidly-induced (from a few minutes to several hours) changes in the cells such as activation of protein kinases, proteases, posttranslational modifications of ion channel proteins, receptors, redox-sensitive proteins and, apparently, immediate early gene transient expression. In the second window (24 h and later) the delayed mechanisms associated with gene expression and *de novo* synthesis of proteins providing long-lasting plasticity and neuronal survival are being activated.

HYPOXIC PRECONDITIONING

Compared to ischemic preconditioning, effects and mechanisms of preconditioning by mild or moderate hypoxic or anoxic episodes (hypoxic preconditioning) have been understudied although this paradigm obviously represents a convenient method of treatment since it does not require surgery. The phenomenon was first described in the 1960s as "a kind of induced tolerance of tissue-cells to hypoxia" (Lu, 1963). The neuroprotective action of brief anoxic pre-exposure was reported in 1964 (Dahl and Balfour, 1964) almost 30 years earlier than cerebral ischemic precondition had been described. Further progress in developing the concept of hypoxic (anoxic) preconditioning and brain tolerance has been achieved in the *in vitro* electrophysiological studies on rat hippocampal slices performed by Schurr and colleagues (Schurr et al., 1986).

One of the earliest models for studying hypoxic preconditioning was autohypoxia. This represents a whole-body preconditioning when anesthetized rodents are placed into an individual sealed container, and authohypoxia is induced by the animal's own oxygen consumption (Lu, 1963). When such a procedure is repeated up to 5 times, the animal becomes more tolerant because the survival time (as judged by the onset of gasping) was found to increase up to 8 times as compared to control non-preconditioned animals (Lu et al., 1999).

Following this paradigm, the most common technique for studying hypoxic preconditioning was the use of 8–13% normobaric hypoxia. To achieve this, animals are usually placed in a hypoxic chamber in which oxygen content is decreased by substituting it with nitrogen. Humans can inhale such a hypoxic gas mixture through a mask (Serebrovskaya et al., 2011), which represents a non-invasive way of application and a more beneficial technique compared to the ischemic methods with regard to their implementation in clinical practice. Although the therapeutic window of the normobaric hypoxia preconditioning appears to be rather narrow lasting only approximately 72 h (Stetler et al., 2014), it has been reported that various regimes of normobaric hypoxic preconditioning significantly protect

animals against injurious effects of subsequent global or focal stroke (Miller et al., 2001), kainic acid-induced seizures and brain edema (Emerson et al., 1999a,b). Numerous developmental studies applying normobaric hypoxic preconditioning at the early stages of ontogenesis demonstrated neuroprotection from ischemic brain injury in different peri- and postnatal periods (Gidday et al., 1994; Vannucci et al., 1998).

In vitro models of cerebral hypoxic preconditioning include studies in hippocampal slices (Pérez-Pinzón et al., 1996; Bickler and Fahlman, 2009) and olfactory cortex (Semenov et al., 2002; Samoilov et al., 2003), as well as primary neuronal cultures (Arthur et al., 2004). It has been demonstrated that preconditioning with a single short (2 min) episode of anoxia in slices of the olfactory cortex or three trials of 1 min anoxia in the hippocampal slicesincreases the resistance of the cells to severe "test" anoxia, preventing depression of the evoked potential amplitudes and calcium overload associated with anoxic injury whereas moderate calcium load is required for induction of tolerance during anoxic preconditioning (Pérez-Pinzón et al., 1999; Semenov et al., 2012).

HYPOBARIC HYPOXIC PRECONDITIONING

Another promising technique of hypoxic preconditioning with high translational potential is exposure to mild hypobaric hypoxia. The revitalizing effects of moderate high altitude occurring in nature have been known for centuries. Currently it is well-established that adaptation (acclimatization) to high altitude both of natural origin and simulated in the hypobaric chamber has a number of beneficial effects on human health, whole body hypoxic tolerance and improvement of physical training (Meerson, 1984; Meerson et al., 1996; Millet et al., 2013). These effects are observed under conditions of chronic mild hypobaric hypoxia (acclimatization mode) and result from reprogramming of the cardio-pulmonary and metabolic processes, including erythropoiesis, vascular remodeling, pulmonary changes and cardiac hypertrophy (Hultgren and Miller, 1967; Savourey et al., 2004; Brito et al., 2015). The integrity of these reactions is called systemic "structural trace of adaptation" due to which the resistance of an organism against any forthcoming hypoxic exposures is achieved along with more general protective effects (Meerson, 1984).

Hypobaric hypoxia is also widely used in the mode of intermittent (multiple, interlaced by normoxic periods) hypoxic training adapted as a therapeutic strategy to treat heart diseases, allergy (Meerson et al., 1987) and for training of athletes to improve their sea-level performance (Levine, 2002). In experimental practice the neuroprotective and anticonvulsant potential of chronic intermittent hypobaric hypoxia has also been demonstrated (Gong et al., 2012; Zhen et al., 2014).

In our laboratory during several years we have been using an original technique of hypobaric hypoxic preconditioning which was developed in early 2000s and consistently validated in subsequent studies (Samoilov et al., 2001; Rybnikova et al., 2005, 2006). With this method, three trials of mild hypobaric hypoxia (360 mm Hg, equivalent to 10% of normobaric oxygen and an altitude of 5 km above sea level, 2 h duration of each episode) spaced at 24 h are necessary and sufficient to achieve the most pronounced neuroprotective effect and significantly protect the brain against subsequent severe global hypoxia. Such a mode of hypoxic preconditioning has greatly improved survival of rats during severe hypoxia (3 h, 180 mm Hg, equivalent to 5% of normobaric oxygen and an altitude of 11 km) and prevented hypoxia-induced injury/loss of vulnerable brain neurons in the hippocampus and neocortex, as well as diminished functional disturbances observed at the behavioral level (Samoilov et al., 2001; Rybnikova et al., 2005). Surprisingly, preconditioning by three episodes of mild hypobaric hypoxia has also demonstrated a potent antidepressant-like and anxiolytic action and protected animals from development of stress-related depressions and anxiety (Rybnikova et al., 2007a). The neuroprotective efficacy of such a preconditioning mode against an ischemic brain injury was further proved by another research group in a model of global cerebral ischemia in gerbils (Duszczyk et al., 2009) and also in a model of cardiac arrest and resuscitation (Xu and Lamanna, 2014).

CEREBRAL MECHANISMS OF HYPOXIC PRECONDITIONING

A central place in the studies of hypoxic preconditioning belongs to identification of its molecular mechanisms with special attention being paid both to their unique character and similarity with the mechanisms of ischemic preconditioning. The process of preconditioning can be divided into several phases starting with the phase of initiation of hypoxic tolerance (Samoilov, 1997; Samoilov et al., 2003) or the immediate phase of adaptation to hypoxia (Lukyanova et al., 2013) which covers the first few minutes following the exposure to moderate hypoxia. This acute stage is clearly linked with the launch of several molecular signaling processes. The important mechanism which significantly contributes to the development of the later phases of brain hypoxic tolerance is obviously associated with the acute changes in neuronal redox state in favor of reduction equivalents that, in turn, can result in modifications of calcium bound to intracellular hydrophobic components (for review see Samoilov et al., 2003). Another key process at this stage is a remodeling of the respiratory mitochondrial chain and switching to the mitochondrial complex II, as well as rapid and transient activation of hypoxia-inducible factor HIF-1 (Lukyanova et al., 2009, 2012). Succinate-induced stabilization of HIF-1α followed by an activation of the transcriptional factor HIF-1 and expression of its target pro-adaptive genes is currently considered as one of the main mechanisms contributing to development of the long-term adaptation to hypoxia (Lukyanova et al., 2009).

The second phase of hypoxic preconditioning, the phase of induction of long-term hypoxic tolerance, involves significant alterations of the intracellular signal transduction processes, primarily modest activation of the glutamatergic, calcium, phosphoinositide, cyclic AMP regulatory systems (Semenov

et al., 2000, 2002; Samoilov et al., 2003) and rapid changes in pro- and antioxidant reactions (Ravati et al., 2000: Furuichi et al., 2005). This is followed by fast protein kinase- and protease-dependent modifications of ion channels, receptors, redox-sensitive proteins, as well as triggering of the third phase—the phase of expression of hypoxic tolerance. The question arises what signal mechanisms are responsible for the transformation of the hypoxic tolerance induction phase into the phase of its long-term expression.

As in cerebral ischemic tolerance, formation of the delayed phase of brain tolerance (*thephase of expression*) induced by hypoxic preconditioning is associated with activation of pro-adaptive genes and expression of their products which affect intracellular plasticity readjustment aimed at retention of vital neuronal functions and structural integrity. A principal role in controlling genome activity belongs to so-called third messengers—transcription factors which are activated in the cytosol, translocated to the nucleus and which bind to the promoters of target genes regulating their transcription (Morgan and Curran, 1991). The transcription factors are activated by intracellular components of calcium, phosphoinositide, cyclic AMP and pro-oxidant regulatory systems and therefore link two sequential phases of hypoxic/ischemic tolerance development, namely the phases of its induction and expression.

The main activators of the late-response genes engaged in the mechanisms of neuronal plasticity and survival include inducible (c-Fos, NGFI-A, HIF-1) and ubiquitous (pCREB, NF-κB) transcription factors. Inducible transcription factors are products of immediate early genes whose expression is driven by signaling cascade-dependent remodeling of chromatin enzymes and modifications of histones, as well as by interaction with ubiquitous transcription factors (Morgan and Curran, 1991; Sng et al., 2004). The hypoxia-inducible factor HIF-1α is stabilized and activated under hypoxic conditions due to hypoxia-dependent inactivation of prolyl hydroxylase reactions and is also controlled by NF-κB, NGFI-A, MAP/ERK (Kallio et al., 1999; Samoilov et al., 2007). Ubiquitous transcription factors CREB, NF-κB are activated by phosphorylation and factors of cellular stress (cytokines, reactive oxygen species, Ca_i^{2+}) (Mattson et al., 2000; Ravati et al., 2000; Kitagawa, 2007).

Until recently, only scattered non-systematic data on the nature of the involvement of various transcription factors in the mechanisms of expression of brain hypoxic tolerance have been reported. Using our model of hypobaric hypoxic preconditioning, a complex study of the expression and activity profiles of the key transcription factors in the rat brain has been performed over the years which allowed us to conclude that neuroprotective hypoxic preconditioning by three episodes of mild hypobaric hypoxia induces persistent (up to 24 h) neuronal overexpression of inducible (c-Fos, NGFI-A, HIF-1α) and activation of ubiquitous (pCREB, NF-kB) transcriptional factors in the most vulnerable brain regions including the fronto-parietal neocortex and various hippocampal fields (CA1, CA3/4, dentate gyrus) (Rybnikova et al., 2008a, 2009). Although we have observed some specific changes characteristic to the neocortex and different areas of the hippocampus (**Figure 1**) it is noteworthy that despite the different levels of expression of the transcription factors their cooperative "cross" activation was observed in all examined brain structures in preconditioned rats.

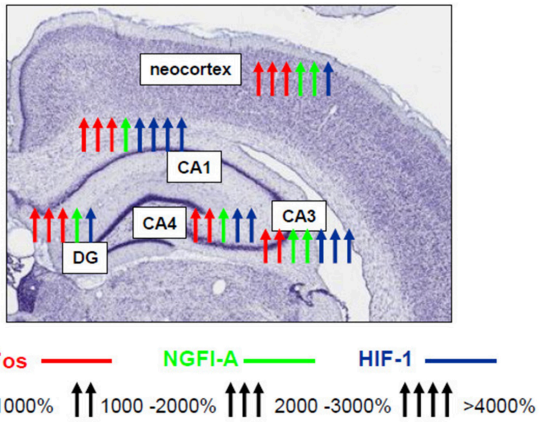

FIGURE 1 | Schematic representation of activating effect of hypobaric hypoxic preconditioning on the transcription factors NGFI-A, c-Fos, HIF-1 in various areas of hippocampus and neocortex (Rybnikova et al., 2009, 2015).

More recent results indicate that the cooperative induction of transcription factors can be attributed to the hypoxia-tolerant phenotype depending on the efficacy and mode of hypoxic preconditioning. Strong activation of neuronal CREB (its phosphorylation) and NF-κB p65 in the neocortex has been observed following the three-trial but not the single-trial preconditioning (Samoilov et al., 2014). In contrast to the three-trial preconditioning, the single preconditioning trial does not result in formation of the hypoxia-tolerant phenotype and provides no protection against the structural and functional injury produced by severe hypoxia.

It is well-known that downstream targets for the transcription factors NGFI-A, c-Fos, HIF-1α, pCREB, and NF-κB include genes encoding pro-adaptive proteins: neurotrophins, antioxidants, anti-apoptotic factors of the *bcl-2* superfamily, heat shock proteins, erythropoietin and others, involved in the processes of neuroplasticity and neuronal survival during harmful exposures (Sheng and Greenberg, 1990; Finkbeiner, 2000; Mattson et al., 2000; Semenza, 2000; Rice et al., 2002; Merelli et al., 2013, etc.). Based on this knowledge it can be hypothesized that the cooperative activation of these transcription factors in the brain ("warning" signaling at the level of the genome) promote the most intense expression of the pro-adaptive genes which are essential for preventing the injury of the susceptible brain neurons caused by damaging factors.

One of the most important common mechanisms of ischemic and hypoxic preconditioning involves reprogramming of apoptosis-related processes. Both types of preconditioning up-regulate expression of anti-apoptotic proteins of the Bcl-2 superfamily and repress the levels of Bax (Rybnikova et al., 2006; Liu et al., 2009). A complex of survival-enhancing, neuroprotective and neuroplasticity processes is induced by hypoxic preconditioning in the phase of expression at the level of late-response genes and *de novo* synthesis of the protein

products. The most substantial contributions to the hypoxia-tolerant phenotype have been ascribed to the up-regulation of mitochondrial and cytosolic antioxidant enzymes (Lin et al., 2003; Arthur et al., 2004; Stroev et al., 2004, 2005; Shao and Lu, 2012), neurotrophin BDNF (Samoilov et al., 2014), erythropoietin (Bernaudin et al., 2000, 2002a; Grimm et al., 2006), adrenomedullin (Tixier et al., 2008), the dampening of the excitotoxicity by down-regulation of AMPA receptors (Chang et al., 2006) and stimulation of group I metabotropic glutamate receptors (Semenov et al., 2012). A target of HIF-1 vascular endothelial growth factor VEGF was also shown to be intensely up-regulated (Bernaudin et al., 2002b) which appears to promote extensive neurovascular remodeling as described for hypoxic preconditioning recently (Boroujerdi and Milner, 2015). Among other protective mechanisms, an induction of heat shock proteins, in particular Hsp70 has also been demonstrated in a model of hypobaric hypoxic preconditioning (Wang et al., 2012). Adenosine content and adenosine A1 receptor affinity in the hippocampus of rats preconditioned by repetitive autohypoxia are markedly higher than those in non-preconditioned animals or animals preconditioned by a single trial (Zhang and Lu, 1997). Comparing the changes in the neuropeptide Y levels in gerbil hippocampus in two models of preconditioning, hypobaric hypoxic and ischemic, Duszczyk and colleagues have demonstrated that ischemic preconditioning caused a 20% rise in the expression of neuropeptide Y which lasted for 4 days, whereas hypobaric hypoxic preconditioning according to our protocol resulted in a two-fold increase in the neuropeptide Y levels that was maintained for at least 7 days (Duszczyk et al., 2009).

Our recent study has also reported that hypoxic preconditioning differentially alters the expression of specific proteins called ADAMs (a disintegrin and metalloprotease). ADAMsare a family of membrane-anchored glycoproteins capable of shedding a multitude of proteins from the cell surface (van Goor et al., 2009). Various ADAMs act as crucial modulators of physiological and pathophysiological processes and are supposed to contribute to control of neuronal death/survival. Hypoxic preconditioning induces prominent overexpression of ADAM17 mRNA and protein in the hippocampus and neocortex and prevented severe hypoxia-induced up-regulation of ADAM 15 linked to the neuronal injury (Rybnikova et al., 2012a). These findings might be of special importance in the focus of neurodegeneration since some of the ADAMs including ADAM10 and ADAM17 (TACE) function as amyloid precursor protein (APP) α-secretases (Allinson et al., 2003). For this reason, the observed up-regulation of ADAM17 corresponded to a higher level of the soluble form of APP produced in response to hypobaric hypoxic preconditioning may predict some therapeutic properties for this technique in prevention of Alzheimer's disease. The restoration of the ischemia-reduced α-secretase activity and expression of the amyloid-degrading enzymes neprilysin and endothelin-converting enzyme has been reported in other models of hypoxic preconditioning (Nalivaeva et al., 2004).

A specific group of ligand-operated transcription factors is represented by receptors of corticosteroid hormones. There are two subtypes of such receptors differing in their characteristics, affinity, localization and functions, namely glucocorticoid and mineralocorticoid receptors. Both receptor subtypes are found in the brain and play important roles in adaptive behavior and endocrine regulation, in particular, in feedback control of the hypothalamic-pituitary-adrenal axis (HPA) exerted by glucocorticoid hormones. To date the functions of gluco- and mineralocorticoid receptors, and especially of their balance, have been expanded to participation in the processes of neuronal death/survival and neuroplasticity (de Kloet et al., 1998; De Kloet et al., 2005; Almeida et al., 2000; Rogalska, 2010). Our studies have demonstrated that three trials of hypoxic preconditioning considerably affected expression of gluco- and mineralocorticoid receptors in the dorsal and ventral hippocampus, achieving their optimal balance for neuronal survival (Rybnikova et al., 2011) while the single-trial preconditioning exhibited such an effect to a much lesser extent.

The preconditioning-induced changes in brain corticosteroid receptors appear to contribute to the improvement of adaptive capabilities of the whole organism. This is supported by the analysis of survival rates in preconditioned vs. non-preconditioned rats under the conditions of acute lethal hypoxia. As mentioned above, hypoxic preconditioning in our model of hypobaric hypoxia significantly decreased the lethality of rats during 3 h of exposure to severe hypoxia (down to 15%). This allowed us to suggest that such preconditioning results in mobilization of systemic mechanisms of adaptation, primarily of those associated to the functioning of HPA responsible for organization of the adaptive stress response (Selye, 1950). Further evidence substantiating this suggestion has been obtained in our experiments showing that effective hypoxic preconditioning enhances the activity and reactivity of HPA to mild stresses and potentiates the negative glucocorticoid feedback (Rybnikova et al., 2007b, 2008b, 2011). No such effect has been seen for the non-effective preconditioning mode. Very recently Feng and Bhatt (2015) have also described similar changes in HPA activity in the model of normobaric hypoxic preconditioning and shown that the glucocorticoid receptor blocker RU486 significantly inhibited hypoxic PC induced neuroprotection in newborn rats (Feng and Bhatt, 2015). Hence it is obvious that one of the most important and universal protective mechanisms launched by the hypoxic preconditioning is the modification of HPA functioning aimed at the optimal mobilization of the pro-adaptive defenses. It is interesting to note that modifications of HPA appear to represent a specific target for the adaptogenic effects of high altitudes, since they are observed also during high altitude acclimatization and training (Meerson et al., 1996).

In addition to normobaric and hypobaric hypoxic preconditioning, molecular mechanisms of neuroprotection have been extensively studied in the autohypoxic model. In contrast to other models of hypoxic or ischemic preconditioning, a number of pro-adaptive factors were down-regulated in the brains of rats preconditioned by episodes of autohypoxia, including pERK1/2, calcium, excitatory amino acids, nitric oxide, expression of alpha synuclein (Shao and Lu, 2012). On the other hand, enzyme antioxidants, CREB-dependent signaling, and expression of HIF-1 and its target molecule VEGF are up-regulated in the vulnerable brain regions as has been reported

for other models. These findings suggest that autohypoxic preconditioning is a model which shares with other models some common mechanisms but differs in its specific features.

An important role of systemic mechanisms for whole-body models of hypoxic preconditioning (autohypoxia, hypobaric hypoxia) might be expected due to an apparent contribution of remote conditioning. The remote preconditioning is a phenomenon in which brief hypoxic/ischemia and reperfusion of one organ increases tolerance in a remote organ (for review see Przyklenk and Whittaker, 2013). In addition, the remote ischemic preconditioning increased resistance of the whole organism to hypobaric hypoxia at high altitude, preventing development of high altitude diseases (Berger et al., 2015). Until recently this phenomenon has been attributed only to the ischemic models (Vijayakumar et al., 2015), however accumulating evidence suggests that in addition to transient ischemia other triggers of remote protection should be introduced, including peripheral nociception, direct peripheral nerve stimulation and electroacupuncture (Heusch et al., 2015). Taking in consideration that during hypoxic preconditioning episodes in the whole-body models all organs and tissues of the body are exposed to the preconditioning hypoxia, systemic mechanisms of remote preconditioning are likely to contribute to the increased tolerance of the brain. A review of the literature gives evidence for both humoral and neural mechanisms of remote preconditioning (Meller and Simon, 2015). Plausible candidates of humoral mediators include plasma microRNA-144 and ATP-sensitive potassium channels of red blood cells (Gopalakrishnan and Saurabh, 2014; Li et al., 2014) but their importance for protecting the brain has not been demonstrated. In contrast to cardioprotection, for neuroprotection resulting from the remote preconditioning there is more evidence on the neural mechanisms, because blocking neural inputs to the central nervous system prevented remote-preconditioning-induced brain protection (Malhotra et al., 2011; Meller and Simon, 2015). Surprisingly, remote ischemic preconditioning, in contrast to our model of hypobaric hypoxia and normobaric hypoxic preconditioning in the newborn, failed to induce a detectable increase in circulating cortisol as recently reported by Birkelund and colleagues (Birkelund et al., 2015).

New and perspective avenues in research into hypoxic preconditioning and brain tolerance involve epigenetic regulation. Recently, it has been reported that methylation of DNA may be involved in the neuroprotection achieved by hypoxic preconditioning (Zhang et al., 2014). The role of other epigenetic mechanisms in the hypoxic preconditioning has not been studied although an involvement of changes at the epigenetic level in cellular responses to hypoxia/ischemia, in particular in the brain, became a subject of several studies (Watson et al., 2010; Perez-Perri et al., 2011; Melvin and Rocha, 2012; Schweizer et al., 2013; Wu et al., 2013; Tsai and Wu, 2014). As noted above, a key role in the formation of preconditioning-induced brain tolerance belongs to the cooperative activation of several transcription factors, HIF-1α being one of the most important. It is known that HIF-1 requires co-activators which modify chromatin structure, opening the access to DNA for the regulators of transcription. HIF-dependent co-activators include histone acetyltransferases, CREB-binding protein (CBP) and p300 (Ema et al., 1999), some histone deacetylases (Kato et al., 2004; Ellis et al., 2009; Seo et al., 2009) and a chromatin remodeling complex SWI/SNF (Wang et al., 2004). Binding of CBP to the CRE elements of gene promoters dependent on pCREB activity is also up-regulated by hypoxic preconditioning, as described above. Thus, involvement of epigenetic mechanisms can be currently expected both at the level of protein-protein interactions with transcriptional regulators and the acetylation of histones followed by chromatin relaxation. However, this hypothesis requires further experimental substantiation, which we are currently working on.

HYPOXIC POSTCONDITIONING

Intensive research into ischemic preconditioning in the 1990s–2000s inspired establishment of a cognate protective phenomenon—ischemic postconditioning. It has been demonstrated that repetitive brief ischemia applied *after* prolonged coronary occlusion, during early reperfusion (such a mode has been termed *postconditioning)* is cardioprotective by attenuating reperfusion after myocardial injury (Zhao et al., 2003). A link to the brain was suggested later, in 2007 (Zhao, 2007), and the first experimental studies of this research group described the neuroprotective effects of ischemic postconditioning in stroke with a putative role suggested for Akt signaling (Gao et al., 2008) that is similar to the mechanisms of ischemic preconditioning. Further studies on cerebral ischemic postconditioning to date provide controversial results and many issues remain to be solved regarding its possible protective effects and feasibility (Leger et al., 2015).

A neuroprotective potential of hypoxic postconditioning has been thoroughly described for the first time using chronic intermittent normobaric hypoxia in mice (Leconte et al., 2009). It has been demonstrated that later application of hypoxia (1 h, 8% O_2, 1–5 days) after the focal ischemia significantly reduced delayed thalamic atrophy. The same study reported that hypoxia *in vitro* (0.1, 1, or 2% O_2) performed 14 h after oxygen glucose deprivation induced neuroprotection in primary neuronal cultures and pointed out the causal mechanistic role for HIF-1α and its target genes erythropoietin and adrenomedullin in this process.

Recently, based on our original studies of hypobaric hypoxic preconditioning, we developed a model of hypobaric hypoxic postconditioning in rats which involves mild hypoxic exposures (360 mm Hg, 2 h) spaced at 24 h applied either 3 or 24 h after severe hypoxia. It was found that both early and delayed applications of the postconditioning episodes remarkably improved recovery from severe hypoxia and attenuated posthypoxic neuronal injury, reducing pyknosis, hyperchromatosis, and interstitial brain edema, as well as the rates of neuronal loss in the hippocampus and neocortex. Besides that, the delayed postconditioning exerted a potent anxiolytic effect on rat behavior, preventing development of posthypoxic anxiety. Both modes of postconditioning had a beneficial effect on the functioning of HPA, but only the delayed postconditioning completely returned HPA to its

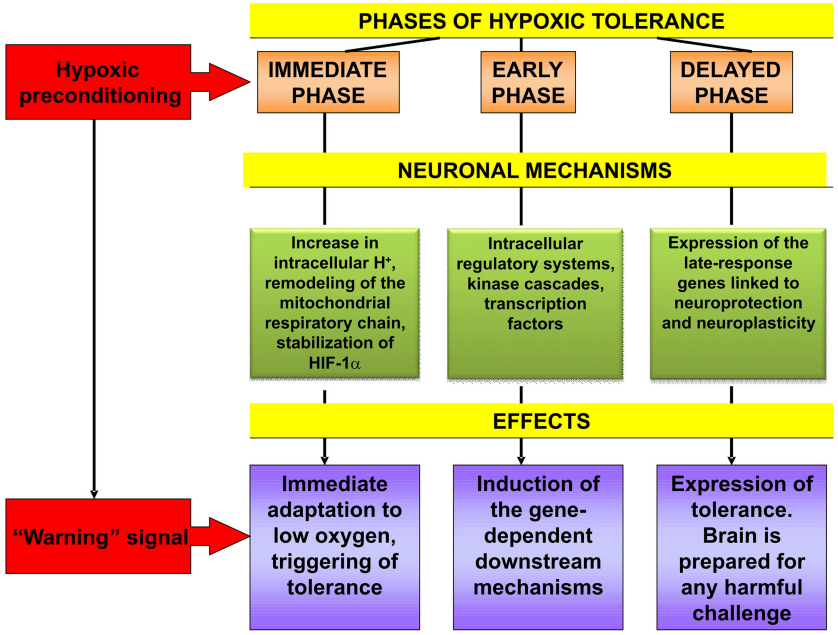

FIGURE 2 | Cascade of molecular mechanisms involved in brain hypoxic tolerance induced by hypoxic preconditioning. See references in the text relevant to this Figure.

baseline activity and reactivity to stress (Rybnikova et al., 2012b). Furthermore, such a mode of postconditioning enhanced expression of HIF-1α and erythropoietin in the hippocampal CA1 neurons of rats surviving after severe hypoxia (Vetrovoy et al., 2014). The modifications of apoptosis-related processes and up-regulation of the neurotrophins (BDNF) has also been implicated in the compensatory mechanisms induced by hypobaric hypoxic postconditioning (Vetrovoy et al., 2015). In parallel to our studies, it has recently been reported that hypobaric hypoxic postconditioning reduces brain damage and improves antioxidative defense in the rat model of birth asphyxia (Gamdzyk et al., 2014).

