MECHANISMS OF INNATE NEUROPROTECTION

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