CONCLUSIONS

To summarize the facts and hypothesis discussed above, it should be stated that hypoxic preconditioning functions as a "warning" signalization which mobilizes evolutionarily acquired genome-determined urgent defense mechanisms of brain neurons and the organism as a whole. The processes resulting in their increased resistance to injurious challenge involve induction of multiple intracellular signal components, as well as adjustment of the neuroendocrine control of HPA function. Hypoxic preconditioning-induced cascade mechanisms of intracellular signaling include changes at the level of intracellular redox status, mitochondrial respiratory chain, key second messenger systems, receptor machinery, transcription factors, early and late-response genes which are sequentially engaged in the initiation, induction and expression of the brain hypoxic tolerance (**Figure 2**). A cooperative activation of the ubiquitous, ligand-operated and inducible transcriptional factors plays the key role for development of the rigorous phase of tolerance expression. Studying the impact of epigenetic regulation in mediating the neuroprotective effects of hypoxic pre- and postconditioning represents a novel perspective trend in this research. Although it is apparent that the main neuroprotective mechanisms of hypoxic pre- and postconditioning might be overlapping this hypothesis needs further investigation.

Based on the notable recent progress in our current knowledge of hypoxic pre- and postconditioning, it is obvious that these techniques possess high translational potential and can inspire development of novel feasible neuroprotective tools for prophylaxis and treatment of neurological, neurodegenerative and stress-related disorders, as well as for increasing of the adaptive capacities of the organism and the brain. It is necessary to consider that adjustment of the particular mode of hypoxic pre- and postconditioing elaborated on the basis of fundamental mechanistic data is principally important for the effective application of their protective potential.

AUTHOR CONTRIBUTIONS

ER wrote the review. MS developed the whole conception of the review and substantially contributed to the editing of the final version.

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REVIEW
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Molecular programs induced by heat acclimation confer neuroprotection against TBI and hypoxic insults via cross-tolerance mechanisms

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Neuroprotection following prolonged exposure to high ambient temperatures (heat acclimation HA) develops via altered molecular programs such as cross-tolerance Heat Acclimation-Neuroprotection Cross-Tolerance (HANCT). The mechanisms underlying cross-tolerance depend on enhanced "on-demand" protective pathways evolving during acclimation. The protection achieved is long lasting and limits the need for *de novo* recruitment of cytoprotective pathways upon exposure to novel stressors. Using mouse and rat acclimated phenotypes, we will focus on the impact of heat acclimation on Angiotensin II-AT2 receptors in neurogenesis and on HIF-1 as key mediators in spontaneous recovery and HANCT after traumatic brain injury (TBI). The neuroprotective consequences of heat acclimation on NMDA and AMPA receptors will be discussed using the global hypoxia model. A behavioral-molecular link will be crystallized. The differences between HANCT and consensus preconditioning will be reviewed.

Keywords: traumatic brain injury and neuroprotection, heat acclimation and cross tolerance, angiotensin AT2 receptor and neurogenesis, AKT-HIF-1 signaling, hypoxia, NMDA and AMPA receptors

Cross-tolerance

Cross-tolerance is the phenomenon whereby exposure to one stressor induces protection from a novel stressor and such cross reinforcement raises the possibility of inducing protection to a stressor without prior exposure to it (Horowitz et al., 2004). It is an important feature of many cellular protective mechanisms and involves the plasticity of interactions of environmental-stressors at one end of the spectrum, to drugs and pharmacological interactions at the other. In this review we will focus on how adjustment to one environmental stressor limits damage caused by exposure to novel stressors i.e., heat-acclimation mediated neuroprotection cross-tolerance (HANCT).

Heat Acclimation

Heat acclimation is the term used to describe the processes occurring when an organism is exposed to environmental heat for a prolonged period. The changes at all levels of body organization lead to a new homeostasis which is achieved by the reprogramming of gene expression.

Heat acclimation (Horowitz, 2014) is achieved by persistent exposure of an organism to an ambient temperature above its normothermic range. Heat acclimation is a "within lifetime" phenotypic adaptation involving adjustments at all levels of body organization to enhance thermotolerance and heat endurance. The physiological criteria for the heat acclimated phenotype are

reduced basal metabolic and heart rates as well as basal body temperature, concurrent with lower temperature-threshold of activation of the heat dissipation effectors. An important additional criterion is delayed thermal injuries, due to an elevated injury temperature threshold. Reprogramming of gene expression and post-transcriptional regulatory mechanisms are essential components of the acclimated phenotype. Major molecular players in the induction of heat acclimation are the Heat Shock Factor 1–Heat Shock Proteins (HSF1–HSPs) cascade and hypoxia inducible transcription factor α (HIF-1α) and its targets (Maloyan et al., 1999, 2005; Shein et al., 2005; Horowitz, 2010; Horowitz and Assadi, 2010). The 1 month needed to induce acclimatory homeostasis augments HSP72 reserves and leads to constitutive elevation of HIF-1α under normoxic environmental conditions. This implies that in the acclimated phenotype, cytoprotection can be accomplished without *de novo* HSP synthesis or abolishment of HIF-1α degradation.

Classical preconditioning (cPC) is a cellular-molecular protective mechanism evoked following exposure to a sub-lethal stress. Initially rapid, transient salvage kinases are activated followed by transcription and translation of cytoprotective proteins. Protection from subsequent exposure to the same stress lasts for 24–48 h.

In cPC, cytoproective molecular reserves are augmented via subjection to intermittent short sub-lethal stresses (i.e., thermal, ischemic, hypoxic), which confer protection to larger stressors, mostly of the same type, when given within 24–48 h (Murry et al., 1986; Obrenovitch, 2008; Thompson et al., 2013). Unlike HA (Section Preconditioning vs. Heat Acclimation Mediated Cross-tolerance below), the short-term sub-lethal stresses of cPC are insufficient to confer long-term protection even though stress-associated genes are immediate "on-demand" responders [namely, induced by preconditioning (or adaptive processes) and remain on "stand by"]. Gene chip bioinformatic analyses of the acclimatome (Tetievsky et al., 2014) showed that heat acclimation induces qualitative changes in molecules such as ion channels, receptor properties and various metabolic pathways, which can also be neuroprotective. In the frontal cortex and the hippocampus, Yacobi et al. (2014) reported significant changes in the properties of the N-methyl-D-aspartate receptor (MNDA-R) and α-amino-3-hydroxy-5-methyl-4-isoazole-propionic acid receptor (AMPA-R), while Schwimmer et al. (2006) provided evidence of changes in cytokines and various pyrogenic neuropeptides and neuropeptide receptors, as well as changes in the angiotensin AT1 and AT2 receptor ratio. These phenotypic changes were found to be neuroprotective, either by "preventive" mechanisms, which attenuate the severity of the damage, or by inducing spontaneous recovery via cytoprotective pathways and are discussed below regarding traumatic brain injury (TBI) and hypoxic stress. For details regarding HANCT following hyperoxic insults the readers are referred to Arieli et al. (2003).

Preconditioning vs. Heat Acclimation Mediated Cross-tolerance

cPC was first reported in 1986, when Murry et al. (1986) discovered that intermittent brief sub-lethal ischemic episodes protect the heart from a subsequent sustained ischemic insult. That phenomenon, defined as "preconditioning" suggested that in cardiac patients, the multiple episodes of angina that often precede myocardial infarction actually delay cell death after coronary occlusion allowing larger areas of the myocardium to survive and function. Less than 10 years later, this preconditioning effect became widely recognized in brain research as a powerful cytoprotective tool and provided an innovative approach for the development of protective strategies (Obrenovitch, 2008). Preconditioning is a part of the universal rapid cellular adaptive mechanisms induced by many physiological and pharmacological stressors (e.g., hypoxia, ischemia, heat stress, anesthetics).

In view of the large number of inducible stress genes responding to heat exposure and/or heat acclimation (Horowitz et al., 2004; Schwimmer et al., 2004; Horowitz, 2014) we postulated that the induction of the consensus heat acclimation players, HSH1-HSP cascade or HIF-1α and targets only is unlikely to be sufficient to confer heat-acclimation-induced cross-tolerance. Gene-cluster analysis of cDNA Atlas arrays of genes representing homeostatic responses and stress-associated genes in the heart (Horowitz et al., 2004), respectively, showed a divergence between genes responding to heat stress and those responding to ischemia/reperfusion insult. The data suggest that "shared signaling cascades" are the underlying mechanism of cross-tolerance *by interacting with those involved in stress-specific or organ specific responses*.

Conceptually, both heat acclimation mediated cross-tolerance and cPC rely on transcriptional activation. However, in contrast to cPC, heat acclimation for 2 days (when apparent acclimation depends on neural activity and cPC is at its peak) impedes tolerance to novel stressors despite the rapid adaptive responses, and this is probably related to phosphorylation processes (detailed in Assayag et al., 2012). Therefore it seems that heat acclimation mediated cross-tolerance depends on long-term translational processes, which establish reserves of cytoprotective proteins (e.g., HSP72, Section Heat Acclimation above) and/or adjust electron transfer and ROS production in the mitochondria (Assayag et al., 2012). Therefore, cross-tolerance can only be tested after acclimatory homeostasis has been achieved. Recent studies from our laboratory (Treinin et al., 2003; Horowitz and Alxander-Shani, 2015) demonstrated that (i) HSF1-HSP72 cascade (at least in the heart and in *C. elegans*) is regulated in a HIF-1α independent manner and that (ii) HSP72 is essential but insufficient to confer protection in our models. It remains unclear whether HIF-1α transcriptional activation alone is sufficient to confer protection.

Traumatic Brain Injury

Epidemiology

TBI affecting about 10 million people worldwide every year, is a leading cause of death and disability and is a major social, economic and health problem (Maas et al., 2008). The World Health Organization estimates that by the year 2020 TBI will become a more common cause of death than other major diseases (Zitnay, 2005). The majority of TBI occurs among young children (0–4 years) and in the 15–45 age group, and 75% of those affected

are male (Bruns and Hauser, 2003; Rutland-Brown et al., 2006; Tagliaferri et al., 2006; Maas et al., 2008). Causes of TBI differ between the age groups. In young children and the elderly, falls are the primary cause of head injury (Bruns and Hauser, 2003). Young adults are more susceptible to TBI through road traffic accidents, fights and contact sports (hockey, football, soccer) (Finfer and Cohen, 2001; Mock et al., 2004). Recently attention has been paid to military personnel suffering from TBI from blast waves (Taber et al., 2006; Hampton, 2011). Almost half of the TBI patients suffer from long-term disabilities such as neurological disorders (e.g., epilepsy and sleep disorders), neurodegenerative and psychiatric diseases, neuroendocrine disorders, and non-neurological disorders (Masel and DeWitt, 2010). In spite of extensive research no effective pharmacological intervention to facilitate recovery after TBI has been found.

Early Harmful Events

The primary injury in TBI is due to mechanical forces on the brain and is accompanied by shearing and tearing of the tissues and blood vessels which trigger immediate and long-term changes in ionic homeostasis, brain metabolism and function. Following the mechanical impact, cellular/molecular mechanisms are set in motion. Many of these mechanisms, such as glutamate-induced excitotoxicity, impaired energy metabolism, oxidative stress and inflammation, are harmful and lead to neuronal cell death, tissue necrosis and functional impairments (Miller, 1993; Werner and Engelhard, 2007; de Lanerolle et al., 2015). Brain hypoxia is one of the most common secondary insults occurring following severe TBI (Chesnut et al., 1993; Jeremitsky et al., 2003). It can be initiated by TBI-induced cerebral hypo-perfusion, or apnea and hypo-ventilation mostly related to brainstem injury (McHugh et al., 2007). Yan et al. (2011) used a combined model of diffused TBI and hypoxia and showed that cerebral hypoxia exacerbates the secondary brain damage following TBI. In their studies Yan et al. (2011) demonstrated increased motor and behavioral deficits along with greater production of pro-inflammatory cytokines and sustained metabolic depression, when compared to TBI alone.

Among the early harmful events that contribute to the pathogenesis of TBI is excessive production of reactive oxygen species (ROS) and NO. The resulting reactive nitrogen species are detected minutes to hours after the insult. Brain tissue is the most vulnerable tissue to oxidative damage due to its high rate of oxidative metabolic activity, intensive production of reactive oxygen metabolites, and relatively low antioxidant capacity (for review e.g., Chong et al., 2005). The brain also contains high levels of transition metals, such as iron, which can catalyze the production of highly toxic radicals via the metal-mediated Haber–Weiss reaction (Halliwell and Gutteridge, 1989). In order to cope with oxidative stress living cells have developed several lines of defense, including antioxidative enzymes and low molecular weight antioxidants.

Endogenous Neuroprotective Mechanisms

In parallel to the harmful cascades, TBI also induces endogenous neuroprotective mechanisms. The ability of cells, tissues and organisms to utilize adaptive self-protective mechanisms is now well recognized in the post-injury phase. The final outcome of TBI is determined by the balance between injury and repair mechanisms (Neary, 2005). Numerous studies have demonstrated the profound protective effect of preconditioning by brief ischemic or thermal exposures on the outcome of ischemic brain injury. The beneficial consequences of such procedures have been demonstrated in several *in vitro* (Liu et al., 2000) and *in vivo* models (e.g., Dirnagl et al., 2003; Glantz et al., 2005; Blanco et al., 2006).

As mentioned above, the functional outcome after TBI depends on the balance between the deleterious and protective mechanisms. Exposure to pre-conditioning stimuli may either inhibit key pathways in harmful cascades or activate key pathways in protective cascades. Identification of these mechanisms may facilitate the design of novel drugs that mimic the self-protective capacity of the brain.

Molecular Mechanisms Underlying HA Mediated Neuroprotection after TBI-inherent Spontaneous Recovery Pathways

The cross-tolerance that HA confers against TBI was investigated extensively by combining HA model (Horowitz, 1976; Maloyan et al., 2005; Tetievsky et al., 2008) with the closed head injury experimental model of TBI (Chen et al., 1996; Flierl et al., 2009). We found that functional recovery of HA rats and mice was greater, with less brain tissue damage, compared to non-HA control animals (Shohami et al., 1994a,b, 1997; Shein et al., 2007). The natural healing processes after TBI are poorly understood, therefore the enhancement of the healing and significantly improved outcomes following TBI after HA prompted us to explore the mechanisms by which HA confers neuroprotection. The accelerated healing of the injured brain in acclimated animals is unusual and provides important information regarding key inherent neuroprotective pathways.

The Importance of Akt Signaling

HANCT confers prolong improved recovery after TBI persisting for at least 6 weeks after injury. This is shown by enhanced motor function, improved cognitive function and reduced edema formation (Shein et al., 2005, 2007; Umschweif et al., 2010, 2013). In order to understand this protection we focused here on Akt (also known as protein kinase B), a master-regulator of pro-survival pathways in many tissues including the brain. Akt activation is enhanced following HA. Moreover, pharmacological inhibition of Akt phosphorylation abolished the beneficial effects of HA in injured mice (Shein et al., 2007), highlighting the importance of this factor in HANCT. Akt activation attenuates the intrinsic apoptosis pathway, which usually causes extensive cell death in neurons (Zhang et al., 2005). Post-injury lesion volume was smaller in the HA group compared with normothermic control mice, possibly due to inhibition of

the intrinsic apoptosis pathway (Umschweif et al., 2010). Akt promotes cellular survival and healing by direct regulation of a variety cellular factors including hypoxia inducible factor 1 alpha (HIF-1α).

HIF-1α—a Key Mediator of HA Mediated Neuroprotection

HIF-1α is the regulatory subunit of the heterodimer HIF-1, a well-known hypoxia induced transcription factor. This subunit responds to many cellular events including hypoxia and is induced by reduced cellular oxygen availability (Singh et al., 2012). The active transcription factor recognizes and binds to the hypoxia-response elements and activates transcription of its target genes, allowing tissue adaptation to hypoxia and ischemia. Secondary ischemia due to reduced oxygen delivery to the injured tissue occurs following TBI; hence HIF-1 activation shortly after TBI fights secondary ischemia and reduces tissue loss. Interestingly, HIF-1α levels are high in HA mice and remain elevated after injury (Shein et al., 2005; Umschweif et al., 2013). The beneficial effect of elevated HIF-1α was lost after inhibition of HIF-1 dimerization with acriflavine (Umschweif et al., 2013). Acriflavine eliminated the spontaneous recovery after TBI and in the first days after TBI no improvement in motor ability was noted, confirming the role of HIF-1α in mediating neuroprotection. Furthermore, in HA mice HIF-1 inhibition not only prevented the enhanced recovery compared to normothermic mice, but motor ability deteriorated, lesion volume was greater as was the death rate of the injured mice (Umschweif et al., 2013). This highlights the importance of HIF-1 in both spontaneous recovery and HANCT. The molecular mechanisms underlying the contribution of HIF-1 to HA mediated neuroprotection are yet to be elucidated, however, there is an evidence that may illuminate events downstream to HIF-1.

Among the cytoprotective and angiogenesis related genes that are upregulated by active HIF-1 is vascular endothelial growth factor (VEGF). VEGF has been described as a potent inducer of neuroprotection and neurogenesis after TBI (Thau-Zuchman et al., 2010, 2012). Therefore it is possible that some of the beneficial effects of HIF-1 in HA mice may be due to the upregulation of VEGF. HA increases VEGF levels and these remain high after TBI. On the other hand, VEGF levels are significantly attenuated following inhibition of HIF-1. It is therefore reasonable to speculate that VEGF is one of the downstream targets mediating HIF-1 induced neuroprotection in HA mice.

Another HIF-1 target gene that may facilitate this effect is the glucose transporter 1 (GLUT1) which is abundant in the brain. Similarly to VEGF, GLUT1 is upregulated in HA brains and this effect is eliminated by HIF-1 inhibition (Umschweif et al., 2013). GLUT1 induction following TBI potentially allows more efficient glucose uptake by brain cells and may fight the energy depletion that results from secondary ischemia. Notably, we demonstrated that HA induces a significant concomitant increase in the expression of nuclear HIF-1α and EpoR prior to and post TBI suggesting the involvement of this pathway in HA-induced neuroprotection (Shein et al., 2005).

Collectively, HIF-1 is a key mediator of HA mediated neuroprotection and elevated HIF-1 levels contribute to the improved outcome of acclimated animals after TBI.

Angiotensin Receptor Type 2: An Upstream Regulator of Neuroprotection and Neurorepair in Injured HA Mice

The crucial role of HIF-1 activation in HA mediated neuroprotection has placed HIF-1 in the spotlight in the search for a new drug target which will successfully mimic the protective effects of HA on TBI. Pharmacological stabilization of HIF-1 independent of cellular oxygen levels may occur following activation of certain growth factor receptors such as insulin like growth factor or epidermal growth-factor (PMID:13130303), which are relevant in the fight against cancer. A different upstream receptor activates HIF-1 in the brain, the angiotensin receptor type 2 (AT2).

AT2 is abundant in the brain during development and under stress (Dumont et al., 1999; Guimond and Gallo-Payet, 2012) including after brain ischemia. It was also elevated in rats following HA (Schwimmer et al., 2004). AT2 activation improves the outcome following brain ischemia and reduces lesion volume (McCarthy et al., 2009, 2012; Guimond and Gallo-Payet, 2012). Taken together, these data promote the hypothesis that increased AT2 levels in the HA phenotype facilitate neuroprotection and improved TBI outcome. Interestingly, AT2 levels were not elevated in HA mice; however, pharmacological blocking of AT2 by a specific antagonist (PD123319) abolished the beneficial effects of HA. HA was found to affect AT2 signaling by increasing levels of another receptor which transactivates AT2, the nerve growth factor receptor, TrkA. HA increased levels of TrkA as well as its endogenous ligand, nerve growth factor (NGF). These events, in combination with the induction of brain derived neurotrophic factor (BDNF) and its receptor TrkB seen in HA mice, may explain the extreme effects of AT2 blocking on TBI outcome. Since both NGF and BDNF increase Akt phosphorylation and in turn activate HIF-1, blocking AT2 inhibits these signals which contribute to neuroprotection in HA mice. Indeed, by blocking AT2 levels in HA mice the levels of NGF and BDNF as well as HIF-1α levels were attenuated. This may explain why the detrimental effects of AT2 blocking were only noted in HA mice, and hardly affected the recovery of control mice after injury. An interesting observation of AT2 blocking in HA mice is the effect on induced neurogenesis seen in HA mice after TBI. HA not only led to TBI induced cell proliferation, but also induced neurogenesis after injury, as seen in the SVZ, the dentate gyrus and in the injury region. These neuroregenerative events are orchestrated and triggered by AT2 signaling—NGF, BDNF, and Akt and were eliminated by blocking AT2, thus, supporting the notion of AT2 as modulator of neurorepair (Umschweif et al., 2014a).

AT2 Activation after TBI Mimics the Beneficial Effects of Long Term Heat Acclimation Prior to Injury

After revealing the role of AT2 signaling and identifying it as an indispensable factor in HA-mediated neuroprotection, we

examined whether these benefits could be mimicked by direct activation of AT2 after TBI.

Using a specific AT2 agonist (CGP42112A), administered for 3 days following TBI we obtained an effect similar to that achieved by HA. The recovery of motor function of the agonist treated mice was somewhat delayed, beginning about 2 weeks after TBI and significantly progressing over the next 3 weeks. Moreover, cognitive function assessed by the ability of the injured mice to recognize a novel object also improved in a dose dependent manner. Lesion volume in the agonist treated mice was smaller than that in the control group (Umschweif et al., 2014b). The molecular mechanism underlying these effects was confirmed. Neurotrophins (NGF, BDNF) and their downstream kinases—Akt and Erk1/2 were induced by the AT2 agonist and blocking the receptor inhibited these molecular effects. These early protective events, occurring within 24 h of the injury, seem to set the stage and probably initiated the neurorepair events seen for weeks after injury. Early AT2 activation induced proliferation and neurogenesis in the neurogenic niches. Interestingly, around the injury site the elevated number of newborn neurons was at the expense of newborn astrocytes (Umschweif et al., 2014b).

To date the molecular mechanisms which were found to underlie the neuroprotective and healing effects of HA, involve Akt, HIF-1, GLUT1, VEGF, NGF, BDNF, and Erk1/2. Pharmacological activation of AT2 after injury partially mimics the beneficial effects of pre-injury HA, and affects all these targets.

Hypoxia from Low PO_2 Partial Pressure: HA Mediated Neuroprotection via NMDA-R and AMPA-R Remodeling-injury "Prevention"

The most sensitive cells to hypoxic/ischemic stress are the neurons of the cornus ammonis 1 (CA1) hippocampal layer and those of layers 3–6 of the frontal cortex (Kirino et al., 1984). Following exposure to hypoxic/ischemic stress there is a massive glutamate discharge which over-activates glutamate-gated ion channels (Choi, 1992; Doble, 1999) and kills these neurons. This is mediated via NMDA-excitotoxicity, free radical formation, lactic acidosis, and inhibition of protein synthesis (Gozal et al., 1998; Lau and Tymianski, 2010). Cell death starts 2–3 days after insult and may continue for several weeks. It is probably caused by calcium influx via glutamate-gated channels during the first hours after the stress (Bochet et al., 1994; Jonas et al., 1994; Osuga and Hakim, 1994; Nadler et al., 1995; Kaur et al., 2012).

In contrast to the previous sections of this review where the impact of heat acclimation on spontaneous recovery signals induced post TBI were discussed, in this section we will describe a hypoxia rat model, which is achieved by exposure of the animals to a mixture of air and nitrogen adjusted to achieve 4.5 ± 0.5% oxygen for 15 min. We will focus on how HA causes hypoxic tolerance via NMDA receptor remodeling. The involvement of NMDA-R in the process of delayed cell death after hypoxic stress is well-established, including its upregulation and increased activity in the post-stress period.

Briefly, the NMDA-R is comprised of several subunits. The GluN1 subunit is present in all NMDA-R assemblies; therefore its levels are used as a marker of NMDA-R on the external cell surface (Kutsuwada et al., 1992; Bochet et al., 1994; Monyer et al., 1994). The GluN2 subunits are located on the external cell membrane and GluN2A and GluN2B are abundant in the hippocampus and frontal cortex, the regions most susceptible to hypoxic stress (Chen et al., 1999; Erreger et al., 2005). Ion channels made of the GluN1/GluN2A combination are four times more permeable to calcium than GluN1/GluN2B. Therefore, (in brain areas where the presence of other NMDA-R subunits is negligible) when the GluN2B/GluN2A ratio is greater than 1, there is a lower channel opening probability and less calcium penetration. In congruence with clinical behavioral tests demonstrating that heat acclimated rats are more able to deal with hypoxia than non-acclimated rats, we showed: (i) that the heat acclimated group had less NMDA-Rs than controls under both basal and hypoxic conditions and (ii) an increased GluN2B/GluN2A ratio in response to hypoxia, with controls showing a reciprocal effect. Collectively, the data indicate that the protective changes in the NMDA-R against hypoxia following long-term heat acclimation are not only the result of quantitative shifts, but qualitative changes in the sub-unit profile of the receptor also occur. Similarly, AMPA-R, another important glutamate-gated ion channels that causes cell damage during hypoxia via alteration of the GluA2 subunit, is also remodeled (Gorter et al., 1997; Carriedo et al., 1998). The HA group responds to hypoxic insult with a significant increase in GluA2 protein levels in contrast to the rapid declines noted in controls. The timing of this decrease, so soon after insult, implies that a damaging rapid increase in inter-cellular calcium concentration occurs in non-acclimated rats.

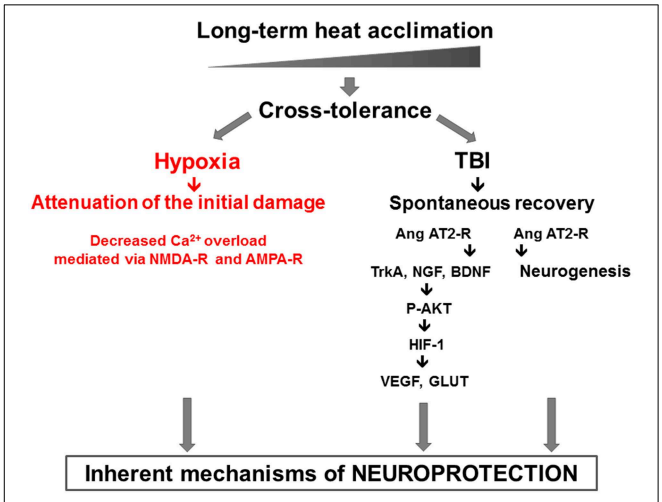

FIGURE 1 | The two pronged approach of heat acclimation mediated neuroprotection: "prevention," or injury attenuation, shown in our hypoxia model via decreased Ca^{2+} permeability and "spontaneous recovery" shown in the TBI model. In the latter, the role of AT2 angiotensin receptors in neurogenesis and P-AKT-HIF-1 cascade are delineated.

Translational Benefits of Heat Acclimation

Studying HANCT improves our understanding of natural, intrinsic mechanisms of neuroprotection. This form of conditioning enhances innate protective mechanisms and enables the organism (or tissue) to combat future insults. HANCT *per se* is impractical as a therapeutic modality as it cannot be applied post-injury.

However the multiple spontaneous signals of recovery, which are evoked by HANCT and minimize neuronal death, can be translated into novel neuroprotective strategies. The inference (i.e., in Section AT2 Activation after TBI Mimics the Beneficial Effects of Long Term Heat Acclimation Prior to Injury above) that pharmacological activation of AT2 (Umschweif et al., 2014b) or treatment with erythropoietin (Shein et al., 2008) is neuroprotective after TBI, stems from our studies on HANCT.

Our translational approach is supported by the interesting similarities between the molecular neuroprotective mechanisms invoked by moderate hypothermia as a therapy (Florian et al., 2008; Dietrich and Bramlett, 2010; Joseph et al., 2012), representing the reciprocal edge of thermal impacts and neuroprotection. Moderate hypothermia reduces excitotoxicity by decreasing extracellular glutamate levels, furthermore apoptosis, inflammation, edema and infarct size are reduced and motor function recovery improves. Similar protective pathways are part of the neuroprotective repertoire conferred by heat acclimation in mice (Shein et al., 2007; Umschweif et al., 2010) and rats (Shohami et al., 1994a,b).

Concluding Remarks

In this review we discussed the neuroprotective properties of phenotypic adaptations to prolonged exposure to environmental heat (heat acclimation—HA) which occur via potentiation of endogenous defense mechanisms. Damage is less severe e.g. via remodeling of NMDA-R and AMPA-R subunit profiles or by attenuating injury processes by recruiting augmented "on demand" constitutive cytoprotective networks.

These are further modulated by dynamic components such as AKT-HIF-1α cascade (Akt, HIF-1, GLUT1, VEGF, NGF, BDNF, and Erk1/2). Our findings confirm the role of enhanced antioxidative, antiapoptotic, and anti-inflammatory capacities (Shein et al., 2007) in HA TBI mice. Upstream pharmacological stabilization of the HIF-1 cascade, independent of cellular oxygen levels, occurs via angiotensin receptor type 2 (AT2) was described. Furthermore, AT2 receptor activation also induces cell proliferation and neurogenesis after TBI, seen in the SVZ, the dentate gyrus and in the injury region of HA mice. *Pharmacological activation of AT2 after injury partially mimicked the beneficial effects of pre-injury HA*. The HA mediated neuroprotective features discussed are presented in **Figure 1**.

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Hypoxia in CNS Pathologies: Emerging Role of miRNA-Based Neurotherapeutics and Yoga Based Alternative Therapies

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Cellular respiration is a vital process for the existence of life. Any condition that results in deprivation of oxygen (also termed as hypoxia) may eventually lead to deleterious effects on the functioning of tissues. Brain being the highest consumer of oxygen is prone to increased risk of hypoxia-induced neurological insults. This in turn has been associated with many diseases of central nervous system (CNS) such as stroke, Alzheimer's, encephalopathy etc. Although several studies have investigated the pathophysiological mechanisms underlying ischemic/hypoxic CNS diseases, the knowledge about protective therapeutic strategies to ameliorate the affected neuronal cells is meager. This has augmented the need to improve our understanding of the hypoxic and ischemic events occurring in the brain and identify novel and alternate treatment modalities for such insults. MicroRNA (miRNAs), small non-coding RNA molecules, have recently emerged as potential neuroprotective agents as well as targets, under hypoxic conditions. These 18–22 nucleotide long RNA molecules are profusely present in brain and other organs and function as gene regulators by cleaving and silencing the gene expression. In brain, these are known to be involved in neuronal differentiation and plasticity. Therefore, targeting miRNA expression represents a novel therapeutic approach to intercede against hypoxic and ischemic brain injury. In the first part of this review, we will discuss the neurophysiological changes caused as a result of hypoxia, followed by the contribution of hypoxia in the neurodegenerative diseases. Secondly, we will provide recent updates and insights into the roles of miRNA in the regulation of genes in oxygen and glucose deprived brain in association with circadian rhythms and how these can be targeted as neuroprotective agents for CNS injuries. Finally, we will emphasize on alternate breathing or yogic interventions to overcome the hypoxia associated anomalies that could ultimately lead to improvement in cerebral perfusion.

Keywords: hypoxia, ischemia, microRNA, yoga, breathing exercise, neuroprotection

INTRODUCTION

Mammalian brain lacks fuel reservoirs and hence needs a constant supply of glucose for ATP generation, cell survival, and production of neurotransmitters. Although it constitutes only 2% of body weight, it consumes nearly a quarter of total body glucose (∼5.6 mg glucose per 100 g human brain tissue per minute) (Erbslöh et al., 1958). Under physiological aerobic conditions, glucose is completely metabolized into carbon dioxide and water resulting in the formation of ATP. Any hindrance in the oxygen supply to the brain may cause catastrophic effects to brain cells. In this review we will first discuss the pathophysiological effects caused due to hypoxia, which has been associated with different neurodegenerative diseases and consecutively, we will discuss miRNA based therapeutic approach to target the hypoxia-associated CNS injuries along with alternate therapies for management of hypoxia.

PATHOPHYSIOLOGICAL RESPONSES TO HYPOXIA

Oxygen is an essential element for cell survival. It acts as a final electron acceptor in oxidative phosphorylation, which ultimately leads to the production of energy in the form of ATP. However, under hypoxia, a cell undergoes prolonged energy deprivation that results in irreversible cellular dysfunction and ultimately leads to cellular demise (Gutierrez, 1991; López-Barneo et al., 2001; Hardie, 2003). Hypoxia may occur in both basal conditions as well as diseased conditions (Brahimi-Horn et al., 2007; Brahimi-Horn and Pouysségur, 2007; Sluimer et al., 2008; Li Z. et al., 2009).

In response to hypoxia, a cell triggers a plethora of reactions, which include disturbance in ion channel homeostasis, energy failure, free radical production, etc. It has been estimated that 60% of ATP is consumed by ATPases, such as the Na^+/K^+ ATPase, and Ca^{2+}ATPase under normal conditions (Zauner et al., 2002). During hypoxia, there is a reduction in the intracellular ATP/ADP ratios, which disrupts the ion concentration gradients by causing an increased efflux of K^+ ions and influx of Na^+ and Ca^{2+} ions. Eventually, membrane depolarization takes place and the accumulated Ca^{2+} ions activate the proteases, which leads to membrane damage, disturbance in mitochondrial metabolism, and generation of reactive oxygen species (ROS) (Boutilier, 2001). Under prolonged exposure to hypoxia, hypoxia-inducible factor (HIF) is upregulated, which is a well-studied transcription factor and serves as a potential endogenous marker for hypoxia. HIF-1 transcriptional complex, a ubiquitously expressed protein made up of two subunits namely HIF-1α and HIF-1β which binds to DNA at hypoxia response elements (HRE) of target genes (Semenza et al., 1994; Semenza, 1999; Kemp and Peers, 2007). More than 200 target genes have been known to be regulated by the HIF complex, which further "turns on" factors involved in erythropoiesis, pro-inflammatory gene expression, energy metabolism or apoptosis, angiogenesis, and cellular survival and proliferation (Kaur et al., 2005; Lundgren et al., 2007; Loor and Schumacker, 2008). Age is a major risk factor for hypoxia and associated clinical outcomes. The vulnerability to hypoxia-induced diseases increases many-fold with age. It has become more evident that with age the oxygen pressure falls which along with a lower cerebral perfusion results in microglial activation and neuroinflammation (Buga et al., 2013; Popa-Wagner et al., 2014; Sandu et al., 2015). Age-related changes in HIF have also been established that have been associated with increased susceptibility toward stroke (Katschinski, 2006). Frenkel-Denkberg et al. observed a difference in DNA-binding efficiency of HIF when compared between young and old mice that were exposed to hypoxia (Frenkel-Denkberg et al., 1999). Different studies have demonstrated that the aging theories of telomere shortening and free-radical generation, both are dependent on the availability of oxygen (Nishi et al., 2004). It has been seen that by conducting aerobic exercise processes including yoga, there is an increase in the expression of brain-derived neurotrophic factor (BDNF) in the body. BDNF is an important mediator of neurogenesis as well as neuronal plasticity. It promotes growth in various areas of the brain such as the hippocampus, the dorsal root ganglions as well as cortical neurons. These levels decrease with age, however an increase in all age groups which performed aerobic exercises, in this case yoga, was observed (Pal et al., 2014). Aerobic exercises increasing blood oxygen levels and thus promoting cerebral perfusion have shown to have a direct impact in the reduction of Sympathetic Adrenal Medulla axis (SAM). Activation of SAM causes an increased release of catecholamines from the adrenal medulla. In acute scenarios, these catecholamines are of great importance. However, if chronically raised they can lead to increased vascular resistance, which in turn can lead to decreased perfusion of organs including cerebral perfusion (Arora and Bhattacharjee, 2008).

Literature indicates that cell differentiation is promoted upon hypoxia in different cell types. It has been demonstrated that molecules playing a critical role in cell differentiation such as Notch, MYC, and Oct-4 in conjunction with hypoxia and HIFs are associated with each other (Simon and Keith, 2008). However, the molecular mechanism and pathways by which hypoxia induces cell differentiation is poorly understood (Gustafsson et al., 2005; Samanta et al., 2017). It is also reported that hypoxia directly regulates the Notch signaling pathway activity in the cell differentiation process (Gustafsson et al., 2005). Additional molecules which are directly influenced by HIF-2α include OCT4 and MYC transcription factors (Dang et al., 2008; Simon and Keith, 2008). By modifying cell growth, cells respond to oxygen deprivation metabolism by activation of hypoxia induced genes. In various cell types the role of HIF1α and hypoxia has been investigated in cellular proliferation. Under hypoxic conditions Notch signaling is also increased so as to maintain progenitor/stem cell state, which is dependent on HIF-1α accumulation (Pear and Simon, 2005). Hypoxia leads to increased Notch1 signaling which further leads to differential expression of regulatory cell cycle proteins and increased proliferation of cells. However, how HIF-1α leads to the activation of these transcription factors are poorly understood and needs further investigations.

Moreover, hypoxia has also been shown to have an impact on circadian rhythms for temperature and oxygen levels. A study by Mortala et al. has demonstrated disruption in temperature rhythms in rats exposed to different levels of oxygen under 12 h light-dark (LD) cycle vs. those under constant light regimen, suggesting a connection between hypoxia and circadian rhythms (Mortola and Seifert, 2000). Likewise, it has also been noted in conditions increasing oxygen transport to the brain such as in aerobic exercise and controlled breathing exercises that there is an increase in alpha wave activity as well as a decreased galvanic skin response. Both of these parameters facilitate a more steady and consistent circadian rhythm, which in turn maintain a more steady oscillation of temperature and oxygen levels (Kumar and Joshi, 2009).

A number of studies have also demonstrated that both pre- and post-hypoxic conditioning could offer protective effects to the tissue and hence may function as a novel therapeutic strategy. Hypoxic/ischemic preconditioning is performed by exposing the tissue to hypoxia/ischemia below dangerous levels for a considerable number of times so that tissue becomes resistant to subsequent insult. This process protects the tissue from further injury. Similarly, post-hypoxic conditioning shields the tissue from damage as it develops tolerance against hypoxia through repeated inductions but is applied after the injury has occurred. The therapeutic effects and mechanisms of hypoxic preconditioning were reported in the 1960s much earlier than cerebral ischemic preconditioning was studied but were not emphasized to that extent as the latter one (Lu, 1963; Dahl and Balfour, 1964). Later, Schurr et al. studied hypoxic preconditioning and brain tolerance in rat hippocampal slices (Schurr et al., 1986). Numerous other *in vitro* models of hypoxic preconditioning have been studied such as primary neuronal cultures, hippocampal slices, olfactory cortex, and transient ischemic attacks in humans showing tolerance effects against the pathological state (Kirino et al., 1996; Perez-Pinzon et al., 1996; Arthur et al., 2004; Steiger and Hanggi, 2007; Obrenovitch, 2008; Shpargel et al., 2008; Bickler and Fahlman, 2009; Gidday et al., 2013).

Hypobaric hypoxia is another means of inducing hypoxic preconditioning and its neuroprotective potential and other beneficial health related effects have already been demonstrated (Meerson, 1984; Meerson et al., 1996; Gong et al., 2012; Millet et al., 2013; Zhen et al., 2014). Rybnikova et al. employed this technique (Samoilov et al., 2001; Rybnikova et al., 2005, 2006) to investigate its mechanisms and therapeutic potential. Recently, same group of investigators used this model in rats and found that both early and delayed applications of the post-conditioning augmented recovery and mitigated neuronal injury caused as a result of hypoxia (Rybnikova et al., 2012). Similarly, Gamdzyk et al. found neuroprotective potential of this technique in the rat model (Gamdzyk et al., 2014). Ischemic post-conditioning is also proven to protect heart and brain tissues (Zhao et al., 2003; Zhao, 2007). All these data suggest the therapeutic potential of hypoxic pre- and post-conditioning and need further investigation. Consequently, the complex nature of pathophysiology associated with hypoxia provides numerous check-points that could be targeted as therapeutic targets.

HYPOXIA/ISCHEMIA IN CNS PATHOLOGIES

The reduced blood supply also deprives the tissue from oxygen and nutrients leading to tissue damage and cell death in a prolonged hypoxic state. Under normal scenario, the body has its own mechanisms to sense oxygen deprivation and to overcome from this. However, permanent damage and pathological outcome may arise in conditions where the tissue under hypoxia is unable to reverse the deficiency. Brain, as it is well-known, has a very high metabolic demand and is completely dependent on oxygen supply for glucose metabolism. Any small disruption in oxygen supply, even for a few seconds, can cause sudden and irreversible damage in the functioning of the brain. Hypoxia in CNS tissues can initiate a series of pathophysiological events that can exert their own impact.

Furthermore, hypoxic injury has been associated with many clinical CNS anomalies including stroke, encephalopathy, ischemic retinopathies, and Alzheimer's (Peers et al., 2007; Zhang and Le, 2010). However, previous studies related to hypoxia and Alzheimer's disease (AD) are meager. With recent investigations it has come into light that the pathology of AD shares common links with hypoxia/ischemia (Peers et al., 2007; Lanteaume et al., 2016; Liu et al., 2016). Studies have demonstrated that hypoxia induces up-regulation of beta-secretase 1 (BACE1) gene expression both *in vitro* and *in vivo,* thereby contributes to the pathology of Alzheimer's disease (AD) (Sun et al., 2006). A recent finding also demonstrates that hypoxia induces epigenetic alteration in AD and suggests that it can acerbate AD progression through demethylation of genes encoding γ-secretase enzyme (Liu et al., 2016). This study revealed that hypoxia exaggerated the neurological outcome and memory dysfunction in AD mice. Patients suffering from stroke or cardiovascular disease have been shown to be more susceptible to cognitive impairment and dementia, such as Alzheimer's (de La Torre, 2008). Zhang et al. demonstrated recently that miR-124 regulates the expression of BACE1 in the hippocampus under chronic cerebral hypoperfusion (Zhang et al., 2017). In case of cerebral ischemia, the susceptibility toward AD was shown to increase (Jendroska et al., 1995; Altieri et al., 2004). Hypoxia can also induce formation of amyloid β plaques on prolonged exposure. Studies conducted in different cell lines exposed to hypoxic conditions demonstrate increase in production of amyloid regardless of the cell line being tested (Wang et al., 2006; Li L. et al., 2009; Muche et al., 2015). The mechanism behind the pathogenesis of AD being linked with hypoxia/ischemia is still unknown. Nonetheless, it has been revealed that the pathways of calcium homeostasis and calcium channel signaling are the connecting link between the AD prognosis and hypoxia (Green and Peers, 2001; Bai et al., 2015). Apart from increasing the production of amyloid β plaques, it has been shown that hypoxia also leads to other pathological hallmarks associated with AD, such as tau phosphorylation, decreasing the degradation, and clearance of plaques (Burkhart et al., 1998; Fang et al., 2010; Zhang and Le, 2010; Zhang et al., 2014).

Recently, Ashok et al. demonstrated that hypoxia-inducible factors may be neuroprotective in Alzheimer's disease (Ashok et al., 2017). Furthermore, hypoxia and ischemia activates processing of amyloid precursor protein and plays a crucial role in the pathogenesis of AD (Salminen et al., 2017). Several investigations using cellular models and *in vivo* studies have revealed that expression and activity of neprilysin (NEP), which is an Aβ-degrading enzyme, declines with age and also under hypoxic/ischemic conditions. This may result in accumulation of Aβ plaques and eventually development of AD pathology (Nalivaevaa et al., 2004; Caccamo et al., 2005; Wang et al., 2005; Fisk et al., 2007; Nalivaeva et al., 2011, 2012; Zhuravin et al., 2011). Furthermore, the levels of caspases such as caspase 3, 8, and 9 are markedly increased in human neuroblastoma NB7 cells, which can cleave APP intracellular domain (Kerridge et al., 2015). These findings indicate that NEP levels in hypoxic/ischaemic brain might be regulated by stimulation of caspases, which may result in loss of Aβ clearance and related to development of AD.

In case of encephalopathy, an obstruction in cerebral blood flow leads to oxygen/glucose shortage in an infant's brain and eventually primary energy failure (Shalak and Perlman, 2004). Simultaneously there is an increase in the production of lactate due to a switch from aerobic to anaerobic metabolism (Hanrahan et al., 1996). A cascade of reactions antecede this event such as disturbance in sodium/potassium (Na^+/K^+) pumps, Ca^{2+} influx, membrane depolarization, glutamate release, and subsequently cellular death (Johnston et al., 2009). Both apoptotic and necrotic cell death have been visualized in primary and secondary energy failure during neuronal injury with the later most prominently seen with primary energy failure (Cotten and Shankaran, 2010). Moreover, during the injury immune infiltrates penetrate the blood-brain barrier (BBB) and enhances neuronal injury but the mechanism remains elusive (Ferriero, 2004). Palmer et al. documented that neutrophils invade the BBB in the initial phase and causes brain edema (Palmer et al., 2004). Therefore, involvement of hypoxia in neuronal degeneration insists the need to explore the therapeutic targets that will target common pathological hallmarks and consequently will help in ameliorating these hypoxia-induced disease conditions.

DEVELOPMENT OF NOVEL THERAPEUTIC INTERVENTION: MIRNA IN NEUROPROTECTION

Hypoxia associated CNS anomalies, such as stroke, Alzheimer's, ischemic retinopathy, and encephalopathy are all linked with some common pathophysiological attributes that could be targeted for therapeutics. With discovery of miRNA-mediated regulation of gene expression, many studies have investigated the miRNA as interventions for human diseases, with few studies now being conducted at preclinical and clinical levels (Christopher et al., 2016).

Over the last 10 years, miRNA have been identified as the 18–25 nucleotides long non-coding RNA molecules responsible behind the translation regulation. These have been observed in organisms from Drosophila to humans (Bartel, 2004). In human genome miRNA have been shown to control various physiological and pathophysiological processes. A functional miRNA is formed in a two-step process, from a primary miRNA or pre-miRNA, which is transcribed through RNA polymerase II. It is then further processed into a hairpin structure (70–100 nucleotide long) by ribonuclease III Drosha that is complexed with RNA binding protein Pasha. Ultimately this hairpin structure is transported to cytoplasm, where it is acted upon by Dicer to form the mature 18–25 nucleotide long miRNA (Lee et al., 2003, 2004). How these miRNAs work to regulate gene expression is a complex process with still many aspects unknown. Mostly these miRNA exert their action through the formation of RNA-induced silencing complex (RISC). Typically the miRNA exert their effect by binding through RISC to the target genes at their 3′ UTR sites and regulating the levels of proteins, either by mRNA degradation, translation repression, or decapping of mRNA (Macfarlane and Murphy, 2010). These negatively regulate the post-transcriptional expression of their target genes either through blocking the translation of concerned mRNA or by degrading them (Ivan et al., 2008). Another aspect essential for the miRNA to function is the sequence complementarity between the miRNA and the mRNA, which decides the fate and mechanism for mRNA regulation. Moreover, a single miRNA can control multiple targets and vice versa, multiple miRNA molecules can collectively regulate a single target mRNA (Bartel, 2004).

miRNAs play a crucial part in development and functioning of brain (Meza-Sosa et al., 2012; Davis et al., 2015). Several brain-specific miRNA have been identified in mouse as well as humans, which include miR9, miR124a, miR124b, miR135, miR153, and others (Sempere et al., 2004). miRNAs serve as vital regulators in the central nervous system (CNS) and are implicated in various neurological diseases (Cao et al., 2016). Indeed, atypical levels of miRNA have been observed in many neurological disorders including those that are hypoxia induced. A few these miRNA that have been identified to play an important role in ischemic injury, include miRNA 15, miRNA 21, miRNA 29, miRNA 124, miRNA 145, miRNA 181, miRNA 200 family, miRNA 497, and others (Xiao et al., 2015). miRNA profiling studies in different animal models of ischemia along with human patients have been conducted, which have revealed patterns of miRNA expression at different time-points post-injury (Jeyaseelan et al., 2008; Tan K. S. et al., 2009). miRNA 210 is one that has shown conservation throughout the evolution. It is expressed in hypoxic tissues and is activated by HIF1α, a hypoxia inducible transcription factor (Chan and Loscalzo, 2010). The expression has also been revealed in middle cerebral artery occlusion (MCAO) model in rat brain as well as blood (Zeng et al., 2011).

Since the role of miRNA has been implicated in neuronal development as well as disease pathogenesis, it becomes necessary to explore the miRNA-associated regulation as a potential to develop novel clinical biomarkers and therapeutics. Recent studies have identified miRNA derivatives, anti-miRNA oligonucleotides and locked nucleic acids, as therapeutic targets to be tested in clinical scenario (Weiler et al., 2006; Love et al., 2008; Nampoothiri et al., 2016). Qiu et al. established the neuroprotective role of miRNA 210 in hypoxia-ischemia injury,

where the carotid artery in rats was permanently ligated at post-natal day 7, followed by exposure to hypoxia for 2 h. miRNA 210 is known to decrease the apoptosis of neuronal cells along with inhibition of caspases (Qiu et al., 2013). miRNA such as these can be targeted as novel therapeutics for ischemic injury. Hu et al. tested the delivery of miRNA 210 through a non-viral vector in ischemic heart injury and demonstrated a decrease in apoptosis (Hu et al., 2010). In different studies it has also been shown that it easily crosses the BBB. Moreover, investigations have also revealed miRNAs that have been implicated to confer neuroprotection in Alzheimer's disease (Liu et al., 2012; Yang et al., 2015). The development of neurodegenerative diseases is associated with the differential expression of miRNAs. Earlier investigations have revealed that ischemic stroke can trigger variations in the miRNA expression (Kocerha et al., 2009; Tan K. S. et al., 2009). A study investigating changes in miRNA profiling showed that 138 miRNAs were upregulated and 19 miRNAs were downregulated in stroke patients (Cao et al., 2012). In addition, miRNAs including hsa-let-7f, miR-126, −1259, −142-3p, -15b, -186, -519e, and -768-5p were found to be downregulated in different subtypes of stroke. Likewise, miRNAs including hsa-let-7e, miR-1184, −1246, −1261, −1275, −1285, −1290, −181a, −25*, −513a-5p, −550, −602, −665, −891a, −933, −939, and −923 showed upregulated expression in the stroke patients (Tan et al., 2013).

An interesting study showed that miRNA expression is sex-specific in ischemic stroke patients (Siegel et al., 2011). A significantly elevated expression of miR-23a was found in females whereas reduced expression was observed in males' post-ischemic event. Furthermore, upregulated expression of miR-223 confers neuroprotection in stroke patients by reducing the expression of the glutamate receptor subunits (Harraz et al., 2012). Alternatively, its decreased expression may aggravate neuronal cell death by elevating glutamate receptor subunits levels thereby indicating that it can prevent neuronal injury by regulating the expression profiles of glutamate receptor subunits. In addition, reduced expression of miR-145 may also prevent neuronal cell death by increasing superoxide dismutase-2 levels (Dharap et al., 2009). Let7f and miR1 was shown to subdue neuroprotection by controlling insulin-like growth factor 1, which protects neurons endogenously (Selvamani et al., 2012). Conversely, treatment with anti-miR1 and anti-Let7f are known to significantly reduce the infarct volume and protect neurons from cell death post-stroke. The findings of Buller et al. (2010) showed that expression of miR-21 is enhanced by three-folds following stroke *in vivo*. miR-21 overexpression inhibits apoptosis and mediates neuroprotection *in vitro*. A study reported that miR-181 expression changes in stroke, and reduced expression of miR-181 confers neuroprotection by manipulating glucose-regulated protein-78 (GRP78) (Ouyang et al., 2012). In addition, miR-124a, miR-210, miR-125b, and anti-inflammatory (miR-26a, miR-34a, miR-145, and let-7b) miRNA show altered expression profiles in stroke patients (Liu et al., 2011; Rink and Khanna, 2011; Zeng et al., 2011). In Alzheimer's disease, expression profiles of several miRNAs have been found to be altered in both the human AD tissue and diseased model (Cogswell et al., 2008; Hebert et al., 2008; Lukiw et al., 2008; Patel et al., 2008; Wang et al., 2008; Croce et al., 2013) suggesting that dysregulated miRNAs are associated with AD pathogenesis. **Table 1** summarizes different miRNA shown to regulate hypoxia/ischemia and thus, their neuroprotective potential in CNS injuries.

Moreover, the function of miRNA as a master regulator of gene expression has made it a potential for clinical translation. For instance, the first clinical trial was with miR 122 antagonists against Hepatitis C virus is under Phase II trial (www.clinicaltrials.gov/NCT01200420) (Gebert et al., 2013). Another clinical trial registered at ClinicalTrials.gov is for miR 34 in hepatic cancers (www.clinicaltrials.gov/NCT01829971). In Alzheimer's disease, a clinical trial investigating correlation between miR 107 and BACE1 levels has been initiated (www.clinicaltrials.gov/NCT01819545). Apart from these, anti-miRNA drugs have also been developed for cardiac as well as muscular disorders (Hydbring and Badalian-Very, 2013). All these clinical trials and drugs have paved way for much more clinical translation to come in near future.

Nonetheless there are some issues that need more investigations such as route of delivery, effects that can be off-target and safety and can be resolved before translation to clinics.

miRNA in Inflammation and Angiogenesis

Hypoxia/ischemia is a complex phenomenon, with multiple events that play a role in the pathogenesis including energy failure, ROS generation, inflammation, and angiogenesis. It has been seen that conditions increasing cerebral perfusion as in aerobic exercises, such as yoga, practiced over a long period of time actually reduce serum IL-6 levels, a pro-inflammatory cytokine found in the body. There was also an increased presence found of C-reactive protein, another inflammatory species in the body, in those who did not perform these aerobic exercises (Kiecolt-Glaser et al., 2010). The two pathways that can be mainly targeted through regulation of miRNA includes angiogenesis and inflammation (Ouyang et al., 2015). Bonauer et al. investigated the role of miR 92a in angiogenesis *in vitro* in endothelial cells as well as *in vivo* using a mouse model of hind-limb ischemia and revealed that overexpressing the miR 92a under ischemic conditions inhibits angiogenesis, therefore, administration of an inhibitor could enhance the blood vessel growth and assist in recovery from ischemia (Bonauer et al., 2009). miRNA-210 is another one that is known to induce angiogenesis in cerebral ischemia. Lou et al. revealed increased expression of miRNA-210 in endothelial cells in a rat model of cerebral ischemia, which was shown to be through Notch 1 signaling, thus, indicating angiogenesis. In another study, overexpressing miR-210 lead to angiogenesis as well as neurogenesis in a mouse brain. miR-210 expression in neuronal cells demonstrated their role in proliferation of endothelial cells and formation of new vascular microvessels (Lou et al., 2012; Zeng et al., 2014). miRNAs are overexpressed in endothelial cells and they have been demonstrated to modulate angiogenesis and endothelial cell function. Angiogenesis is a process in which new blood vessels emanate from preexisting vessels. It has been reported by several studies that Dicer enzyme, if inactivated may result in abnormal endothelial cell function and blood vessel formation. Dicer is an enzyme involved in

TABLE 1 | Different miRNA known to regulate hypoxia/ischemia associated genes and how these can play a neuroprotective role in CNS pathologies.

miRNA	Associated target/Role in CNS injury	Neuroprotective role	References
miR 21	Blocks Fas ligand; increased after ischemia	Increases neuronal survival post ischemia	Buller et al., 2010
miR 30a, miR 383, miR 320a	Aquaporin proteins; downregulated in ischemia	Overexpression can modulate cerebral edema	Jeyaseelan et al., 2008
miR-106	Regulates the transporter ABCA1 involved in ApoE production	In AD patients downregulated in the temporal cortex	Kim et al., 2012
miR-107	Up regulation of BACE1	Downregulated in temporal cortex of AD patients which could impact upon Aβ production	Goodall et al., 2013
miR-124	Regulates the Expression of BACE1	In the hippocampus under chronic cerebral hypoperfusion	Long et al., 2014
miR-125b	Cell cycle regulator	Glial cell and astroglial proliferation	Pogue et al., 2010
miR 126, miR 130a, miR 296, miR 424	Promote angiogenesis	Modulation can increase angiogenesis after ischemia and maintain vascular integrity	Würdinger et al., 2008; Ghosh et al., 2010; Caporali and Emanueli, 2012
miR-134	Heat-shock proteins; increased in ischemia	Downregulation can decrease apoptosis and cellular damage, improve neurological outcomes	Chi et al., 2014
miR-142 -3p	Promotes the IL-1β-dependent glutamate dysfunction	Upregulated in the CSF of MS patients and in experimentl autoimmune encephalomyelitis cerebellum	Mandolesi et al., 2017
miR-145	Targets SOD2; Upregulated after ischemia	Antagonists can increase SOD2 expression and decrease ROS	Dharap et al., 2009
miR-146a	Complement activation repressor	Altered innate immune response and neuroinflammation	Alexandrov et al., 2011
miR-146a	Transmembrane protein; regulator of βAPP cleavage	Aberrant βAPP processing and amyloidogenesis	Yanez-Mo et al., 2011
miR 181	Glucose-regulated protein 78 (GRP78); increased in ischemic injury	Reduction can increase neuronal survival	Ouyang et al., 2012
miR 155	Regulates inflammation	Regulates $CD4^+$ and $CD8^+$ T cell accumulation, NK cell maturation and expansion, T cell cytokine production, $CD8^+$ T cell-mediated cytotoxicity, astrogliosis, macrophage polarization, expression of receptors necessary for viral entry, and expression of viral proteins	Dickey et al., 2017
miR 210	Decreased in ischemic stroke	Overexpression induces angiogenesis	Fasanaro et al., 2008; Zeng et al., 2011; Lou et al., 2012; Ma et al., 2016
miR-339-5p	Regulates BACE1 expression and is most likely dysregulated in AD patients	Triggers the amyloidogenic pathway	Long et al., 2014
miR 497	Anti-apoptotic genes—Bcl2; upregulated in ischemia	Inhibition increases neurological function and decreases infarcts	Yin et al., 2010

miRNA biogenesis and functions in regulation of vascular development (Yang et al., 2005; Kuehbacher et al., 2007; Suarez et al., 2007, 2008). Yang et al. showed that mice hypomorphic for Dicer die after few days and possess abnormal blood vessel formation suggesting that Dicer is required for normal mouse development (Yang et al., 2005). Several other groups observed profound consequences resulted by knockdown of Dicer, and concluded that molecule is necessary for normal mouse development (Bernstein et al., 2003). Giraldez et al. found that there were significant abnormalities in gastrulation phase, brain development, and blood circulation in zebrafish offspring model which was deprived of both maternal and zygotic Dicer (Giraldez et al., 2005).

It has been seen that angiogenic regulators such as angiopoietin-2 receptor, Tie-1, VEGF, and its receptors VEGFR1 and VEGFR2, are differentially expressed and linked with abnormal Dicer ex1/2 embryos and yolk sacs (Yang et al., 2005; Suarez and Sessa, 2009). Several other *in vitro* studies have determined the significant role of miRNAs in endothelial cells (Otsuka et al., 2007, 2008). Knockdown of Dicer in human endothelial cells lead to the formation of defective capillary-like structures and stunted cell growth (Kuehbacher et al., 2007; Suarez et al., 2007, 2008). In addition, expression of Tie-2/TEK, VEGFR2, endothelial nitric oxide synthase, IL-8, and angiopoietin-like 4 regulator proteins is found to be altered in Dicer silenced endothelial cells (Suarez et al., 2007). Interetingly, the expression of thrombospondin-1 remained elevated in these cells (Kuehbacher et al., 2007; Suarez et al., 2008).

Numerous *in vivo* studies demonstrated the crucial role of Dicer in postnatal angiogenesis. Findings of Kuehbacher et al. (2007) showed sprout formation was abridged when nude mice was subcutaneously injected for Dicer silencing in HUVECs (suspended in a Matrigel plug). Similarly, Suarez et al. (2008) generated two Dicer knockout mouse cell lines, which were endothelial specific (Suarez et al., 2008). These findings suggest that the role of miRNA in modulation of

distinct aspects of angiogenesis might prove to be worthwhile in many human pathological diseases, especially those involving the vasculature.

Inflammation is an important impact as well as a cause in ischemia-reperfusion injury. Eisenhardt et al. in their study investigated the role of miRNA-155 in a mouse model of myocardial ischemia-reperfusion and revealed its involvement in exacerbation of inflammation as well as ROS generation post-injury (Eisenhardt et al., 2015). miRNAs play a crucial role in regulating both innate and adaptive immunity (Lu et al., 2009; Dalal and Kwon, 2010). Innate immunity is the first line of defense and protects the organism from invasion of foreign pathogens. On the contrary, adaptive immune system is highly specific to a particular antigen and elicits B and T lymphocytes to combat microbes. The former immune response neutralizes the effect of pathogens through the pathogen associated molecular patterns (PAMPs), recognition process by the toll-like receptors, or TLRs (Takeda and Akira, 2015). TLRs are transmembrane proteins expressed by cells participating in innate immune system, which recognize invading microbes and induce downstream signaling pathways that triggers immune responses to kill foreign pathogens.

It is well-documented that miRNAs are implicated in regulating inflammation and immune responses. A number of miRNAs have been induced by lipopolysaccharide (LPS) mediated TLR signaling such as miR-146a, miR-155, and miR-132 (Taganov et al., 2006). Studies have shown that cytokines like interleukin-1β, LPS, and tumor necrosis factor (TNF-α) stimulates the expression of miR-146a. Consecutively, miR-146a, suppresses the expression of IL-1 receptor associated kinase and TNF receptor-associated factor-6, components of the TLR4 signaling pathway (Taganov et al., 2006). Hence miR-146a negatively controls TLR signaling pathway. Similarly, interferon-β, and LPS induces the expression of miR-155 in murine macrophages (O'connell et al., 2007; Tili et al., 2007). Once miR-155 are activated, cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are triggered (Tili et al., 2007). miR-155 regulates suppressor of cytokine signaling (SOCS)-1 demonstrating its involvement in regulation of innate immune system (Rodriguez et al., 2007; Tili et al., 2007; Lu et al., 2009). Ceppi et al. showed that silencing of miR-155 in dendritic cells derived from human myeloid cells significantly elevated protein levels of the pro-inflammatory cytokine interleukin1 (IL-1) (Ceppi et al., 2009). In addition, miR-155 knockdown in these antigen-presenting cells directly suppressed pro-inflammatory TAK1-binding protein 2 levels, indicating its anti-inflammatory property (Ceppi et al., 2009). On the contrary, several studies have reported that miR-155 can augment inflammation. Overproduction of miR-155 in mouse bone marrow results in a myeloproliferative phenotype (O'connell et al., 2007).

While the expression of both miR-146 and miR-155 is elevated in macrophages in response to LPS stimulation, the expression of miR-125b is decreased. Tili and colleagues showed that reduced expression of miR-125b results in increased expression of TNF-α and eventually augments inflammation (Tili et al., 2007). Several other miRNAs such as miR-9, miR-21, and miR-147 are induced by TLR signaling and are expressed considerably and hence regulate immune responses in response to microbial invasion (Bazzoni et al., 2009; Liu et al., 2009; Sheedy et al., 2010).

Similarly, miRNAs are implicated in adaptive immune system by regulating the development of both T cell and B cell lymphocytes. Numerous studies have shown the involvement of miRNAs in controlling signaling networks in T cells (Monticelli et al., 2005; Wu et al., 2007; Merkerova et al., 2008). Wu et al. demonstrated that some overexpressed miRNAs are dynamically regulated during antigen-specific T-cell differentiation when miRNA profiling was performed in naive, effector and memory CD8+ T cells (Wu et al., 2007). Other studies showed that there were abnormally developed and fewer mature T-cells in Dicer deficient mice as compared to normal mice (Cobb et al., 2005; Muljo et al., 2005).

Reports have revealed the involvement of both miR-17 and miR-92 in T cell development (Xiao et al., 2008). Lately, it has been reported that miRNAs are also involved in the differentiation of T cells into distinct effector T helper cells. Du et al. reported that miR-326 regulates differentiation of TH17 cells both *in vitro* and *in vivo* (Du et al., 2009). Other miRNAs such as miR-181a can facilitate the intensity of T cell receptor signaling (Li et al., 2007) whereas miR-155 is implicated in regulatory T (Treg) cell formation and function (Zheng et al., 2007; Chong et al., 2008; Liston et al., 2008; Zhou et al., 2008). Furthermore, role of several miRNAs such as miR-150, miR-155, and miR-34a has also been described in humoral immunity involving B-lymphocytes (Chen et al., 2004; Monticelli et al., 2005; Vigorito et al., 2007; Xiao et al., 2007; Zhou et al., 2007; Basso et al., 2009; Tan L. P. et al., 2009; Xiao and Rajewsky, 2009; Rao et al., 2010).

Studies have indicated toward miRs that could regulate the immunological processes as well as nervous system, which have been termed as NeurimmiRs. NeurimmiRs such as miR 132 and miR 124 have shown to play a role in cross-talk between the two, at both local and peripheral levels. Furthermore, investigating such miRs that regulate the neuroimmunological processes could lead to exploring their potential as therapeutics (Soreq and Wolf, 2011).

miRNA and Circadian Rhythms in Hypoxia/Ischemia

Superchiasmatic nucleus (SCN) located in the anterior hypothalamus of a mammalian brain is the central switch for circadian rhythms. Apart from SCN, it has been demonstrated that each cell in the body has its own clock which controls the molecular and cellular functions. The proteins associated with the molecular clock include, Brain, Muscle ARNT-Like protein 1 (Bmal1), Period (Per1, Per2), Cryptochrome (Cry), and Circadian Locomotor Output Cycles Kaput (CLOCK) (Shearman et al., 1997; Zylka et al., 1998). All these proteins belong to the bHLH-PAS protein family which is characterized by the presence of basic helix-loop-helix domain. The members of this protein family are known to play an important role in response to low oxygen as well as diurnal changes in light. Another protein that

belong to this same family includes HIF1α that is involved in the maintenance of oxygen homeostasis. Another new proteins have been recently identified, MOP3 and MOP9, which are associated with both the pathways, i.e., circadian and hypoxia (Bunger et al., 2000; Hogenesch et al., 2000).

The variables that display circadian patterns include body temperature, metabolic rate etc. Any condition that causes prolonged hypoxia ultimately disturbs the oscillations of these essential parameters and their associated body functions. Under normal physiological conditions, these events follow well-defined oscillations which help in maintenance of homeostasis. Studies have demonstrated the existence of a cross-talk between these two pathways (Ghorbel et al., 2003). Chilov et al. in their study observed increase in the level of PER1 and CLOCK along with co-immunoprecipitation of HIF1α with PER1 under hypoxia in mice (Chilov et al., 2001).

Circadian rhythms have also been associated with stroke occurrence in humans with the temporal and seasonal pattern of occurrence (Marler et al., 1989; Argentino et al., 1990; Elliott, 1998; Raj et al., 2015). Higher risk for hemorrhagic stroke has been reported in evening (Elliott, 1998). In another study, higher frequency of ischemic stroke was noted in 6 a.m. to noon quarter as compared to other quarters of the day (Argentino et al., 1990). These studies show that the circadian variation in time of occurrence can depend on the sub-type, which can also help in planning therapeutics.

miRNA regulation of circadian rhythms has also been implicated through different studies (Chen and Rosbash, 2016; Gao et al., 2016). It binds to 3′ UTR region of the target genes and control different processes such as its mRNA stability and/or translation. The miRNA that control the circadian rhythms, such as miR219, miR132, also show rhythmic expression in SCN. Cheng et al. in their study have demonstrated increase in rhythmicity as an outcome of administration of antagonists against these miRs (Cheng et al., 2007). In another study by Shende et al. investigated the miRNAs that target the clock gene, Bmal1, in mouse which were exposed to 12 h LD cycles. The authors identified miR 152, miR 494, which showed bimodal pattern of expression, following diurnal oscillations along with the levels of Bmal1. These miRs were also shown to interact with Clock (Shende et al., 2011).

Many physiological features, especially cardiovascular, follow defined circadian cycles. Therefore, the circulating levels of miRNA play an important role in circadian control associated with different pathology. The different identified miRNAs can also act as targets as well as biomarkers for any disturbances from normal physiology. The knowledge about the miRNA involved in the clock genes is still limited and needs further detailed investigations for their translation to clinics.

OVERCOMING HYPOXIA WITH YOGA BASED ALTERNATE THERAPIES

With increasing evidence in the past years, Yoga is gaining recognition as a therapeutic intervention in the management of non-communicable diseases like hypertension, coronary artery disease (Cramer et al., 2014), diabetes (Innes and Vincent, 2007), obesity, back pain (Cramer et al., 2013), and also as an adjunct in the management of cancer. The reduction in respiratory rate, heart rate, vagal predominance as a general response following yoga practices possess a considerable role in managing hypoxia and its associated pathophysiological responses. This understanding might be beneficial in its application as a targeted therapy. While meditation's any effect on Alzheimer disease (AD) is hypothetical, meditation has received significant consideration as a tool that possibly will have positive medical and psychological benefits. Studies indicate improvement in cognitive functions in elders with dementia (Oken et al., 2006). Kirtan Kriya (KK), a type of meditation was also investigated in subjects with memory impairment, Newberg et al. demonstrated positive effects of KK on cerebral blood flow and cognitive function. A difference in the activation of the anterior cingulate gyrus and prefrontal cortex, was seen in the subjects who practiced KK (Newberg et al., 2010). Neuroimaging studies following Yoga practices in individuals with mild cognitive impairment suggest increased memory performance. Enhanced memory performance was correlated with increased neuronal connectivity between default mode networks and frontal medial cortex, pregenual anterior cingulate cortex, right middle frontal cortex, posterior cingulate cortex, and left lateral occipital cortex suggesting that yoga might be helpful in enhancing memory recall, specifically visual memory encoding (Eyre et al., 2016). Meditation improves sleep and sleep problem is also a risk factor for AD (Devore et al., 2014; Innes and Selfe, 2014). Froeliger et al. revealed that upon conducting controlled breathing exercises such as pranayama and meditation, there was a significant decrease in cognitive failures as evaluated by Voxel-Based Morphometric analysis, which showed a positive co-relation with increased GMV (Gray matter volume) in the regions of cerebellar, temporal, occipital, limbic, and frontal lobe of the brain. There was also a positive correlation seen with the amount of increase in GMV and the duration of practice of the breathing exercises and meditation (Froeliger et al., 2012). Long term meditation practice is associated with larger overall gray matter volume and increased regional enlargement of areas associated with sustained attention, introspection, and autonomic function surveillance, indicating the possible role of Yoga practices in facilitating neuroplasticity (Hernández et al., 2016). It has also been seen in studies that upon activities that increase cerebral perfusion as well as increase oxygen inhalation, such as performing yogic asanas (yogic poses) and pranayama (yogic breathing exercises), there is an increase in the amplitude in the P3 wave evoked potential (Tripathi and Bharadwaj, 2013). This escalation in P3 amplitude means that there is an increased neuronal pool recruitment, which can be seen as increased attention as well as memory updating. The study revealed that those who took part in activities which, increased cerebral perfusion showed a significantly improved functioning in comparison to the control group which was only on oral Alzheimer medications. Sutton et al. first of all identified the P3 wave in 1965 and since then it has been used extensively in the area of event-related potentials (ERPs) (Sutton et al., 1965). ERPs are electrophysiological responses

produced in the brain as a result of specific events or stimuli (Blackwood and Muir, 1990). The latency range for most adults for auditory stimuli is 250–400 ms. The latency is generally explained on the basis of the speed of stimulus of one event from another. Shorter latencies signify exceptional mental execution compared to longer ones. The P300 can be employed as a diagnostic tool to discriminate dementias of different origin and can be elicited by a wide variety of sensory or motor events. In addition, P300 is used to evaluate and monitor Alzheimer's disease (AD). Lengthening of latency is the most pronounced variation in the auditory modality in AD and other cortical dementias, which is related to debilitated memory (Polich et al., 1990). Latency lengthening is also observed in AD with visual modality stimulation and in the auditory modality in healthy individuals with increased risk of having AD. This may be used in early monitoring for this disease (Saito et al., 2001). In related investigation by Caravaglios et al. it was revealed that P300 latency was higher in AD elderly subjects as compared to controls (Caravaglios et al., 2008). A similar study found significant differences in P300 latency between AD subjects and control (O'mahony et al., 1996). Lai et al. determined P300 latency as well as amplitude in AD group, group with mild cognitive impairment, and the control group (Lai et al., 2010). The authors found that P300 latency was higher in AD group followed by those with cognitive involvement as compared to controls, while no difference in P300 amplitude was observed among the three groups. Similarly in another study Yamaguchi et al. compared P300 latency and amplitude in Alzheimers' with vascular dementia and the controls and found that both the diseased groups had a higher latency as compared to the control group (Yamaguchi et al., 2000). Several other studies evaluated P300 latency and its subcomponents, called P3a and P3b in AD patients and compared them with controls and found significantly higher latency in patients compared to controls (Frodl et al., 2002; Bennys et al., 2007; Juckel et al., 2008). Telles et al. assessed whether practicing alternate nostril yoga breathing (*nadisuddhi pranayama*) has any influence on P300 auditory evoked potentials and found a dramatic elevation in the P300 peak amplitudes suggesting that it unequivocally affects intellectual processes (Telles et al., 2013; Mccaffrey et al., 2014), conducted a study and found the possibility of older adults with AD to complete the Sit "N" Fit Chair Yoga Program with positive changes across each physical process (Mccaffrey et al., 2014). In healthy individuals, controlled breathing at a rate of 5.5 breaths per minute evoked activity at the brainstem, across the dorsal length of pons, in hypothalamus, hippocampus, lateral cortices and regions of striatum. Interestingly, studies on meditation techniques (Cyclic Meditation and Transcendental Meditation) in healthy regular practitioners demonstrated hypometabolic states with significant reductions in oxygen consumption (Wallace, 1970; Telles et al., 2013). Long-term Yoga practices are independently associated with decreased chemoreflex hypoxic and hypercapnic responses and better baroreflex sensitivity (Spicuzza et al., 2000). This might be achieved as a result of adaptation of peripheral and central chemoreceptors and pulmonary stretch receptors to the regular practice of slow breathing during yoga practices, followed by a decrease in the vagal afferent discharge to the bulbopontine centers. Hypometabolic state in yoga practitioners could enable tolerating hypoxic conditions. Another study designed to understand the efficacy of Yoga practices in promoting physiological recovery showed that Yoga practitioners with 2 years of experience practicing yoga atleast twice a week produced 41% less lipopolysaccharide stimulated IL-6 in response to the laboratory stressor than novices (Kiecolt-Glaser et al., 2010). Investigations are required to understand the regulatory role of Yoga on miRNA. However, based on the literature available, we speculate that miRNA associated in the process of inflammation, angiogenesis and stress response might be differentially regulated in long term practitioners, whereas, the same is expected be the process through which the therapeutic effects are moderated by Yoga. Therefore, Yoga practices might be a promising tool to understand the inherent ability of the body in managing hypoxia induced CNS injuries and also as a possible therapeutic tool.

FUTURE DIRECTIONS

With advancement in awareness about the profiling and expression patterns of different miRNA associated with hypoxia/ischemia-induced CNS injuries, it has led to translation of this information in clinical set-ups. The miRNA levels could now be utilized as clinical biomarkers for ischemia. Moreover, since a single miRNA could regulate a whole network with multiple targets, controlling the levels of individual miRNA species could assist in designing therapeutics for complex diseases. Unlike other exercises, Yoga practices appear to be unique and the present day findings indicate that yoga can restore or maintain homeostasis. However, the underlying mechanisms need to be understood in detail. Further investigations pertaining to miRNAs linked with ischemic injuries and the role of Yoga practices in regulating them could benefit in management and therapy for CNS pathologies in future.

AUTHOR CONTRIBUTIONS

GM wrote the manuscript and contributed in writing content related to hypoxia in CNS pathologies, miRNA in neuroprotection, and circadian rhythms in hypoxia etc.; DM contributed in writing content related to hypoxia pathophysiological responses and the role of miRNA in inflammation, angiogenesis, and neuroprotection; BR contributed content for the Yoga based therapeutics; VP added yoga related information in the manuscript; NS added information related to correlation between cerebrovasculature and AD pathogenesis, yoga related studies, and hypoxia in promoting cell differentiation; AA conceptualized, designed, and edited the manuscript.

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Sinomenine Provides Neuroprotection in Model of Traumatic Brain Injury via the Nrf2–ARE Pathway

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The neuroprotective effect of sinomenine (SIN) has been demonstrated in several brain injury models. However, its role and molecular mechanism in traumatic brain injury (TBI) remain unknown. In this study, we investigated the neuroprotective effects of SIN in the weight-drop model of TBI in male ICR mice. Mice were randomly divided into the sham and TBI groups, SIN (10 mg/kg, 30 mg/kg and 50 mg/kg, administered intraperitoneally) or equal volume of vehicle was given at 30 min after TBI. Treatment with 30 mg/kg SIN significantly improved motor performance and alleviated cerebral edema. However, treatment with 10 mg/kg or 50 mg/kg SIN did not exhibit a better outcome. Therefore, we chose 30 mg/kg SIN for our subsequent experiments. SIN significantly increased the expression of Bcl-2 and decreased that of cleaved caspase-3, indicating that SIN is anti-apoptotic. This was confirmed by the observation that SIN-treated animals had fewer apoptotic neurons. Cortical malondialdehyde content, glutathione peroxidase (GPx) activity and superoxide dismutase (SOD) activity were restored in the group that received SIN. Furthermore, Western blot and immunofluorescence experiments showed that SIN enhanced the translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) to the nucleus. SIN administration also significantly upregulated the expression of the downstream factors heme oxygenase 1 and NAD(P)H:quinone oxidoreductase 1 at pre- and post-transcriptional levels. Together, these data demonstrate that SIN exerts a neuroprotective effect in a model of TBI, possibly by activating the Nrf2–antioxidant response element (ARE) pathway.

Keywords: traumatic brain injury, nuclear factor erythroid 2-related factor 2, sinomenine, neuroprotection, reactive oxygen species

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Abbreviations: TBI, traumatic brain injury; Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; SIN, sinomenine; NQO1, NAD(P)H:quinone oxidoreductase 1; HO1, heme oxygenase 1; MDA, malondialdehyde; GPx, glutathione peroxidase; SOD, superoxide dismutase; ROS, reactive oxygen species; RT-PCR, real-time quantitative polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Bcl-2, B-cell lymphoma 2; CNS, central nervous system; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffer solution; PVDF, polyvinylidene fluoride; DAPI, 4′,6-diamidino-2-phenylindole; i.p., intraperitoneally.

INTRODUCTION

Traumatic brain injury (TBI) remains a major public health problem in modern society, leading to high medical costs, morbidity and mortality (Zipper and Mulcahy, 2003; Tsai et al., 2013). The pathological process of TBI involves primary and secondary injury. After a primary brain insult, a complex series of endogenous events are triggered, including oxidative stress, glutamate excitotoxicity, activation of the inflammatory response, loss of ionic homeostasis, and increased vascular permeability (Werner and Engelhard, 2007; Bell et al., 2009; Cornelius et al., 2013), leading to further neuronal degeneration and apoptosis. These subsequent pathological events are referred to as secondary brain injury. Despite the efforts focused on seeking effective ways to alleviate the secondary injury, to date, most approaches to the treatment of TBI targeting a single injury mechanism have failed in clinical trials (Sun et al., 2015).

Oxidative stress plays an important role in secondary injury (Bains and Hall, 2012). It occurs when the production of reactive oxygen species (ROS) exceeds the cell ability to detoxify. The excessive production of ROS damages cellular components including lipids, proteins, and DNA, leading to a decline in physiological function and cell death (Ansari et al., 2008; Adibhatla and Hatcher, 2010).

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is a key protein in the reduction of oxidative stress (Yan et al., 2008). Under normal conditions, kelch ECH associating protein 1 (KEAP1), a cytosolic repressor protein that binds to Nrf2, retains Nrf2 in the cytosol and facilitates its proteasomal degradation. Once the cell encounters stimulations such as oxidative stress, Nrf2 dissociates from KEAP1 and translocates into the nucleus (de Vries et al., 2008). By binding to the antioxidant response element (ARE), Nrf2 induces the production of a battery of endogenous enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), nicotinamide adenine dinucleotide phosphate, quinine oxidoreductase-1 (NQO1), and heme oxygenase-1 (HO-1). Together, these free radical scavenging enzymes represent a powerful antioxidant defense mechanism (de Vries et al., 2008; Ma, 2013). The Nrf2-ARE signaling pathway is activated in several central nervous system (CNS) diseases, including TBI (Wang et al., 2007; Chen et al., 2011), and is considered a protective molecular mechanism against TBI (Yan et al., 2009).

Sinomenine (7,8-didehydro-4-hydroxy3,7-dimethoxy-17-methyl-9α, 13α, 14α-morphinan-6-one; SIN) is an active alkaloid isolated from the Chinese medicinal herb *Sinomeniumacutum*, and is used in China for the clinical treatment of rheumatoid arthritis and mesangial proliferative nephritis (Xu et al., 2008; Cheng et al., 2013). It has a variety of pharmacological properties including immunosuppression, anti-inflammation and cytoprotection (Qian et al., 2007; Cheng et al., 2009; Wang and Li, 2011). SIN exerts neuroprotection in several CNS disease models, including cerebral ischemia (Wu et al., 2011), intracerebral hemorrhage (Yang et al., 2014), and neurodegenerative diseases (Qian et al., 2007). However, few studies have addressed the neuroprotective effect of SIN in TBI. Therefore, the purpose of the present study was to determine whether SIN administration after TBI could attenuate brain injury in a TBI model.

MATERIALS AND METHODS

Animals

Male ICR mice weighing 28–32 g were obtained from the Animal Center of Jinling Hospital. The animals were housed in a controlled environment with a reversed 12/12 h light/dark cycle and free access to food and water and were acclimatized for at least 4 days before any experiment. All procedures involving animals were approved by the Animal Care and Use Committee of Southern Medical University, Guangzhou, China, and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Model of TBI

The model of TBI used in the present study was based on Marmarou's weight-drop model with some modifications as previously described by Flierl et al. (Flierl et al., 2009; Zhang et al., 2016). Mice were anesthetized in a closed container on a wire mesh over ether-soaked cotton and then placed onto the platform directly under the weight of the weight-drop device.

The impact area (left anterior frontal region, 1.5 mm lateral to the midline on the midcoronal plane) was located, and a 200 g weight was released and dropped onto the skull from a height of 2.5 cm. Mortality rate from apnea was reduced by early respiratory support. The scalp wound was closed with standard suture material. After recovering from anesthesia, mice were returned to their cages with food and water provided *ad libitum*. Sham-injured animals underwent the same procedure except for the weight drop.

A total of 214 mice were used in our experiment. No animals died in the sham-injured group and sham + SIN group. Ten (TBI, $n = 3$; TBI + vehicle, $n = 3$; TBI + 10 mg/kg SIN, $n = 1$; TBI + 30 mg/kg SIN, $n = 2$; TBI + 50 mg/kg SIN, $n = 1$) TBI mice died before sacrifice, and the mortality rate was 6.7%.

Experimental Groups and Drug Treatment

Animals were randomly assigned to the following groups: (1) sham ($n = 42$); (2) sham + SIN (50 mg/kg i.p.) ($n = 12$); (3) TBI ($n = 42$); (4) TBI + vehicle ($n = 42$); (5) TBI + SIN (10 mg/kg i.p.) ($n = 12$); (6) TBI + SIN (30 mg/kg i.p.) ($n = 42$) and (7) TBI + SIN (50 mg/kg i.p.) ($n = 12$). SIN was purchased from Sigma-Aldrich (St. Louis, MO) and freshly prepared in saline containing 1% dimethylsulfoxide (DMSO) just before injection. Animals in the TBI + SIN and TBI + vehicle groups received SIN or equal volumes of 1% DMSO, respectively, 30 min after TBI. Animals in the sham + SIN and sham + vehicle groups received SIN or vehicle intraperitoneally 30 min after surgery. The mice were sacrificed 24 h after TBI for biochemical and histological analyses. The doses used in this study were based on a study of neuroprotection of

SIN in a middle cerebral artery occlusion model (Wu et al., 2011).

Neurological Evaluation

Neurological function was evaluated by the grip test which was developed on the basis of the test of gross vestibulomotor function as described elsewhere (Bermpohl et al., 2006; Xu et al., 2014). Briefly, mice were placed on a thin, horizontal, metal wire (45 cm long) that was suspended between two vertical poles 45 cm above a foam pad and were allowed to traverse the wire for 60 s. The latency that a mouse remained on the wire within a 60-s interval was measured, and wire grip scores were quantitated using a 5-point scale (**Table 1**). The grip test was performed in triplicate, and a total value was calculated for each mouse. The test was performed by an investigator who was blinded to experimental grouping.

Brain Water Content

Brain water content was measured according to a previous study (Xu et al., 2013). In brief, the brain was removed and placed on a cooled brain matrix 24 h after TBI. The brainstem and cerebellum were removed, and the remaining ipsilateral tissue was weighed immediately to obtain the wet weight (ww). Then the hemisphere was dried at 80°C for 72 h and weighed to obtain the dry weight (dw). We calculated water content as a percentage using the following formula: (ww − dw)/ww × 100%.

Tissue Processing

For Western blot and real-time quantitative polymerase chain reaction (RT-PCR) analysis, animals were anesthetized with chloral hydrate 24 h after TBI and perfused through the left cardiac ventricle with 0.9% normal saline solution at 4°C. Ipsilateral cerebral cortex tissue 3 mm from the margin of the contusion site was dissected on ice, immediately frozen in liquid nitrogen, and stored at −80°C until use. For immunofluorescence staining and terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nickend labeling (TUNEL), after being deeply anesthetized with chloral hydrate 24 h after TBI, animals were perfused with 0.9% normal saline solution followed by 4% buffered paraformaldehyde, and the brains were immersed in 4% buffered paraformaldehyde overnight (all at 4°C).

TABLE 1 | Behavior scores.

Behavior	Points
Unable to remain on the wire for less than 30 s	0
Failed to hold on to the wire with both fore paws and hind paws together	1
Held on to the wire with both fore paws and hind paws but not the tail	2
Used its tail along with both fore paws and both hind paws	3
Moved along the wire on all four paws plus tail	4
Scored four points also ambulated down one of the posts used to support the wire	5

Determination of Malondialdehyde (MDA), Superoxide Dismutase (SOD), and Glutathione Peroxidase (GPx)

Tissue samples were homogenized in 2 ml of phosphate buffer (10 mM; pH 7.4). After centrifugation at 12,000 rpm for 15 min at 4°C, MDA, SOD, and GPx content in the supernatant was measured using a spectrophotometer (Nanjing Jiancheng Biochemistry Co., Nanjing, China) according to the manufacturer's instructions. Protein concentrations were determined using the Bradford method. MDA content was expressed as nmol/mg protein, and SOD and GPx activity were expressed as U/mg protein.

Tunel Analysis

Apoptosis was determined using TUNEL, and included analysis of DNA fragmentation assays based on 3H-thymidine and 5-bromo-2-deoxyuridine (In Situ Cell Death Detection Kit, TMR red; Sigma-Aldrich), according to the manufacturer's instructions. The slides were then washed with PBST three times for 30 min prior counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) for 15 min. After three more washes, the slides were coverslipped with anti-fade mounting medium for further study. The TUNEL-positive cells were counted by an observer who was blind to the experimental groups. To evaluate the extent of cell apoptosis, the apoptotic index was defined as the average number of TUNEL-positive cells in each section counted in six microscopic fields.

Total/Nuclear Protein Extraction and Western Blot Analysis

Proteins were extracted using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotech Inc., Nantong, China) according to the kit instructions, and protein concentrations were determined using the Bradford method. Equal amounts of protein per lane (50 μg) were separated by 10 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene-difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated in blocking buffer (Tris buffered saline/0.05% Tween 20 [TBST] containing 5% skim milk) for 2 h at room temperature, then overnight at 4°C with primary antibodies (all raised in rabbit), as follows: anti-Nrf2, anti-HO-1 and anti-NQO-1 (1:1000, all from Abcam, Cambridge, MA, USA), anti-Bcl-2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cleaved caspase-3 and anti-Histone 3 (1:1000, Cell Signaling Technology, Beverly, MA, USA), anti-β-actin (1:5000, Bioworld Technology, St. Louis Park, MN, USA). After three 10 min washes with TBST, the membranes were incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (1:5000, Bioworld Technology) for 2 h at room temperature. Protein bands were visualized by enhanced chemiluminescence western blot detection reagents (Millipore) and quantification was performed by optical density methods using ImageJ software (NIH). Proteins of interest were normalized to β-actin or histone 3.

qRT-PCR

Total RNA was extracted from the ipsilateral cortex with RNAiso Plus (Takara Bio, Dalian, China). The concentration and purity of total RNA were determined with a spectrophotometer (OD260/280 1.8–2.0) and 1% agarose gel electrophoresis. To avoid RNA degradation, some of the RNA was immediately reverse-transcribed to cDNA using the PrimeScript RT reagent kit (Takara Bio); surplus RNA was kept at 80 °C. The primers were designed according to PubMed GenBank and synthesized by Invitrogen Life Technologies (Shanghai, China). The sequences were as follows: NQO1: F, 5′-CATTCTGAAAGGCTGGTTTGA-3′; R, 5′-CTAGCTTTG ATCTGGTT-GTCAG-3′; OH-1: F, 5′-ATCGTGCTCGCAT GAACACT-3′; R, 5′-CCAACACTGC-ATTTACATGGC-3′; β-actin: F, 5′-AGTGTGACG-TTGACATCCGTA-3′; R, 5′-G C-CAGAGCAGTAATCTCCTTCT-3′. qRT-PCR analysis was performed using the Mx3000P System (Stratagene, San Diego, CA, USA), applying real-time SYBR Green PCR technology. All samples were analyzed in triplicate. β-actin was used as an endogenous reference "housekeeping" gene.

Immunofluorescence for Nrf2

Cryostat frozen sections (8 μm thick) were mounted on gelatin-coated slides, which were warmed at room temperature for 30 min. Slides were washed three times in PBS for 10 min each time before immunofluorescence staining. Based on the established immunostaining protocol, slides were incubated in blocking buffer (10% normal goat serum in PBS containing 0.1% Triton X-100) for 2 h followed by overnight incubation at 4°C with rabbit anti-Nrf2 (1:100, Abcam) and anti-NeuN (1:100, Millipore). The next day, after three more 5 min washes in PBS, the slides were incubated with appropriate secondary antibodies (Alexa Fluor 488, 1:200) for 2 h at room temperature. The slides were washed three times in PBS, counterstained with DAPI for 2 min, rinsed with PBS, and coverslipped with mounting medium. Fluorescence microscopy imaging was performed using a Zeiss HB050 inverted microscope system and handled by Image-Pro Plus 6.0 software (Media Cybernetics, USA) and Adobe Photoshop CS5 (Adobe Systems, USA). The specificity of the immunofluorescence reaction was confirmed using a negative control in which the primary antibody was replaced with PBS. Six random fields of vision (200×) were chosen for each coronal section. Four sections from each animal were used for quantification. The final average number of positive cell in the four sections was used as the data for each sample. Data are presented as the mean fluorescence intensity per 200× magnification field. The entire process was conducted by two pathologists blinded to the grouping.

Statistical Analysis

All data used for statistical analysis are expressed as the mean ± SEM. One-way ANOVA and Tukey's test were used to analyze differences between groups except for the neurobehavioral scores, which were analyzed using nonparametric tests (Kruskal–Wallis followed by Dunn's test). SPSS 20.0 was used for statistical analysis (IBM Corp., Armonk, NY, USA). Statistical significance was inferred at $P < 0.05$.

RESULTS

SIN Improves Recovery of Motor Performance and Alleviates Cerebral Edema after TBI

To examine whether SIN provides neuroprotection after TBI, animals were grouped as follows: (1) sham; (2) sham + SIN (50 mg/kg); (3) TBI; (4) TBI + vehicle; (5) TBI + SIN (10 mg/kg); (6) TBI + SIN (30 mg/kg) and (7) TBI + SIN (50 mg/kg). Motor performance was evaluated using the grip test 1 day, 3 and 7 days after TBI. All animals were trained on the task 24 h before TBI. The sham group showed no difference between different time points and there was no difference between the TBI group and the vehicle-treated group. All groups exhibited an improved motor performance over time after TBI. Within 3 days after TBI, the performance of the groups that received SIN was significantly better than those that received vehicle. Larger doses such as 50 mg/kg, however, did not exhibit a better outcome than 30 mg/kg ($P > 0.05$; **Figure 1A**).

We then measured brain water content to confirm the neuroprotective effect of SIN. Brain water content after TBI was markedly lower in all three SIN-treated groups than in animals that received vehicle ($P < 0.01$, $P < 0.001$, and $P < 0.01$, for 10, 30, and 50 mg/kg, respectively; **Figure 1B**). Consistent with the grip test, the 30 mg/kg dose had a slightly, but not significantly, greater effect in reducing TBI-induced brain edema than the other doses ($P > 0.05$; **Figure 1B**). These data confirm that SIN is neuroprotective against TBI, 30 mg/kg provided better recovery when comparing the results of these two tests, maybe in further tests other doses would have different dynamics. Therefore, we used this dose in the subsequent studies.

Sinomenine Administration Attenuated Neuronal Apoptosis in the Brain after TBI

To determine whether the neuroprotective effects of SIN can be detected at a histopathological level, NeuN/TUNEL double immunofluorescence staining was performed to evaluate neuronal apoptosis. The total number of TUNEL and NeuN double-stained cells was significantly greater in the TBI and TBI + vehicle groups 24 h after TBI (**Figures 2A,B**). However, the number of TUNEL-positive neurons was reduced after treatment with SIN. To investigate the effect of SIN on neuronal apoptosis, we have measured expression of the apoptosis-related proteins Bcl-2 and the level of cleaved caspase-3. Bcl-2 is a major anti-apoptotic member of the Bcl-2 family, and protects cells against a variety of insults such as exposure to calcium ionophores, glutamate, free radicals and withdrawal of trophic factors (Reed, 1998; Strauss et al., 2004). Cleaved caspase-3 is an essential component of the apoptotic machinery in many cell types (Yuan and Yankner, 2000; Engel et al., 2011).

Bcl-2 expression was lower in the TBI and TBI + vehicle groups than in the sham group 24 h after TBI, but elevated after SIN administration (**Figure 2C**). Content of cleaved caspase-3 was elevated in the TBI and TBI + vehicle groups and reduced

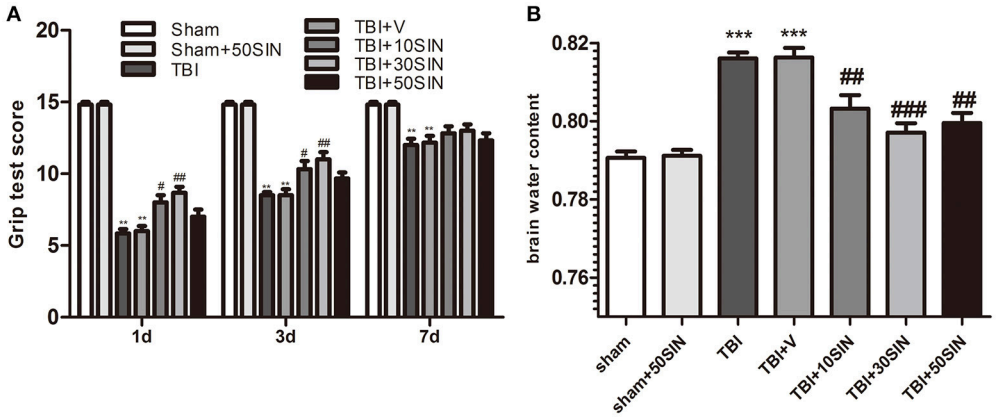

FIGURE 1 | SIN protects against secondary brain injury in a mice model of TBI. Mice were subjected to TBI and then received 10, 30, and 50 mg/kg of SIN or vehicle (dimethyl sulfoxide) 30 min later. Grip test score were examined at 1 day, 3 days, and 7 days after TBI while brain water content were evaluated at 1 day after TBI. **(A)** Within 3 days after TBI, performance of the 10 and 30 mg/kg SIN-treated groups was significantly better than in the vehicle group; however, there was no significant difference between the 50 mg/kg group and the vehicle group. This effect was no longer significant 7 days after TBI between all groups treated with different doses of SIN and the vehicle-treated group. $n = 6$ per group. **(B)** Brain water content was significantly greater in the TBI and TBI + vehicle groups than in the sham or sham + SIN groups. SIN significantly attenuated brain water content compared with the TBI+vehicle group, with no significant difference between doses. $n = 6$ per group. Data are presented as mean ± SEM. $^{**}P < 0.01$ vs. sham; $^{***}P < 0.001$ vs. sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. TBI + vehicle group. TBI, qtraumatic brain injury; SIN, sinomenine.

after treatment with SIN (**Figure 2D**). These data demonstrate that SIN successfully inhibited neuronal apoptosis induced by TBI.

SIN Attenuates Oxidative Stress Caused by TBI

To evaluate whether the neuroprotective effect of SIN was derived from its ability to reduce TBI-induced oxidative stress, levels of MDA, GPx and SOD, indicators of lipid peroxidation and antioxidant enzyme activity, were measured in brain tissue. The TBI + vehicle group had a higher level of MDA than the sham group ($P < 0.001$) (**Figure 3A**), and there was no difference between the TBI and TBI + vehicle groups ($P > 0.05$). However, the level of cortical MDA in mice treated with SIN was significantly lower than in the TBI + vehicle group ($P < 0.001$) (**Figure 3A**). Activity of GPx and SOD, antioxidant enzymes responsible for scavenging metabolites generated by free radicals, was significantly lower after TBI than after sham injury (both $P < 0.001$), whereas the SIN-treated group showed significant upregulation of GPx and SOD activity ($P < 0.05$ and $P < 0.01$, respectively) (**Figures 3B,C**).

SIN Markedly Promotes Translocation of Nrf2 from Cytoplasm to the Nucleus in the Cortex at 24 h after TBI

The data obtained demonstrated that SIN has significantly reversed the parameters of oxidative stress induced by TBI (**Figure 3**). Because Nrf2 is a key protein in the reduction of oxidative stress, it was reasonable to hypothesize that SIN might activate Nrf2, thereby enhancing the activity of the antioxidant enzymes. Compared with the sham-injured group, both TBI and SIN administration induced Nrf2 nuclear translocation (**Figure 4A**). In addition, the SIN-treated group showed significantly greater nuclear expression of Nrf2, and lower cytoplasmic expression, than the TBI group that received vehicle ($P < 0.01$ and $P < 0.001$, respectively; **Figure 4A**), which demonstrated that Nrf2 was activated and SIN promoted Nrf2 nuclear translocation.

This effect was confirmed by immunofluorescence. Nrf2 expression in the sham-injured group was weak compared with the TBI or vehicle-treated groups and was predominately detected in the neuronal cytoplasm. Compared with the sham-injured group, there were more Nrf2-immunoreactive neurons in the TBI and vehicle-treated groups, with some translocation of Nrf2 from the cytoplasm to the nucleus being observed. In the SIN-treated group, there was more Nrf2-immunoreactivity detected in cell nuclei 24 h after TBI than in the vehicle group (**Figure 4B**). Together, this provides abundant evidence that SIN promotes Nrf2 translocation from the cytoplasm to the nucleus, thus improving its ability to bind to downstream genes.

SIN Upregulates the Expression of Nrf2 Downstream Factors

Because SIN was able to activate Nrf2 and provide neuroprotection against TBI, we hypothesized that it might also regulate downstream factors in the Nrf2 pathway. We therefore measured the expression of NQO-1 and HO-1. At the protein level, NQO-1 and HO-1 were both upregulated after TBI ($P < 0.001$ and $P < 0.05$, respectively; **Figure 5A**). Additionally, administration of SIN further enhanced protein expression compared with vehicle ($P < 0.05$ and $P < 0.001$, respectively; **Figure 5A**). At the mRNA level, consistent with the protein changes, SIN enhanced the expression of

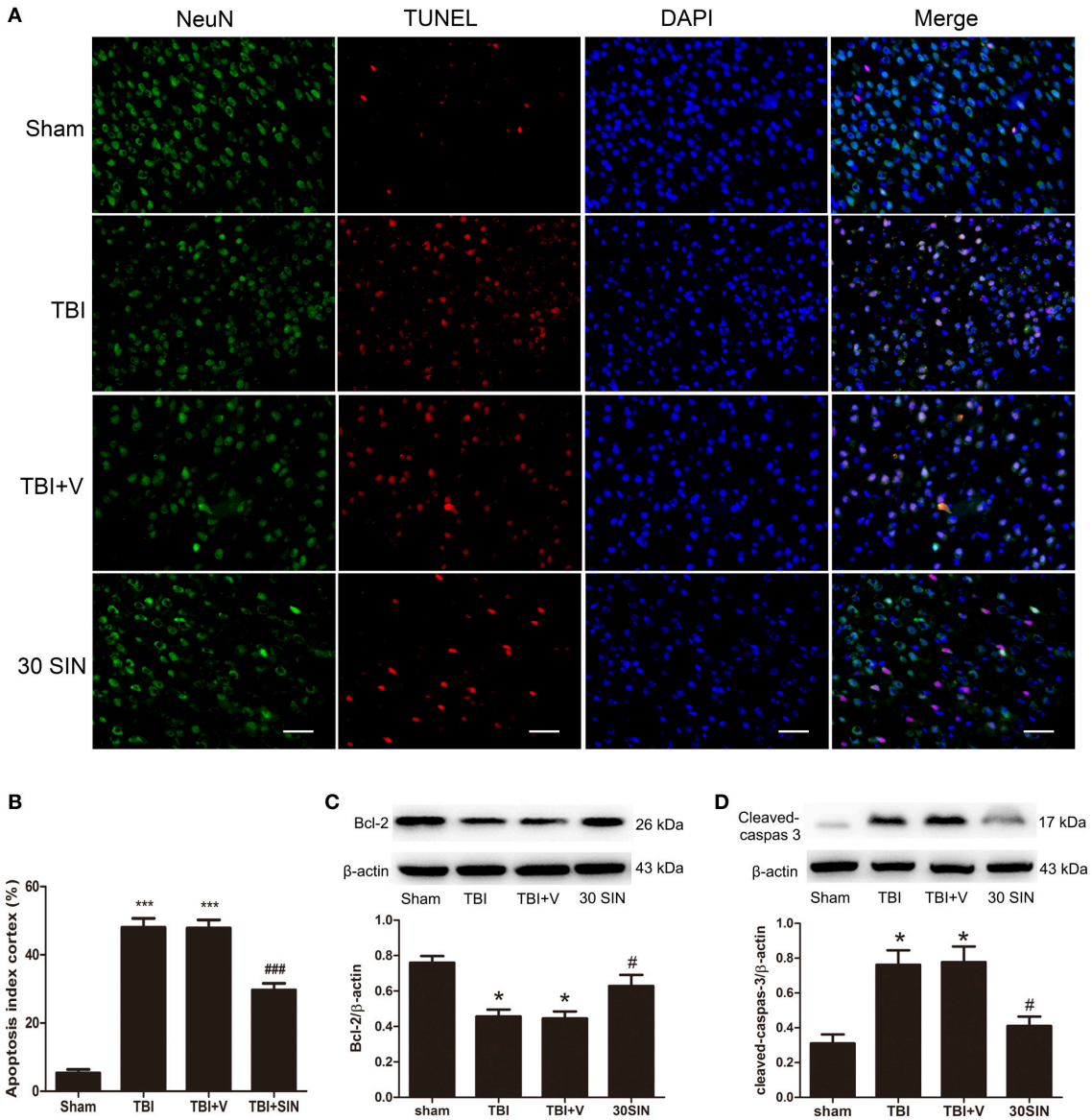

FIGURE 2 | SIN treatment attenuated TBI-induced neuronal apoptosis 24 h after TBI. (A) Representative TUNEL staining of brain sections of sham, TBI, vehicle-treated and SIN-treated mice at 24 h post-injury. **(B)** SIN treatment significantly decreased the number of TUNEL-positive neurons after TBI. Quantification showed that SIN-treated mice had significantly fewer TUNEL-positive neurons than the vehicle-treated mice. Representative immunoblots showing the protein levels of Bcl-2 **(C)** and cleaved caspase-3 **(D)** in the sham, TBI, TBI+vehicle, and TBI+SIN groups at 24 h after TBI. $n = 6$ per group. Data are presented as mean ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. sham group, $^{\#}P < 0.05$, and $^{\#\#\#}P < 0.001$ vs. TBI + vehicle group.

NQO-1 and HO-1 compared with vehicle (both $P < 0.01$; **Figure 5B**). These results demonstrate that SIN induced the expression of factors downstream of Nrf2 in terms of protein and mRNA levels, via activation of the Nrf2 and the Antioxidant Responsive Element (Nrf2-ARE) signaling pathway.

DISCUSSION

In the present study, we evaluated the neuroprotective effects of SIN on TBI-induced brain injury in a mouse model and the underlying molecular mechanisms. The main findings of this study are as follows. (1) Administration of SIN improved neurological function, alleviated cerebral edema, and inhibited neuronal apoptosis. (2) SIN mitigated TBI-induced oxidative stress, which is represented by the level of MDA and the activity of GPx and SOD. (3) The translocation of Nrf2 from the cytoplasm to the nucleus after TBI, and SIN further promoted this translocation, subsequently increased the expression of downstream factors on mRNA and protein levels. These results suggest that SIN provides neuroprotection in a mouse model of TBI via the Nrf2-ARE pathway.

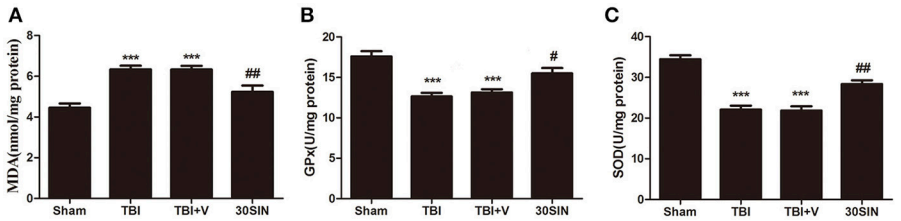

FIGURE 3 | SIN reduces oxidative stress in brain tissue following TBI. (A–C) Oxidative stress was evaluated by the level of malondialdehyde (MDA) and the activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD). Mice were subjected to TBI and then treated with SIN (30 mg/kg) or vehicle 30 min after TBI. As shown, **(A)** the level of MDA in the cortex increased remarkably 24 h after TBI. SIN treatment significantly suppressed the production of MDA after TBI. **(B,C)** The activities of GPx and SOD were decreased after TBI, while SIN treatment increased the activities of GPx and SOD. $n = 6$ per group. Data are presented as mean ± SEM. $^{***}P < 0.001$ vs. sham group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. TBI + vehicle group.

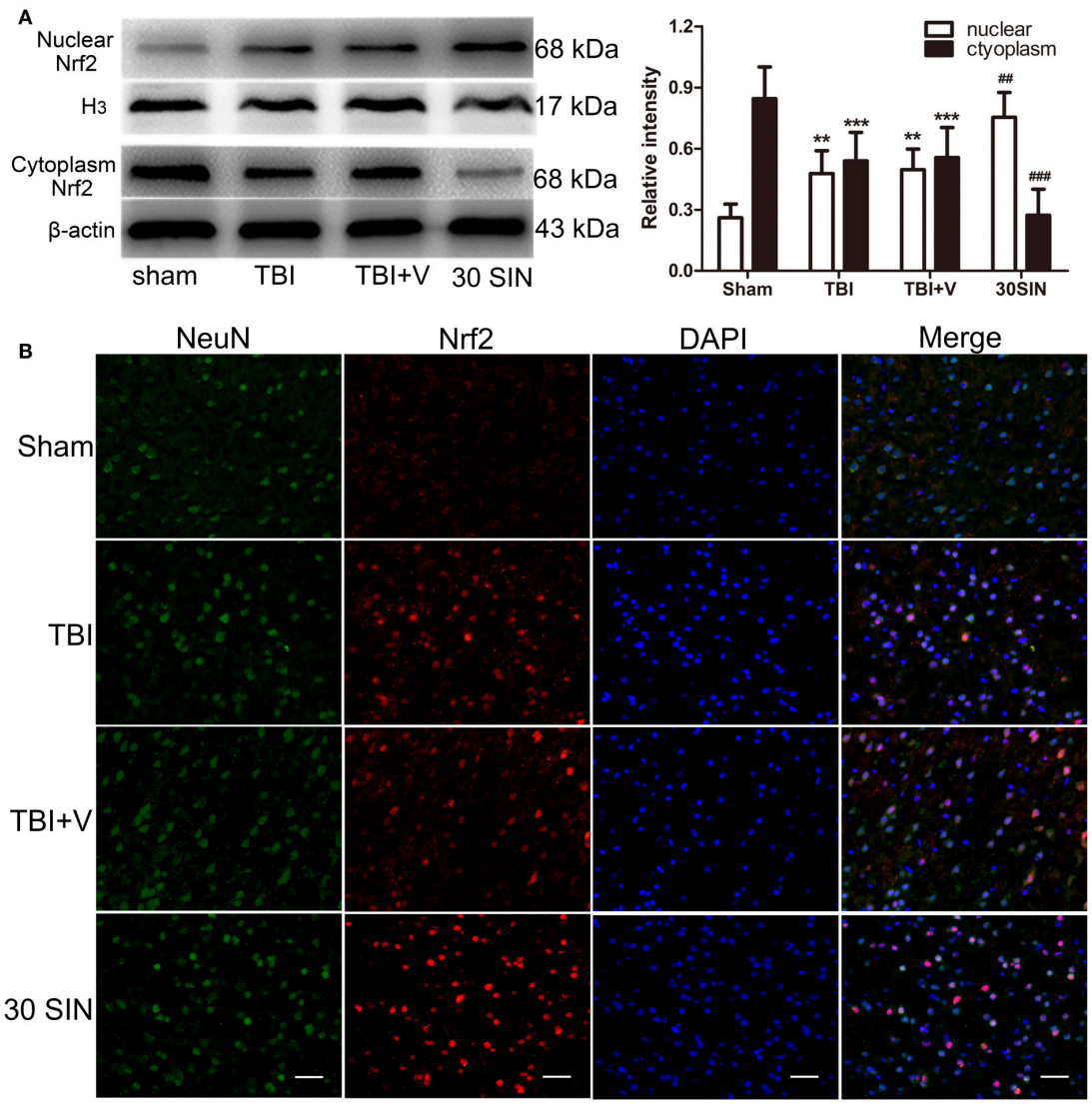

FIGURE 4 | SIN promoted translocation of Nrf2 from cytoplasm to the nucleus. (A) Mice brain tissues were collected 1 day after TBI in different groups and the Nrf2 levels in both cytoplasm and nucleus were measured by Western blot. SIN significantly increased the level of Nrf2 in the nucleus and decreased the level of Nrf2 in the cytoplasm. **(B)** Representative immunofluorescence staining of Nrf2 after SIN treatment in mice with TBI. Double immunofluorescence analysis was performed with Nrf2 antibodies (red) and neuronal marker (green), and nuclei were fluorescently labeled with DAPI (blue). Scale bar = 20 μm. $n = 6$ per group. Data are presented as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. sham group; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. TBI + vehicle group.

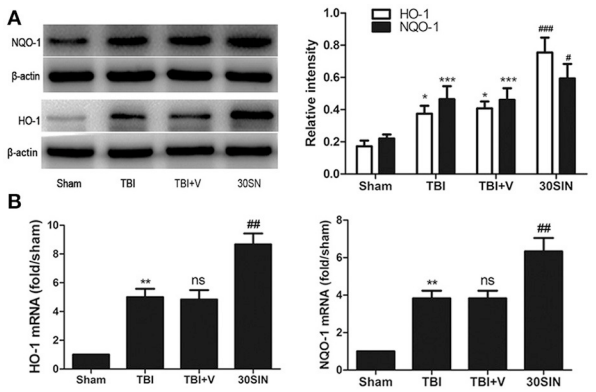

FIGURE 5 | SIN upregulated the expression of Nrf2 downstream factors in mice at both protein and mRNA levels. Mice were subjected to TBI and treated with vehicle or SIN (30 mg/kg) 30 min after TBI. Protein and mRNA expression was measured by Western blot and RT-PCR, respectively. **(A)** Both HO-1 and NQO-1 proteins were upregulated after TBI; additionally, SIN further increased their expression in brain tissue. Actin was used as a loading control. Relative intensity means NQO-1 or HO-1/β-actin. **(B)** NQO-1and HO-1 mRNA were elevated after TBI and were further increased with administration of SIN. $n = 6$ per group. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. sham group; $^{ns}P > 0.05$ vs. TBI group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. TBI + vehicle group.

Secondary brain injury after TBI represents consecutive pathological processes involving oxidative stress, glutamate excitotoxicity, loss of ionic homeostasis, inflammatory response, and increased vascular permeability (Werner and Engelhard, 2007; Bell et al., 2009; Cornelius et al., 2013). Accumulating evidence shows that oxidative stress is a crucial contributor to the pathophysiology of TBI and mediates subsequent histopathology and neurobehavioral deficits (Bains and Hall, 2012). The enhanced production of reactive oxygen and nitrogen species though several different cellular pathways plays a pivotal role in TBI (Lewén et al., 2000; Cornelius et al., 2013; Radak et al., 2013). Moreover, these radicals can lead to lipid peroxidation, protein nitration and oxidation (Ansari et al., 2006), and DNA damage (Shao et al., 2006; Ansari et al., 2008). Oxidative stress may be a novel therapeutic target in TBI. MDA levels reflect lipid peroxidation (Hou et al., 2012; Cornelius et al., 2013), and begin to increase immediately after TBI, remaining elevated 48 h after injury (Hou et al., 2012). SOD and GPx are antioxidant enzymes responsible for scavenging metabolites generated by free radicals, catalyzing the conversion of peroxides into nontoxic forms (Miller et al., 2009; Cornelius et al., 2013). GPx is an intracellular antioxidant enzyme, converting peroxides into nontoxic forms (Miller et al., 2009; Cornelius et al., 2013). In the present study, SIN treatment after TBI reduced MDA level and increased GPx and SOD activity, suggesting that SIN could attenuate TBI-induced oxidative stress.

Increasing evidence indicates that Nrf2 is indispensable in the induction of antioxidant enzymes, as revealed in various CNS conditions (Wang et al., 2007; Yan et al., 2009; Chen et al., 2011) and Nrf2 and phase II enzymes, such as NQO1 and HO-1, were activated after TBI (Yan et al., 2008, 2009). Besides, Nrf2-/- mice exhibited poorer outcomes than the wild-type mice, while activation of Nrf2 could protect against brain injury after TBI (Wang et al., 2012). Our results also showed that Nrf2 translocated from the cytoplasm to the nucleus after TBI. These evidences indicate that activation of the Nrf2–ARE pathway is beneficial for TBI.

SIN has been shown to exert neuroprotection in several CNS disease models. Zhao and colleagues (Yang et al., 2014) reported that SIN attenuates brain injury from intracerebral hemorrhage by inhibiting activation of microglia. Wu et al. (2011) demonstrated that SIN is potently neuroprotective against ischemic brain injury when administered before or after the injury. In a recent study, Qin et al. (2016) showed that SIN upregulates Nrf2 and phase-II enzymes to exert renoprotective effects in mouse fibrotic kidney. We therefore hypothesized that SIN could also activate the Nrf2-ARE pathway in the brain after TBI to alleviate brain injury by inhibiting oxidative stress secondary to the upregulation of antioxidant enzymes. In the present study, we investigated the changes in the Nrf2-ARE signaling pathway after SIN administration. The data showed that Nrf2 translocated from the cytoplasm to the nucleus after TBI, and SIN further promoted this translocation. Compared with the TBI and TBI + vehicle groups, SIN administration also significantly upregulated the expression of HO-1 and NQO1 at the pre- and post-transcriptional levels 24 h after TBI. HO-1 and NQO1 are potent antioxidant and detoxifying enzymes. HO-1 protects against a number of pathophysiological insults including oxidative stress (Miller et al., 2009). NQO1 is able to protect cells against the adverse effects of quinones and related compounds (Radjendirane et al., 1998). Accordingly, SIN administration reduced the oxidant damage and alleviated brain injury in the TBI models in this study. However, the mechanism of activation of the Nrf2–ARE pathway by SIN has not been completely elucidated. A number of kinases, such as phosphoinositol-3 kinase and extracellular signal-regulated protein kinase reportedly phosphorylate Nrf2 directly and affect its cellular location or stability in response to some stimuli (Zipper and Mulcahy, 2003; Tsai et al., 2013). Furthermore, protein kinase C (PKC) could disrupt the interaction between Nrf2 and KEAP1 by directing phosphorylation of Nrf2 (Huang et al., 2000). These mechanisms may be involved in the activation of the Nrf2–ARE pathway by SIN after TBI, and need to be investigated in future studies.

Our study has several limitations. First, SIN was only administered once; we do not know whether multiple treatments with different time courses would be as effective. Regarding the clinic application, the therapeutic window of 30 min which was employed in this study is narrow. Thus, other time points later than 30 min post-TBI should be tested. Second, SIN may have other protective effects against TBI that were not evaluated in this study, such as anti-inflammatory and immunomodulatory properties. Lastly, we did not use Nrf2 gene knockout mice, which would confirm whether the observed effects of SIN are due to activation of the Nrf2–ARE pathway. Further comprehensive studies are warranted.

CONCLUSION

To the best of our knowledge, the present study is the first to demonstrate the effects of SIN on the Nrf2–ARE

signaling pathway in a model of TBI. Our data show that SIN treatment 30 min after TBI ameliorates secondary brain injury by improving neurologic function, reducing brain edema, combating oxidative stress and attenuating neuronal apoptosis. These effects might correlated with translocation of Nrf2 from the cytoplasm to the nucleus and activation of downstream proteins.

AUTHOR CONTRIBUTIONS

YY: Designed the study, performed the TBI model and biochemical analysis and wrote the manuscript. LL and XL: Prepared the drug solutions and performed histological examination. QW, HD, and XW: Performed the TUNEL staining and the animal studies. ZY, LW, and XZ: Contributed to the Western blotting. MZ and HP: Designed the animal studies. HW: Contributed to the design and analysis of the study and wrote the manuscript. All authors approved the final version of the manuscript.

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Effects of Cannabidiol and Hypothermia on Short-Term Brain Damage in New-Born Piglets after Acute Hypoxia-Ischemia

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Hypothermia is a standard treatment for neonatal encephalopathy, but nearly 50% of treated infants have adverse outcomes. Pharmacological therapies can act through complementary mechanisms with hypothermia improving neuroprotection. Cannabidiol could be a good candidate. Our aim was to test whether immediate treatment with cannabidiol and hypothermia act through complementary brain pathways in hypoxic-ischemic newborn piglets. Hypoxic-ischemic animals were randomly divided into four groups receiving 30 min after the insult: (1) normothermia and vehicle administration; (2) normothermia and cannabidiol administration; (3) hypothermia and vehicle administration; and (4) hypothermia and cannabidiol administration. Six hours after treatment, brains were processed to quantify the number of damaged neurons by Nissl staining. Proton nuclear magnetic resonance spectra were obtained and analyzed for lactate, N-acetyl-aspartate and glutamate. Metabolite ratios were calculated to assess neuronal damage (lactate/N-acetyl-aspartate) and excitotoxicity (glutamate/Nacetyl-aspartate). Western blot studies were performed to quantify protein nitrosylation (oxidative stress), content of caspase-3 (apoptosis) and TNFα (inflammation). Individually, the hypothermia and the cannabidiol treatments reduced the glutamate/Nacetyl-aspartate ratio, as well as TNFα and oxidized protein levels in newborn piglets subjected to hypoxic-ischemic insult. Also, both therapies reduced the number of necrotic neurons and prevented an increase in lactate/N-acetyl-aspartate ratio. The combined effect of hypothermia and cannabidiol on excitotoxicity, inflammation and oxidative stress, and on cell damage, was greater than either hypothermia or cannabidiol alone. The present study demonstrated that cannabidiol and hypothermia act complementarily and show additive effects on the main factors leading to hypoxic-ischemic brain damage if applied shortly after the insult.

Keywords: hypothermia, neonatal brain, neurodevelopment, neuroprotection, hypoxic-ischemic encephalopathy, newborn animal, cannabidiol

INTRODUCTION

Excitotoxicity, inflammation and oxidative stress are the "deadly triad" leading to hypoxia-ischemia (HI)-induced brain damage (Johnston et al., 2011; Drury et al., 2014; Juul and Ferriero, 2014). Only pleiotropic therapies acting on all these processes provide successful neuroprotection (Cilio and Ferriero, 2010; Johnston et al., 2011; Juul and Ferriero, 2014). Therapeutic hypothermia is an example of a pleiotropic neuroprotective therapy (Drury et al., 2014). Studies in immature an adult animals have demonstrated antiexcitotoxic, anti-oxidant, and anti-inflammatory effects of hypothermia (Thoresen et al., 1997; Mueller-Burke et al., 2008; Jenkins et al., 2012; Joseph et al., 2012; Sandu et al., 2016) which become the gold standard for treating infants with hypoxic-ischemic encephalopathy (HIE; Jacobs et al., 2013). Despite treatment with hypothermia, some infants with neonatal HIE present adverse outcomes. Further trials to determine the appropriate techniques of cooling, including refinement of patient selection, duration of cooling and method of providing therapeutic hypothermia, will improve our understanding of this therapy (Jacobs et al., 2013). In addition, a recent meta-analysis reported that hypothermia is not protective and can even be harmful in low- and middle-income countries in Africa and Asia (Pauliah et al., 2013). In this context, complementary therapies can be combined with hypothermia to enhance its neuroprotective properties and/or extend its therapeutic time window. These therapies might even become an alternative to hypothermia when needed (Cilio and Ferriero, 2010). On the other hand, a successful treatment for HIE neuroprotection may not be complementary to hypothermia if its mechanisms of action overlap with those of hypothermia without any improvement of the outcome. Thus, IGF-1 (George et al., 2011) or SOD mimetic EUK-134 (Ni et al., 2012) therapies failed to demonstrate additive neuroprotective effects when combined with hypothermia. By contrast, other therapies such as xenon or melatonin (Chakkarapani et al., 2010; Faulkner et al., 2011; Robertson et al., 2013), have been observed to augment hypothermic neuroprotection at least in some situations, showing additive protection with mild hypothermia.

Cannabidiol (CBD), the main non-psychoactive component of Cannabis sativa, has recently been included among pleiotropic therapies, as it has been shown to reduce HI-induced brain damage in newborn rats and piglets by modulating excitotoxicity, inflammation and oxidative stress (Alvarez et al., 2008; Castillo et al., 2010; Lafuente et al., 2011; Pazos et al., 2012, 2013). Some of the properties of CBD underlying these effects include its antioxidant properties, the inhibition of calcium transport across membranes, inhibition of endocannabinoid uptake and enzymatic hydrolysis, activation of serotonin 5HT1A receptors and inhibition of NF-κB activation (Alvarez et al., 2008; Castillo et al., 2010; Lafuente et al., 2011; Pazos et al., 2012, 2013). The neuroprotective action of CBD was not associated with significant side effects, and additional benefits were demonstrated if immediate rescue treatment was provided (Alvarez et al., 2008; Lafuente et al., 2011; Pazos et al., 2012, 2013). In summary, these studies support the role of CBD as a non-artificial drug for newborns with HIE. However, no evidences of how CBD might work together with hypothermia have been provided so far.

Our aim was to assess the early effects of combining cannabidiol with therapeutic hypothermia in neonatal hypoxic-ischemic piglets using clinical biomarkers of brain injury shortly after the injury.

MATERIALS AND METHODS

All experimental procedures and euthanasia of the animals were conducted in strict compliance with European and Spanish regulations on the protection of animals used for scientific purposes (European Directive 2010/63/EU and Spanish Royal Legislative Decree 53/2013). The protocols were approved by the Committees on the Ethics of Laboratory Animal Welfare of Biocruces Health Research Institute and the Research Institute Puerta de Hierro Majadahonda (Permit Numbers: SEP#009_09 and SEP#012_11) and performed in its experimental surgical theaters. Using G*Power 3.0 (Faul et al., 2007), the change in the area under the curve for lactate/N-acetyl aspartate from baseline at 6 h was used as the primary outcome to calculate the sample size. Our previous work in this model suggested that the change in lactate/N-acetyl aspartate ratio after 6 h varied between vehicle-treated and cannabidiol-treated groups by 50 ± 20%. Assuming similar magnitude of additional effect for cannabidiol-augmented hypothermia (vs. hypothermia or cannabidiol alone) and with an alpha risk of 0.05 and a beta risk of 0.2, eight subjects would be necessary in each group. It was anticipated a drop-out rate of 5%. All experimental procedures were designed and carried out by personnel qualified in Laboratory Animal Science, following FELASA recommendations (Category B and C). All surgery was performed under adequate anesthesia and analgesia, and great efforts were made to minimize suffering.

Experimental Protocol

Animals were daily obtained from a local vendor with specific pathogen free status for international swine breeding program (TOPIG, B-line) and for *in vitro* genetic reproduction (Arri-Turri farm, Alava, Spain). In short, 1- to 2-day-old male piglets were intubated under 5% sevoflurane anesthesia and managed by controlled mechanical ventilation (VIP Bird, Bird Corp., Palm Springs, CA, USA). A marginal ear vein was cannulated to maintain intravascular anesthesia and analgesia by continuous infusion of 3 mg/kg/h propofol, 0.5 mg/kg/h midazolam, and 4 μg/kg/h fentanyl. Once adequate analgesia was achieved and confirmed, respiratory paralysis was induced with 3 mg/kg/h atracurium to prevent spontaneous breathing. Then, the two common carotid arteries were exposed and elastic bands were placed loosely around each one. A non-invasive ultrasonic probe (Transonic Systems Inc., NY) was placed in the right common carotid artery to measure the instantaneous blood flow. Indwelling catheters (5 Fr, PiCCO Plus, Pulsion Medical Systems, München, Germany) were inserted into the right jugular vein, to infuse dextrose (at a rate of 4 mg/kg/min), and into the right femoral artery to continuously monitor cardiac output, heart rate, mean arterial blood pressure and central temperature (Omnicare

CMS 24, HP, Göblingen, Germany). Further, brain activity was monitored by amplitude-integrated electroencephalography (aEEG; BRM2; BrainZ Instruments, Auckland, New Zealand). The "raw" EEG traces were manually reviewed for electrical seizures. Body temperature was maintained between 37.5 and 38.5°C using an air-warmed blanket. Arterial blood gases were monitored throughout the experiment. Dopamine infusion (10–20 μg/kg/min) was used as needed to maintain mean arterial blood pressure over 40 mmHg (Chakkarapani et al., 2010; Faulkner et al., 2011; Robertson et al., 2013). After surgical instrumentation, each animal was left to stabilize for 30 min (baseline).

After the surgery, HI brain injury was induced in the piglets by total interruption of the carotid blood flow (tightening the elastic bands around the arteries, and confirmed by the ultrasonic probe) and reducing the fraction of inspired oxygen to less than 10%. The hypoxic-ischemic conditions were maintained for 30 min, measured from the point at which there was evidence of reduced brain activity on the aEEG (flat traces < 4 μV). After this period of injury (end of HI), carotid blood flow was restored and the inspired fraction of oxygen was returned to 21%.

After 30 min, control and HI-injured piglets were first randomized by sealed envelope to normothermia or hypothermia. In normothermic animals, rectal temperature was maintained at 38°C (range: 37.5–38.5°C) using the air-warmed blanket. In hypothermic animals, based on previously reported studies for combination of therapies in hypoxic-ischemic piglets (Chakkarapani et al., 2010; Faulkner et al., 2011; Robertson et al., 2013), rectal temperature was reduced within 10 min to 33–34°C using a circulating water mattress (Recirculating Chiller 1171MD, VWR International, CA, USA).

Then, HI-injured piglets treated with normothermia or hypothermia were again randomized for drug administration by sealed envelope. CBD (a generous gift by GW Pharma Ltd, Mambridge, UK) was prepared in a 5 mg/mL formulation of ethanol/solutol/saline (2:1:17), as reported in previous studies (Alvarez et al., 2008; Lafuente et al., 2011; Pazos et al., 2012, 2013). Doses were selected following previous *in vivo* experiments by our group (Pazos et al., 2012, 2013) and HI-injured animals received i.v. 1 mg/kg (0.2 mL/kg) of CBD or an equivalent volume of vehicle (ethanol/solutol/saline), further diluted to 10 mL in saline before administration as slow bolus.

After 6 h of treatment, anesthetized piglets were euthanized by cardiac arrest with potassium chloride (∼150 mg/kg), and the brains were immediately removed from the skull and sliced. Brain slices were placed into 4% paraformaldehyde, for histological analysis (left hemisphere), or frozen in isopentane and conserved at −80°C, for spectroscopy and biochemical analysis (right hemisphere). For both types of analysis, we used parietal cortex samples from the same brain section, corresponding to 5 mm of the posterior brain region identified according to the stereotaxic atlas of the pig brain (Felix et al., 1999).

Accordingly, there were four experimental groups of HI piglets: normothermic vehicle-treated group (NV; $n = 9$), normothermic cannabidiol-treated group (NC; $n = 11$), hypothermic vehicle-treated group (HV; $n = 8$) and hypothermic cannabidiol-treated group (HC; $n = 8$). Normothermic and hypothermic sham operated groups of animals without HI are included in supplemental files as referenced data.

Histological Analysis

Neuronal necrosis was identified by Nissl staining in 4-μm thick coronal sections obtained from fixed brain hemispheres, as described previously (Alvarez et al., 2008; Lafuente et al., 2011; Pazos et al., 2013). Random areas were examined in the three central lobes of the parietal cortex, focusing on 1-mm^2 areas in the layers II-III. This analysis was conducted by a pathologist investigator blinded to the experimental groups using an optical microscope at 400x and a grid of 50 compartments, with the mean of three compartments being calculated. The parietal cortex was selected to illustrate histological brain damage because this is the most vulnerable area in the brain to HI in piglets, and because the damage seen in this area correlates with that observed in other vulnerable areas such as the hippocampus or striatum (Peeters-Scholte et al., 2003; Schubert et al., 2005). Normal neuronal cells were recognized by the presence of the typical nuclei with clear nucleoplasm and distinct nucleolus surrounded by purple-stained cytoplasm containing Nissl substance. Neurons were classified as damaged cells (pyknotic or necrotic) when we were unable to ascertain a well-defined nuclear and cytoplasmic boundary.

Proton Magnetic Resonance Spectroscopy (H^+-MRS)

Ex vivo proton magnetic resonance spectroscopy (H^+-MRS) has been demonstrated to provide metabolic information with higher sensitivity and spectral resolution than *in vivo* magnetic resonance spectroscopy (Jiménez-Xarrié et al., 2015). As previously described (Pazos et al., 2013), H^+-MRS was performed in the MRI Unit of the Instituto Pluridisciplinar (Universidad Complutense, Madrid, Spain) at 500.13 MHz using a Bruker AMX500 spectrometer at 11.7 T, operating at 4°C on frozen parietal cortical samples (5–10 mg weight) placed within a 50-μL zirconium oxide rotor with a cylindrical insert and spun at a rate of 4000 Hz. H^+-MRS spectra were acquired using a standard solvent-suppressed pulse/acquire sequence based on the start of a Nuclear Overhauser Effect Spectroscopy pulse sequence, with 16,000 data points, averaged over 256 acquisitions. Data acquisition time was approximately 14 min. The NOESY preset pulse sequence employed a mixing time of 150 ms with t1 fixed at 3 μs, during which time the effects of B0 and B1 field inhomogeneities were suppressed (relaxation delay-90°-t1-90°-tm-90°-acquire free induction decay signal). A secondary radio-frequency field was applied at the water resonance frequency during the relaxation delay of 2 s.

A spectral width of 8333.33 Hz was used. All spectra were processed using TOPSPIN software, version 1.3 (Bruker Rheinstetten, Germany). Prior to Fourier transformation, the free induction decay values were multiplied by an exponential weight function corresponding to a line broadening of 0.3 Hz. Spectra were phased, baseline-corrected and referenced to the sodium (3-trimethylsilyl)-2,2,3,3-tetradeuteriopropionate singlet at $\delta = 0$ ppm.

After curve fitting was performed (SpinWorks ver. 3.1.7.0, University of Manitoba, Canada), all spectra were analyzed for lactate (Lac), N-acetyl-aspartate (NAA), and glutamate (Glu). Later, several ratios were calculated that are predictive of neurodevelopmental outcome after hypoxic-ischemic brain damage in piglets (Vial et al., 2004; Munkeby et al., 2008): lactate/N-acetylaspartate (Lac/NAA) and glutamate/N-acetylaspartate (Glu/NAA).

Western Blot Studies

Levels of oxidized proteins were quantified by Western blot analysis to assess protein carbonylation in brain tissue. A detection kit (Millipore Iberica; Madrid, Spain) was used according to the manufacturer's protocol, and brain samples containing 15 μg of total protein were subjected to the derivatization reaction with 2,4-dinitrophenylhydrazine (DNPH). Two aliquots of each sample to be analyzed were treated: one aliquot was subjected to derivatization reaction, and the other aliquot, served as a negative control, by substituting the derivatization control solution with DNPH solution. The treated samples were fractionated in a 12% dodecyl-sulfate–polyacrylamide gel and electroblotted onto PVDF membranes (GE Healthcare; Buckinghamshire, UK). DNPH-BSA standards (Millipore Iberica, Madrid, Spain) were loaded on each gel and used as a reference. Membranes were incubated in Tris/glycine/methanol transfer buffer at 4°C and proteins separated under constant voltage (2 h at 250 mA). The resulting blots were blocked by overnight incubation in phosphate-buffered saline-Tween (PBST) containing 5% nonfat dried milk at 4°C. Primary antibody incubation (Rabbit Anti-DNPH 1:150; Millipore Iberica, Madrid, Spain) was performed in PBST containing 5% nonfat dried milk for 1 h at room temperature. After washing with PBST, the membranes were incubated with the secondary antibody (Goat anti-rabbit IgG HRP conjugated 1:300; Millipore Iberica, Madrid, Spain) for 1 h at room temperature. Finally, an enhanced chemiluminescent substrate detection system (GE Healthcare, Buckinghamshire, UK) was used to visualize the blots. The films were scanned and analyzed with ImageJ software (NIH, USA). Oxidized protein levels were quantified via measurement of the optical density, using the NIH Image J analysis software (Bethesda, MD, USA). Results were normalized by total protein loading (Red Ponceau staining) and expressed as OxyBlot/Red Ponceau ratio.

TNFα and caspase-3 Western blot assays were performed with brain samples containing 20 μg of total protein. In this case, proteins were electrophoresed in an 18% sodium dodecyl-sulfate–polyacrylamide gel and then electroblotted onto PVDF membranes (GE Healthcare, Buckinghamshire, UK) in Tris/glycine/methanol transfer buffer under constant voltage (2 h at 250 mA). The resulting blots were blocked in PBST containing 5% nonfat dried milk at room temperature for 1 h, followed by primary antibody incubation (Rabbit anti-TNFα 1:1000, and rabbit anti-Caspase-3 1:500; Abcam Plc., Cambridge, UK) in PBST containing 5% nonfat dried milk at 4°C overnight. After washing with PBST, the membranes were incubated with the secondary antibody (Goat anti-rabbit IgG HRP-Conjugated for TNFα and goat anti-mouse IgG HRP-Conjugated for caspase-3, 1:2000; Bio-Rad Lab., Hercules, CA, USA) for 1 h at room temperature. Finally, as above, the ECL system was used, and the films were scanned and analyzed using ImageJ software. Protein levels were quantified using densitometric analysis, normalized by β-actin (1:500, Abcam Plc., Cambridge, UK) loading and expressed as TNFα/β-actin and caspase-3/β-actin ratios.

Statistical Analyses

SPSS 19.0.0 software (IBM Software, NY, USA) was used for statistical analyses. All data are expressed as mean ± standard error of the mean (SEM). Comparisons between groups were performed by Kruskall-Wallis one-factorial analysis of variance by ranks with Dunn's *post-hoc* test. A $P < 0.05$ was considered to be statistically significant.

RESULTS

Normothermic and hypothermic sham operated groups of animals without HI are included in the Supplementary Materials. Piglets from all experimental groups were similar in terms of age, weight and physiological parameters (**Table 1**), except for mean arterial blood pressure and aEEG (**Figure 1**). The hypoxic-ischemic insult induced a decrease in pH in all injured animals (H interval, **Table 1**). After hypothermic therapy begins, pH levels significantly recovered toward baseline levels at the drug treatment (D interval, **Table 1**) in both HV and HC groups. At the end of experiment (E interval, **Table 1**), mean pH level in HC group was significantly higher than in the other groups. In normothermia-treated animals, the hypoxic-ischemic insult led to a decrease in mean arterial blood pressure that spontaneously recovered in the following 30 min.

In the NV group mean arterial blood pressure values dropped again throughout the post-HI period (**Figure 1A**); in a half of the animals (5 animals), dopamine infusion (at 10–20 μg/kg/min) was needed to maintain mean arterial blood pressure over 40 mmHg. Hypothermia was associated with a decrease in mean arterial blood pressure in all groups (**Figure 1A**), although dopamine infusion was not administered since the mean arterial blood pressure did not fall under 40 mmHg in any of the piglets. No such decline in the mean arterial blood pressure was observed in the HI-injured piglets treated with normothermia and CBD, and these animals did not require dopamine.

After injury, basal aEEG amplitude was decreased below 4 μV in all normothermic and hypothermic groups (**Figure 1B**). The depressed activity remained unchanged in both NV, HV and HC groups during all experiment, but was partially recovered at the end of the experiment in NC group. Electrical seizures were detected in one piglet from the HV group and one from the HC group.

Effects of the Treatments on the Characteristics of Excitotoxicity

The Glu/NAA ratio was higher in the NV group than in the HV group (**Figure 2**). Cannabidiol treatment alone (NC group) reduced Glu/NAA ratio to a similar extent compared to the levels in HV group. The administration of both therapies (hypothermia

TABLE 1 | General and physiological parameters.

Group		NV	NC	HV	HC
Sample size		(*n* = 9)	(*n* = 11)	(*n* = 8)	(*n* = 8)
Age (days)		1.8 (0.1)	1.9 (0.1)	1.6 (0.1)	1.7 (0.1)
Weight (kg)		1.7 (0.1)	1.9 (0.1)	1.6 (0.1)	1.6 (0.1)
CO (ml/min/100 g)	B	339 (35)	357 (25)	315 (11)	301 (41)
	H	355 (34)	321 (66)	410 (28)	402 (32)
	D	369 (41)	334 (41)	317 (15)	262 (26)
	E	344 (42)	336 (43)	324 (27)	292 (20)
pH	B	7.32 (0.02)	7.35 (0.02)	7.36 (0.01)	7.41 (0.03)
	H	7.17 (0.04)	7.20 (0.04)	7.23 (0.04)	7.24 (0.03)
	D	7.21 (0.03)	7.20 (0.05)	7.36 (0.03)**	7.40 (0.03)**
	E	7.32 (0.03)	7.32 (0.02)	7.34 (0.02)	7.42 (0.03)***
pCO_2 (mm Hg)	B	39.4 (1.9)	40.7 (2.6)	40.5 (1.5)	39.4 (2.6)
	H	39.2 (2.9)	40.1 (3.3)	43.4 (1.5)	44.4 (2.9)
	D	42.4 (2.2)	43.4 (2.3)	37.4 (3.2)	38.0 (3.0)
	E	42.2 (2.9)	42.3 (1.6)	43.3 (1.8)	37.2 (2.2)

Mean values ± SEM (given in brackets) obtained from 1- to 2-day-old piglets after hypoxic-ischemic insult, treated with normothermia or hypothermia and administered with either vehicle or cannabidiol. B, Baseline; H, end of HI, D, Drug; E, End of the experiment (**) $P < 0.05$ vs. both normothermic groups (NV and NC) by Kruskall-Wallis one-factorial analysis of variance; (***) $P < 0.05$ vs. all groups by Kruskall-Wallis one-factorial analysis of variance.

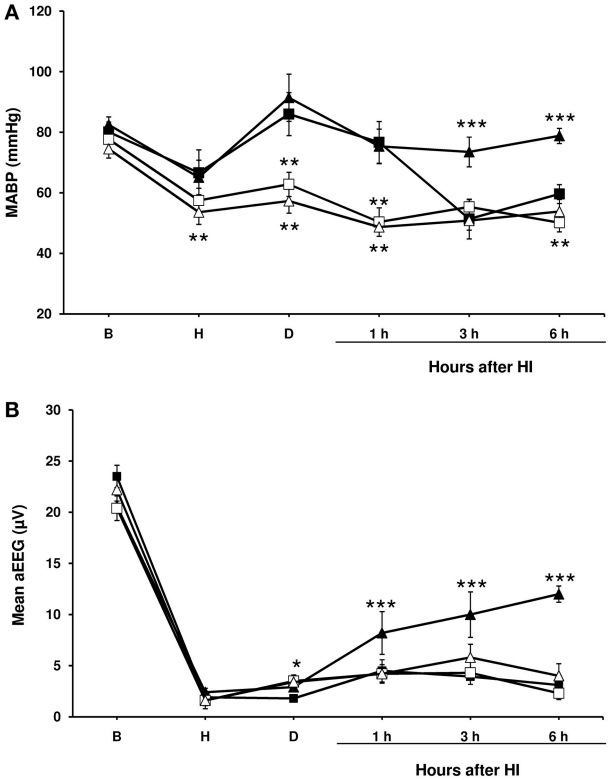

FIGURE 1 | Mean arterial blood pressure and amplitude-integrated electroencephalography (aEEG) in experimental groups of piglets. Line draws represent the mean arterial blood pressure **(A)** and the mean aEEG **(B)**, obtained from 1- to 2-day-old piglets after hypoxic-ischemic insult, treated with normothermia (shaded symbol) or hypothermia (empty symbol) and administered with either vehicle (square) or cannabidiol (triangle). Data are represented as mean ± SEM. (*) $P < 0.05$ vs. NV group by Kruskall-Wallis one-factorial analysis of variance; (**) $P < 0.05$ vs. both normothermic groups (NV and NC) by Kruskall-Wallis one-factorial analysis of variance; (***) $P < 0.05$ vs. all groups by Kruskall-Wallis one-factorial analysis of variance.

and CBD) together produced additive effects, so the Glu/NAA ratio in the HC group was lower to that observed in HV or NC groups.

Effect of the Treatments on the Characteristics of Oxidative Stress

A lower mean value of OxyBlot/Red Ponceau ratio in the analyzed proteins from the parietal cortex was observed in the HV group than in NV group (**Figure 3**), indicating a reduction of protein carbonylation. A single dose of CBD after HI (NC group) induced a similar effect on this ratio as in the HV group (**Figure 3**). The mean levels of OxyBlot/Red Ponceau ratio were lower in the HC group than in the HV or NC groups, suggesting that administration of hypothermia together with CBD led to an additive effect.

Effect of the Treatments on the Characteristics of Neuroinflammation

Hypothermia treatment led to a reduction of TNFα content in the parietal cortex of animals from the HV group as compared to the NV group (**Figure 4**). With the administration of CBD in normothermia, brain TNFα concentration was almost 18% lower than in the NV group. Again, the combination of two treatments resulted in an additive effect: TNFα levels in the HC group were lower than those in the NC or HV groups.

Effects of the Treatments on the Characteristics of Brain Damage

HI-induced brain damage and treatment-induced effects were both observed in the histological and biochemical analyses. At the end of the experiment, about a quarter of neurons in the cortex of the NV group animals appeared to be necrotic (**Figure 5A**). Either hypothermia treatment (HV group) or CBD administration (NC group) alone reduced this percentage to a similar extent. Administration of both therapies together produced an additive effect bringing the per cent of necrotic neurons in the HC group down compared to the NC or HV animals.

The expression of caspase-3 was reduced in the HV group in comparison to NV group (**Figure 5B**). Moreover, the NC group showed a significant reduction of caspase-3 levels in comparison to the NV group. In this case the effect was so strong that caspase-3 levels in HV group were similar to those in HC, with no additive effect shown by combining hypothermia and CBD.

The Lac/NAA ratio was reduced in the HV group in comparison to NV group (**Figure 5C**). Also, the effect of hypothermia alone (HV group) was slightly greater than that of CBD alone (NC group) after HI. Administering the two treatments together resulted in an additive effect, the Lac/NAA ratio in the HC group being lower than that in the NC and HV groups.

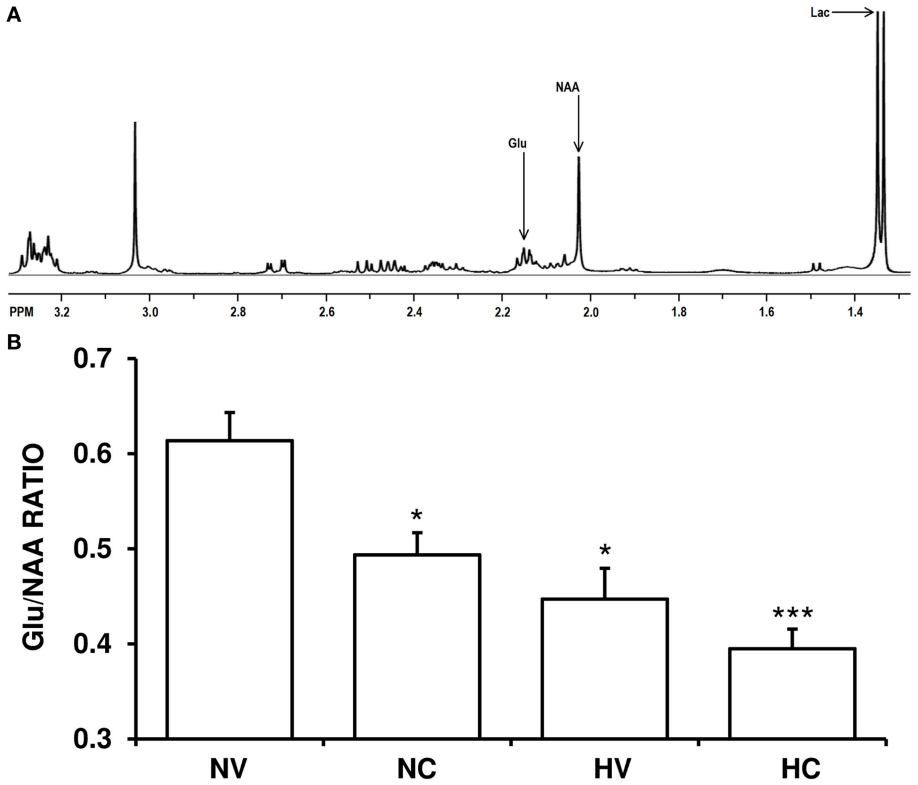

FIGURE 2 | Effect of the treatments on the characteristics of excitotoxicity. (A) A representative brain spectra from a hypoxic-ischemic animal managed with normothermia (NV group). Arrows show the metabolite peaks of glutamate (Glu), N-acetylaspartate (NAA) and lactate (Lac). **(B)** Bars represent the results of Glu/NAA ratio (mean ± SEM), in brain samples from 1- to 2-day-old piglets after hypoxic-ischemic insult, treated with normothermia or hypothermia and administered with either vehicle or cannabidiol. (*) $P < 0.05$ vs. NV group by Kruskall-Wallis one-factorial analysis of variance; (***) $P < 0.05$ vs. all groups by Kruskall-Wallis one-factorial analysis of variance.

DISCUSSION

Although not completely conclusive because of the short-term follow-up, this study suggests that CBD and hypothermia could complement each other in their protective mechanisms resulting in a reduced brain injury in hypoxic-ischemic newborn piglets. In fact, the combination of the two therapies leads to better results in terms of anti-excitotoxicity, anti-inflammation and anti-oxidation than those observed with either CBD or hypothermia alone.

Excitotoxicity, inflammation and oxidative stress, the "deadly triad" leading to HI-induced brain damage, are particularly damaging for the immature brain (Johnston et al., 2011; Drury et al., 2014; Juul and Ferriero, 2014). In particular, the high concentration and activity of glutamate receptors aggravate the deleterious effects of this excitatory amino acid in the immature brain (Johnston et al., 2011; Drury et al., 2014; Juul and Ferriero, 2014). Among various approaches to quantify the increase in glutamate content in the immature brain after a hypoxic-ischemic insult, the Glu/NAA ratio has been observed to be closely related to excitotoxic brain damage (Groenendaal et al., 2001). The relative paucity of antioxidants and excess of pro-oxidant substances (in particular, iron) determine the particular susceptibility of the immature brain to oxidative stress (Johnston et al., 2011). HI-induced increases in oxidative stress can be assessed by quantifying protein carbonylation, an irreversible type of oxidative damage leading to functional loss observed in brain cells after ischemia/reperfusion (Oikawa et al., 2009) and detectable very shortly after HI in the piglet brain (Mueller-Burke et al., 2008; Pazos et al., 2013). There is increasing evidence supporting the importance of inflammation in the pathophysiology of acute brain damage and its long-lasting impact on the immature brain after HI insults (Allan and Rothwell, 2001; Johnston et al., 2011; Drury et al., 2014; Juul and Ferriero, 2014). In particular, the increase in TNFα production after HI, which occurs as early as 1 h after insult, correlates with the extent of injured tissue and with clinical outcomes (Allan and Rothwell, 2001).

In this study, for the first time, we have presented evidence of hypothermia acting simultaneously on these three factors in HI-injured piglets. There are few studies directly demonstrating the anti-excitotoxic effect of hypothermia in HI-injured piglets. Using microdialysis studies, it was previously shown that hypothermia reduces the glutamate increase seen after HI (Thoresen et al., 1997). Here, we have reported that hypothermia attenuates the HI-induced increase in the Glu/NAA ratio. As for

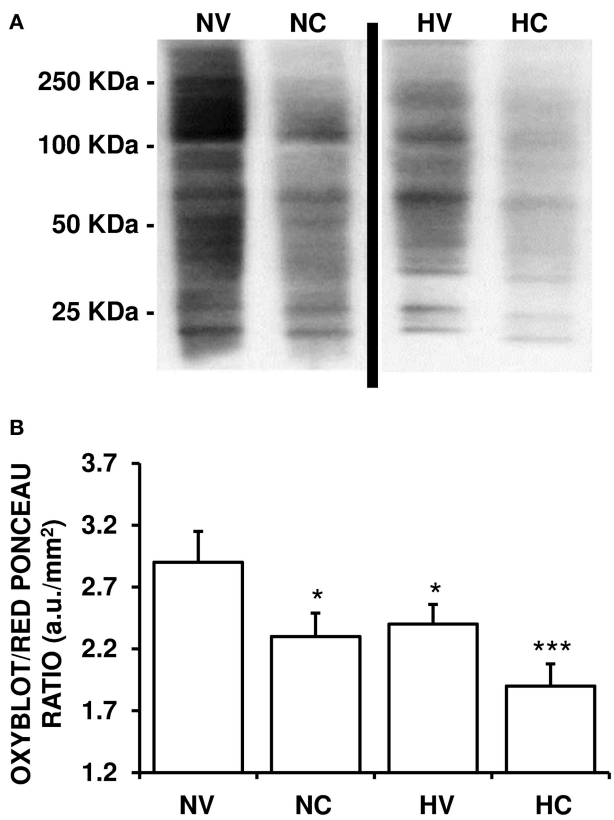

FIGURE 3 | Effect of the treatments on the characteristics of oxidative stress. (A) A representative image of immunoblotting indicating protein carbonyl formation (Oxyblot) using anti-DNPH antibody. Samples were obtained using brain tissue from 1- to 2-day-old piglets after hypoxic-ischemic insult, treated with normothermia or hypothermia and administered with either vehicle or cannabidiol. **(B)** Relative protein carbonyl levels were quantified by densitometric analyses of the blots. The content of oxidized proteins was normalized by total Red Ponceau-stained protein loading and expressed as OxyBlot/Red Ponceau ratio (see Materials and Methods for details). Bars represent mean ± SEM of 6–8 experiments. (a.u.) arbitrary units; (*) $P < 0.05$ vs. NV group by Kruskall-Wallis one-factorial analysis of variance; (***) $P < 0.05$ vs. all groups by Kruskall-Wallis one-factorial analysis of variance.

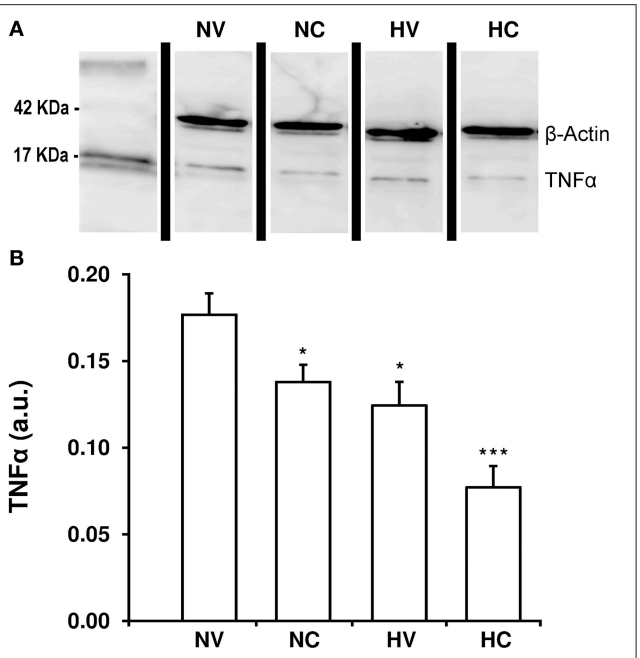

FIGURE 4 | Effect of the treatments on the characteristics of neuroinflammation. (A) A representative image of immunoblotting using anti-TNFα antibody, carried out on the samples from 1- to 2-day-old piglets after hypoxic-ischemic insult, treated with normothermia or hypothermia and administered with either vehicle or cannabidiol. **(B)** Densitometric analysis of relative TNFα contents. β-actin was used to normalize for the differences in protein loading between lanes in the blot. Bars represent mean ± SEM of 6-8 experiments. (a.u.) arbitrary units; (*) $P < 0.05$ vs. NV group by Kruskall-Wallis one-factorial analysis of variance; (***) $P < 0.05$ vs. all groups by Kruskall-Wallis one-factorial analysis of variance.

oxidative stress, it has been reported that hypothermia reduces the HI-induced increase in protein carbonyl formation seen in the piglet brain 6 h after HI (Mueller-Burke et al., 2008). This effect was confirmed in our experiments. In the present study we have found that hypothermia reduces TNFα production in the HI-injured piglet brain. There are no previous reports on the effects of hypothermia on TNFα production in the brain of newborn pigs *in vivo*. Other authors have reported that hypothermia reduces the LPS-induced increase in TNFα production seen in cultured microglial cells (Schmitt et al., 2007). It was found that gaseous hypothermia can modulate inflammation and phagocytosis in aged rat brain after cerebral ischemia (Joseph et al., 2012; Sandu et al., 2016). In addition, it is known that hypothermia modulates the serum cytokine increase observed in HI infants (Jenkins et al., 2012).

This work confirms that CBD is also a pleiotropic substance that modulates excitotoxicity (blocking the increase in Glu/NAA ratio), oxidative stress (preventing the increase in carbonylated proteins) and inflammation (reducing the increase in TNFα production) in HI-injured animals at short-term outcome. CBD reduces both the *in vitro* and *in vivo* release of glutamate, as previously demonstrated in slices of newborn mice forebrain exposed to oxygen-glucose deprivation (Castillo et al., 2010), and in the brain of HI rats (Pazos et al., 2012). This compound is a potential antioxidant either due to its molecular properties (Hampson et al., 1998) or because it can modulate the expression of iNOS and free radicals after HI (Johnston et al., 2011; Juul and Ferriero, 2014). In previous studies, we have shown that the significant release of free radicals associated with iNOS after HI, is especially harmful in newborn mice (Castillo et al., 2010). Further, it reduces the release of cytokines (IL-1, IL-6, TNFα) after an HI insult, as demonstrated in newborn rodents *in vitro* (Castillo et al., 2010) and *in vivo* (Pazos et al., 2012).

Interestingly, the combination of hypothermia and CBD produced an evident accumulative effect acting on the same characteristics of excitotoxicity, oxidative stress and inflammation compared to either CBD or hypothermia alone. This finding suggests that the mechanism by which CBD modulates those factors does not interfere with that of hypothermia. On the contrary, it suggests that CBD and hypothermia probably act complementarily on brain-damaging

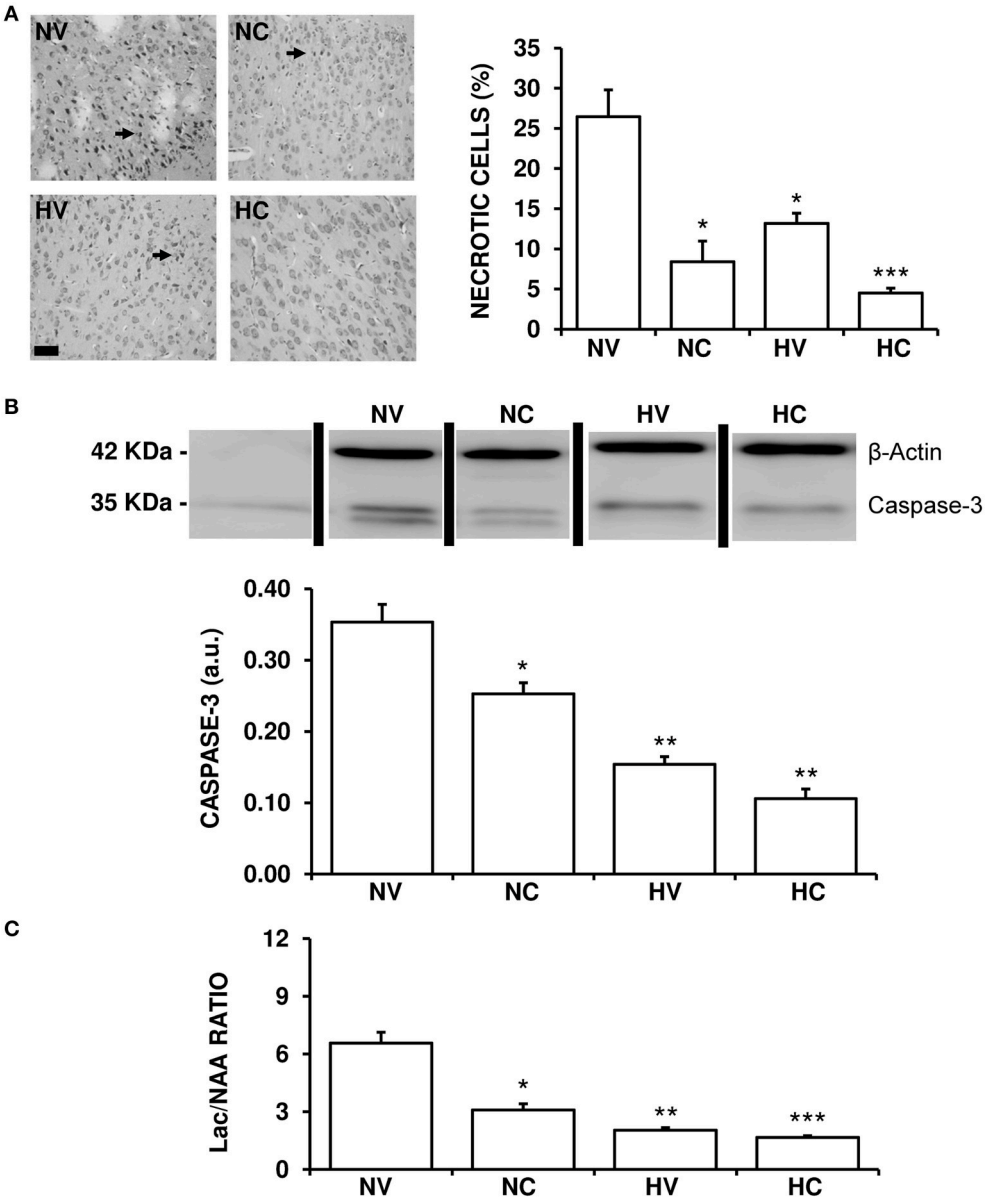

FIGURE 5 | Effect of the treatments on the characteristics of brain lesion. (A) Representative light microphotographs of Nissl-stained brain sections, obtained from 1- to 2-day-old piglets after hypoxic-ischemic insult, treated with normothermia or hypothermia and administered with either vehicle or cannabidiol. The number of pyknotic cells was significantly higher in the NV group (arrows), while it was lower in the hypothermia and cannabidiol-treated groups. Original magnification x200, white bar: 100 μm. **(B)** Top: A representative image of immunoblotting using an anti-Caspase-3 antibody, carried out on the samples from 1- to 2-day-old piglets after hypoxic-ischemic insult, treated with normothermia, or hypothermia and administered with either vehicle or cannabidiol. Bottom: Densitometric analysis of relative caspase-3 contents. β-actin was used to normalize for differences in protein loading between lanes of the blot. **(C)** Changes in Lac/NAA ratio obtained by H^+-MRS analysis of brain samples from 1- to 2-day-old piglets after hypoxic-ischemic insult, treated with normothermia or hypothermia and administered with either vehicle or cannabidiol. Lac: lactate; NAA: N-acetylaspartate. In all figures, bars represent mean ± SEM of 6–10 experiments. (a.u.) arbitrary units. (*) $P < 0.05$ vs. NV group by Kruskall-Wallis one-factorial analysis of variance; (**) $P < 0.05$ vs. both normothermic groups (NV and NC) by Kruskall-Wallis one-factorial analysis of variance; (***) $P < 0.05$ vs. all groups by Kruskall-Wallis one-factorial analysis of variance.

pathways during the same phase or at different time intervals. This indicates that CBD is potentially a good partner for hypothermia treatment. In addition, CBD showed a very similar profile to hypothermia regarding its effects on the parameters characterizing brain damage after HI. This indicates that CBD might be, in addition to an important adjunct to hypothermia, a valuable alternative whenever the latter is not feasible.

Previously, we have reported that HI injury induced an increase in the number of necrotic neurons in the cortex. Both hypothermia (Mueller-Burke et al., 2008) and CBD alone

(Alvarez et al., 2008; Pazos et al., 2013) reduced the neuronal death in hypoxic-ischemic piglets, as early as 6 h after the injury. Caspases, including caspase-3, are good markers of the activation of apoptotic pathways (Drury et al., 2014). It has been demonstrated that CBD reduces the efflux of caspase-9 as observed in newborn mice forebrain slices exposed to oxygen glucose deprivation (Castillo et al., 2010), and hypothermia reduces caspase-3 expression in newborn rats and fetal sheep (Drury et al., 2014). However, hypothermia alone did not significantly reduce the number of TUNEL+ and caspase-3 immunoreactive cells in the cortex of piglets, 48 h after a hypoxic-ischemic insult (Faulkner et al., 2011). Although no additive effect on caspase-3 was observed, the disagreement between these and our results may be due to the different follow-up periods and/or the different methods used to induce HI brain damage.

Lactate accumulates in the brain after HI as a result of mitochondrial dysfunction, whereas NAA content decreases when the number of viable neurons and oligodendrocytes decreases; thus, the Lac/NAA ratio is an excellent indicator of hypoxic-ischemic brain injury (Vial et al., 2004; Munkeby et al., 2008). In hypoxic-ischemic piglets, Lac/NAA ratio is closely correlated with the results from TUNEL and microglial ramification studies (Faulkner et al., 2011). In our study, both CBD and hypothermia alone attenuated the HI-induced modification in Lac/NAA in the cortex, in agreement with previous reports (Faulkner et al., 2011; Pazos et al., 2013). Moreover, the combination of hypothermia and CBD produced the best protective effects, being the most effective treatment to reduce histological brain damage and the Lac/NAA ratio. Well-known neuroprotective agents, such as xenon (Chakkarapani et al., 2010; Faulkner et al., 2011) or melatonin (Robertson et al., 2013), have also been demonstrated to augment hypothermia neuroprotection in the brain of injured piglets. We cannot rule out the possibility that brain damage was somehow mitigated in NV animals, since the use of dopamine infusion could reduce brain damage in some experimental models of newborn pigs (Park et al., 2003). However, neither CBD nor the combination of the therapies were associated with acute hypotensive episodes that would require inotropic support. In addition, a remarkable recovery of aEEG amplitude was observed in HC but not in HV animals. A similar effect has been previously described for CBD in asphyxiated piglets (Alvarez et al., 2008; Pazos et al., 2013) in association with the improvement of brain metabolism and the reduction of brain edema and histological damage. In this case a possible additive effect of CBD and hypothermia could not be assessed since hypothermia led to the flattening of aEEG trace throughout the experiment, as previously reported in similar models (Robertson et al., 2013).

Although providing encouraging results, the main limitation of our work is the short duration of the follow-up after the HI insult. In immature brain, the initial HI insult is usually followed by a latent period lasting for 6–24 h. During this period the aforementioned "deadly triad" (excitotoxicity, inflammation and oxidative stress) initiates various pathological processes that eventually lead to a secondary deterioration due to delayed energy failure (Johnston et al., 2011; Drury et al., 2014; Juul and Ferriero, 2014). Once initiated, most of these processes are thought to be irreversible (Drury et al., 2014). Theoretically, therefore, neuroprotective strategies must be applied prior to the onset of such irreversible processes (Drury et al., 2014). That is why the window for initiating neuroprotective strategies, such as hypothermia, is up at the start of the latent period, which usually is 6 h following HI insult. Hence, studying whether a neuroprotective treatment can effectively modulate excitotoxicity, inflammation and oxidative stress before the latent phase is a necessary early step to establish whether such treatment can be considered potentially useful (Mueller-Burke et al., 2008). In this context, our data suggest that CBD combined with hypothermia has beneficial modulatory effects when applied at short term. However, since the aforementioned processes are still working beyond the latent phase spreading and aggravating the damage (Drury et al., 2014), neuroprotective strategies are administered for at least 48 h in experimental conditions (Chakkarapani et al., 2010; Faulkner et al., 2011; Robertson et al., 2013) or, as for hypothermia (Johnston et al., 2011; Drury et al., 2014; Juul and Ferriero, 2014), in the clinical practice. It has been demonstrated, for instance, that gaseous hypothermia has different therapeutic effects on aged rats after cerebral ischemia depending whether the treatment was administered for 24 or 48 h (Joseph et al., 2012; Sandu et al., 2016). Another limitation of our study is the short lapse between the end of the HI insult and the initiation of the treatment. This short lapse is useful in terms of studying the effects of the treatments on the different cellular processes initiated by HI but is unrealistic in clinical terms. Therefore, before considering this treatment for clinical use it would be necessary to determine whether the neuroprotective effects of cannabidiol combined with hypothermia still act throughout the secondary deterioration period, which will require further experiments extending the monitoring period beyond 48 h after HI and with a more prolonged lapse between HI and treatments. Finally, other interventions, like anesthesia, can also significantly modulate the damaging processes and interact in exactly the same way in all anesthetized animals acting as potential neuroprotective agents. However, ethical aspects preclude the elimination of the anesthesia/analgesia and their effects cannot be specified.

In conclusion, CBD and hypothermia applied at short term act on the same processes related to HI brain damage, modulating excitotoxicity, inflammation, and oxidative stress. These two therapies in combination do not compete which each other in modulating these processes, but rather produce additive effects, resulting in greater overall benefit. Whether or not this combination produces effective long-term additive neuroprotection should be tested using longer follow-up studies and a "realistic" delay of treatment.

AUTHOR CONTRIBUTIONS

JM and FA have designed the experimental design in accordance with international ARRIVE guidelines. HL, MP, NM, MS, MA, JM, and FA have performed the *in vivo* experimental

phase. HL, MP, AA, and MA have performed the *in vitro* experimental phase. AA, JM, and FA have contributed with reagents/materials/analysis tools. HL, JM, and FA have been responsible for data analysis. HL, MP, AA, NM, MS, MA, JM, and FA have collaborated and approved the final manuscript version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2016.00323

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Therapeutic benefits of nanoparticles in stroke

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Stroke represents one of the major causes of death and disability worldwide, for which no effective treatments are available. The thrombolytic drug alteplase (tissue plasminogen activator or tPA) is the only treatment for acute ischemic stroke but its use is limited by several factors including short therapeutic window, selective efficacy, and subsequent haemorrhagic complications. Numerous preclinical studies have reported very promising results using neuroprotective agents but they have failed at clinical trials because of either safety issues or lack of efficacy. The delivery of many potentially therapeutic neuroprotectants and diagnostic compounds to the brain is restricted by the blood-brain barrier (BBB). Nanoparticles (NPs), which can readily cross the BBB without compromising its integrity, have immense applications in the treatment of ischemic stroke. In this review, potential uses of NPs will be summarized for the treatment of ischemic stroke. Additionally, an overview of targeted NPs will be provided, which could be used in the diagnosis of stroke. Finally, the potential limitations of using NPs in medical applications will be mentioned. Since the use of NPs in stroke therapy is now emerging and is still in development, this review is far from comprehensive or conclusive. Instead, examples of NPs and their current use will be provided, as well as the potentials of NPs in an effort to meet the high demand of new therapies in stroke.

Keywords: blood-brain barrier, ischemic stroke, nanoparticles, drug delivery, diagnostic tool

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Introduction

Stroke is a disease, which occurs unexpectedly and has a disastrous outcome. Approximately 15 million people will experience a stroke episode every year worldwide of which 33% is left with a permanent disability whereas ∼40% of the cases will result in death (Go et al., 2014). Due to the high impact of the disease around the world, stroke is ranked as the second deadliest disease for individuals surpassing 60 years of age, and fifth among individuals of ages 15–59 (The European Stroke Initiave Executive Committee and the Eusi Writing Committee, 2003). The major issue concerning stroke is the lack of effectiveness of the current diagnostic tools. The coexisting etiological factors make it more difficult to determine the direct cause of the disease, and although the routine hematological and biochemical tests regarding stroke patients are carried out, hematological disorders cannot be accurately assessed. According to recent meetings of the American Heart Association, about 87% of strokes, appear to be cases of ischemic stroke (Go et al., 2014).

Abbreviations: BBB, Blood-brain barrier; NPs, Nanoparticles; CNS, central nervous system; GLUT1, glucose transporter 1; TEER, Trans-endothelial electrical resistance; PEG, polyethylene glycol; ROS, reactive oxygen species.

At the event of an ischemic stroke, the affected locus shows apoptosis of the cells. Despite the complexity of the events which occur during a stroke, the surrounding area (penumbra) can be rescued, as long as the appropriate drugs are administered in an early time frame (Zhang et al., 2015). In the molecular level, the oxidative stress caused by an ischemic event significantly affects the BBB. When the barrier's integrity is compromised the junctional complex formed by the endothelial cells allows for vascular fluid components (white cells, prostaglandins, amino acids etc.), to cross to the abluminal side of the brain. As these cells were never meant to exist within the central nervous system (CNS), it is believed that they contribute toward the progression of a vasogenic edema (Pillai et al., 2009).

The damage from an ischemic stroke does not apply only to the barrier's junctional complex, but also to the cells which form and support the blood-brain barrier itself. Lack of oxygen around the area of the endothelial cells (cell hypoxia) revealed up-regulation of the glucose transporter 1 (GLUT1) (Yeh et al., 2008). The endothelial cells are fortified by the presence of pericytes, which are important for the formation of new microvasculature, both during embryogenesis as well as in adult life (Bergers and Song, 2005). Additional support to the BBB comes from the astrocytes found in the brain. The astrocytic end-feet from these cells showed important Trans-Endothelial Electrical Resistance (TEER) increase when co-cultured with endothelial cells (Abbott et al., 2006). Chemical compromise of the astrocytes leads to a chain reaction where pericytes are immediately affected, occludin is reduced within the junctional complex, and the BBB is presented with increased permeability (Willis et al., 2004; Bonkowski et al., 2011).

Treatments of Today

The most important parameter during the event of a stroke is time. The activation of cells, which respond to brain injury or even cell death signaling, occurs in a matter of minutes. As previously mentioned the surrounding area which was affected by stroke can be salvaged and that is due to the indirect effect that the disease has on neighboring cells (Arai et al., 2011).

Drug administration to the area is desirable but less blood covering the area would also translate to less quantities of drug being delivered to the cells. Moreover, the BBB breakdown during a stroke is not an irreversible event. Mechanisms of cell repair are activated in order to restore the barrier's function (Liu et al., 2010) thus the direct administration of drugs in the brain is not ideal. At the moment there are developments in research in order to tackle the issue of drug delivery during stroke events, but the only commercially-available pharmaceutical agent is recombinant tissue plasminogen activator (rt-PA) (Jahan and Vinuela, 2009). The plasminogen activator allows the oxygenation of the damaged locus as well as partial restoration of the blood flow. This therapy is again highly depended on time in order to be effective (not as beneficial if it exceeds a 3–4 h window) but also increases the risk of bleeding if the damage is excessive (Messe et al., 2012). Provision of anticoagulants to the patients is the only other commercially available option but again anticoagulants are entirely used as precaution to future or suspected episodes, and in no circumstances can repair or shield the affected area from further damage (Chen et al., 2000).

BBB Breakdown after Stroke and Secondary Neuronal Damage

It has been previously shown that in an event of stroke, the BBB is severely compromised and incapable of re-establishing regular function for as long as 7 days post-trauma (Lakhan et al., 2013). The extended period of this breakdown could lead to further neuronal damage both from host as well as foreign components. During stroke a complex cascade of events is present (i.e., disruption of ion channels, excitotoxicity, inflammation, activation of apoptotic pathways). The occurrence of these events could lead to further neuronal damage (secondary neuronal damage) (Chen, 2012). The majority of this damage will not be detected in the early stages of a stroke; neuronal injury/cell death can progressively continue even months after the initial event (Dihne et al., 2002; Baron et al., 2014). Bacterial infection, head injury, stroke, or even autoimmune diseases can be involved in this secondary damage and lead to severe disorders such as motor impairments and neuropsychiatric illness (Chen et al., 2014). It has been previously demonstrated that the BBB may not be the only accessible barrier for effective brain injury treatment. According to Chen Y., monoisonitrosoacetone (which can cross the BBB with ease) does not reinstate the normal levels of aceltylcholinesterase (AChE) in the cholinergic nervous system. During an organophosphate (OP)-induced damage event, administration of therapeutic substances which are BBB-specific could lead to progression of a secondary neuronal damage and irreversible neurological deficits (Chen, 2012).

Crossing the BBB during Stroke

Due to the type of the disease, treatment needs to be target-specific where the administrated drugs will be able to preserve their molecular structure thus therapeutic abilities (bioavailability). The BBB has always been a constant boulder against efficient drug delivery. Usually "BBB-proof" drugs are characterized by increased lipophilicity and a small molecular structure/weight; regardless of the increased probability to successfully pass the BBB, studies have shown that drug delivery across the BBB has a crossing success rate of 4–7% (Sierra et al., 2011). In parallel with the issue of poor drug delivery, additional problems rise such as lack of systematic drug dosage, decomposition or even chemical absorption by the host's metabolic system (Ghosh et al., 2010). Increased rates of drug delivery can be achieved with vasodilation drugs. Stretching of the BBB junctional complex occurs which allows to molecules of higher molecular weight or different surface charge to cross to the abluminal side of the brain with minimal restrictions. Complete dissociation of the junctional complex also allows other molecules to penetrate the brain locus which are considered toxic to the brain and may cause more damage rather than protect during a cerebral edema (Sasaki et al., 1986; Beletsi et al., 1999). So according to the issues raised above, developing a drug for

stroke disease must have high sustainability rates in order to go against the host's metabolism and natural defenses. Like in daily life, the transportation of goods by appropriately conditioned means, allows the product to stay in its initial form as well as in its optimum condition for a longer period of time. A similar delivery of goods into the brain can be achieved by the use of NPs.

Drug Delivery and NPs

A NP which is designed for drug delivery purposes can derive from materials with artificial characteristics or materials with an organic background. Micelles, inelastic spherical shells, nanotubular particles, liposomes, golden NPs, and polymers fall in the category of NPs (Mc Carthy et al., 2014). The use of the NP as an agent carrier can be achieved in various ways (encapsulation or conjugation with some of the host's components). Enhancement of the NP as a drug delivery element is accomplished by the determination of the NP's polarity, the introduction of a surface receptor recognized by endothelial cells/immunity cells (phagocytes), or the prolongation of their lipoid acid chain in an effort to increase lipophilicity (Mc Carthy et al., 2014). The thickness of the NP's capsule and the capsule's size are significant factors for increased therapeutic abilities. The sizes of NPs can range between 1 and 300 nm regardless of the type that is used. With variation in sizes the core to surface ratio changes. For example, smaller NPs have a smaller core to surface ratio, which allows a drug to be immediately released once the NP's membrane is breached. Larger NPs are not preferred due to the uneven drug release that may occur; inefficient drug delivery would take place if the NP is preventing its complete release due to either slow NP decomposition or entrapment of the drug within the particle's compartment (Singh and Lillard, 2009). The time of release for a drug is important, since an early release into the blood-stream results in decomposition by the host's metabolism and clearance from the host's system (Desai et al., 1997). Increased specificity regarding the destination of a drug can be achieved through the natural process of transcytosis. The versatility to adapt with other cellular components or be fused with them (i.e., antibodies, peptides) puts the NPs in a favorable position regarding their use as drug carriers.

Examples of Chemical Agents that Enhance NP Efficiency

The surfacing of NPs with the polyethylene glycol (PEG) polymer creates a protective layer, which increases the lifespan of the NP in the circulatory system, and also enhances the NP's abilities to follow a transcytosis route (Xie et al., 2012; Alyautdin et al., 2014). NPs with a polysorbate coat have shown enhanced abilities to cross the BBB through endocytotic routes by binding to specific lipoproteins found on the surface of endothelial cells. The use of different polymers in combination, showed enhanced control regarding drug delivery and release. The use of polyethylene oxide (PEO) with lactic acid-*co*-glycolic acid (PGLA) showed that the desired drug can be found within the host's nervous system (Singh and Lillard, 2009). Targeting the transporters that are associated with the BBB is one of the best ways to deliver a drug in the CNS with high accuracy. For example, conjugation of liposomes with different molecular weights of PEG leads to the linkage of glucose with cholesterol; unavoidably the glucose-part of the fusion molecule is recognized by the brain endothelial cell transporter GLUT1 which grants access to the liposomes in a "Trojan horse" manner (Xie et al., 2012). The use of NP's is not limited to encapsulation of drugs or conjugation with surface receptors. Recent studies show that specific NP's can function as scavengers of reactive oxygen species (ROS). These free radicals increase during cerebral ischemia, especially after reperfusion. For example, platinum nanoparticles (nPts) appear to scavenge superoxide anions as well as hydrogen peroxide, when tested *in vivo* (Takamiya et al., 2012). The same scavenging properties are presented by ceria NPs; during cerium's oxidative changes (reduction—oxidization), oxygen binding occurs in a way that is similar to the biological antioxidants. These NPs can also be efficient in ultra-small scales (i.e., 4 nm) making them excellent candidates for treatments against stroke (Kim et al., 2012).

NPs as a Diagnostic Tool in Stroke

From the research that was recently performed, we can observe that future perspectives in regards to NPs and stroke therapy are promising. With the increasing number in types of NPs (Mc Carthy et al., 2014), parameters such as time of drug release, NP size, tolerance against degradation by blood components, will not be an obstacle toward drug delivery in an ischemic brain. Drugs that showed promising results *in vitro* such as statins or candesartan can now be encapsulated in NPs, which can use endocytotic or transcytotic routs in order to cross the BBB (Sierra et al., 2011; So et al., 2014). A combination of old and new ideas can be combined where labeled antibodies of CNS injury biomarkers such as S100 calcium binding protein B (S100B), vascular cell adhesion molecule (VCAM), glial fibrillary acidic protein (GFAP) etc., can be conjugated with NPs in order to quickly detect them using a computed tomography (CAT) scan or magnetic resonance imaging (MRI) (Jickling and Sharp, 2011). The ability of NPs to endure degradation could be used in order to circulate NPs, conjugated with blood clotting biomarkers in an effort of early diagnosis or even the disruption of a molecular cascade that leads to thrombosis (Jickling and Sharp, 2011). Steps toward the diagnostic use of NP's have been made by Kevin Y. Lin and his colleagues who have conjugated thrombin-sensitive peptide substrates on the surface of the NPs and can detect the changes of thrombin levels in the circulatory system providing this way constant monitoring for coagulation (Lin et al., 2013).

Stroke Therapy and NPs

Until very recently the NPs were used for experimental therapies, preclinical studies as well as early phase clinical trials (Dobrovolskaia et al., 2008; Jain et al., 2012). Despite of the current developments in NP research, the use of NPs in regards to CNS diseases (as well as stroke) is still under development. Studies show that cytidine 5′ diphosphocholine has neuroprotective abilities regarding reperfusion and ischemia

TABLE 1 | Modification of nanoparticles (NPs) to improve specificity against the central Nervous System (CNS).

Modification of NPs	Target of modification	Use of NPs
Introduction of a surface receptor	Adjusted polarity and lipopholicity	Drug delivery
Adjustment of size and thickness	Core to surface ratio	Drug delivery/release
Surfacing with PEG	Indurance and transcytotic pathways	Drug delivery
Liposome conjugation	Regognition by the GLUT1 transporter	Drug delivery
Vasolidation drugs (indirect)	Increased drug delivery in the CNS	Drug delivery
Compromised BBB (indirect)	Increased drug delivery in the CNS	Drug delivery
Specificity in the type (i.e., nPts)	Binding of ROS	Therapeutic
Conjugation with labeled antibodies	Detection through CAT/MRI	Diagnostic
Conjugation with clotting biomarkers	Monitoring thrombin levels	Diagnostic/Therapeutic

(Hurtado et al., 2011). Encapsulation of this drug in the appropriate NPs could allow the drug to travel through the circulatory system into the CNS. Current studies show that encapsulation of the basic fibroblast growth factor (bFGF) or the caspase-3 peptide inhibitor (z-DEVD-FMK) into chitosan-NPs, showed high numbers of NPs crossing through the BBB(Yemisci et al., 2014). This was achieved by inducing a receptor-mediated transcytosis of the transferrin receptor-1, which is found on the endothelial cells forming the BBB. The great numbers of bFGF or z-DEVD-FMK found within the brain showed reduce blood loss after a 2-h artery occlusion in the middle cerebral locus. High success rates of the administration of the drug gave access to a 3-h therapeutic window (Yemisci et al., 2014). A more recent study by Alice Gaudin and associates showed that squalenoyl adenosine (SQAd) which is rapidly metabolized and cleared from the bloodstream, can preserve 75% of the initial administration intact in mouse plasma. Adenosine's hydroxyl groups are protected by the use of tert-Butyldimethylsilyl chloride (TBDMSCl) in order for the squalenoyl group to connect with the amino group of the adenosine. De-protection of the hydroxyl ends leads to nanoprecipitation and the formation of squalenoyl adenosine nanoassemblies (SQAd NAs). The ability of SQAd NAs to breach the CNS showed promising improvements of the neurologic deficit score in mice, which relates to cerebral ischemia (Gaudin et al., 2014).

Summary

Although promising, the use of NPs in drug delivery is still under development and as previously mentioned, stroke initiates a series of events which can occur simultaneously or even for prolonged periods of time. While a compromised (open) BBB is not desirable, the 7 days post-trauma window where the BBB remains open can be used to cross NPs which could act as neuroprotective agents or even as diagnostic tools. From the studies that have been carried out, it was shown that NPs lose their efficiency as their size increases. Large NPs will either not be absorbed by the desired tissue, or the polymeric chain forming the NPs may end up blocking the recognition point of the NP from the desired surface receptor. Another obstacle is the undefined amount of drug that can be absorbed by a NP. If a drug is greatly absorbed, the amount of drug released or even the time of its release might not be beneficial in regards to drug delivery. As expected, inability of the NP to absorb the drug will result in the release of a significant amount which might not be beneficial if the desired drug is meant to be released as part of a chronic therapy (Singh and Lillard, 2009; Xie et al., 2012). Various alterations of the NPs could improve their efficacy in regards to drug delivery or their potential use in diagnostics/therapeutics (**Table 1**).

The latest developments in NP research show more types of polymers being used as NPs. One of the variables that can be altered in order to resolve the problem of low drug amounts crossing the BBB is the increased amount of NPs carrying the desirable drug. Increase of the dose will eventually result in increased toxicity toward the cells. M. Kolter et al., showed that significant reduction in cell viability won't occur unless dosages of 500 μg/ml or higher are used. Nevertheless, TEER is vastly affected with dosages as little as 15 μg/ml. By allowing the dissociation of the BBB we are also allowing the entrance of foreign (to the CNS) cells (i.e., white cells). This may lead toward a worsen outcome instead of an improvement (Kolter et al., 2015). Further adjustments of the parameters involved in NP research will eventually result in an optimized model, where NPs will be used as drug delivery vessels with minimum complications as well as high success rates.

To conclude, stroke is a time-sensitive disease and early diagnosis determines the patient's outcome. Unfortunately, stroke therapy is bounded by many limitations and its therapeutic window is significantly small. An effective compound should be able to provide neurological protection not just by targeting the BBB route but also through other CNS barriers in order to increase the potential of a neuroprotective agent in the brain. A spherical view of therapeutics should be adopted and take into account both primary and secondary neuronal damage. The advancements in NPs research can lead to their use as a diagnostic tool, a therapeutic tool or even the combination of both in order to prevent stroke from developing or leaving permanent neurological deficits.

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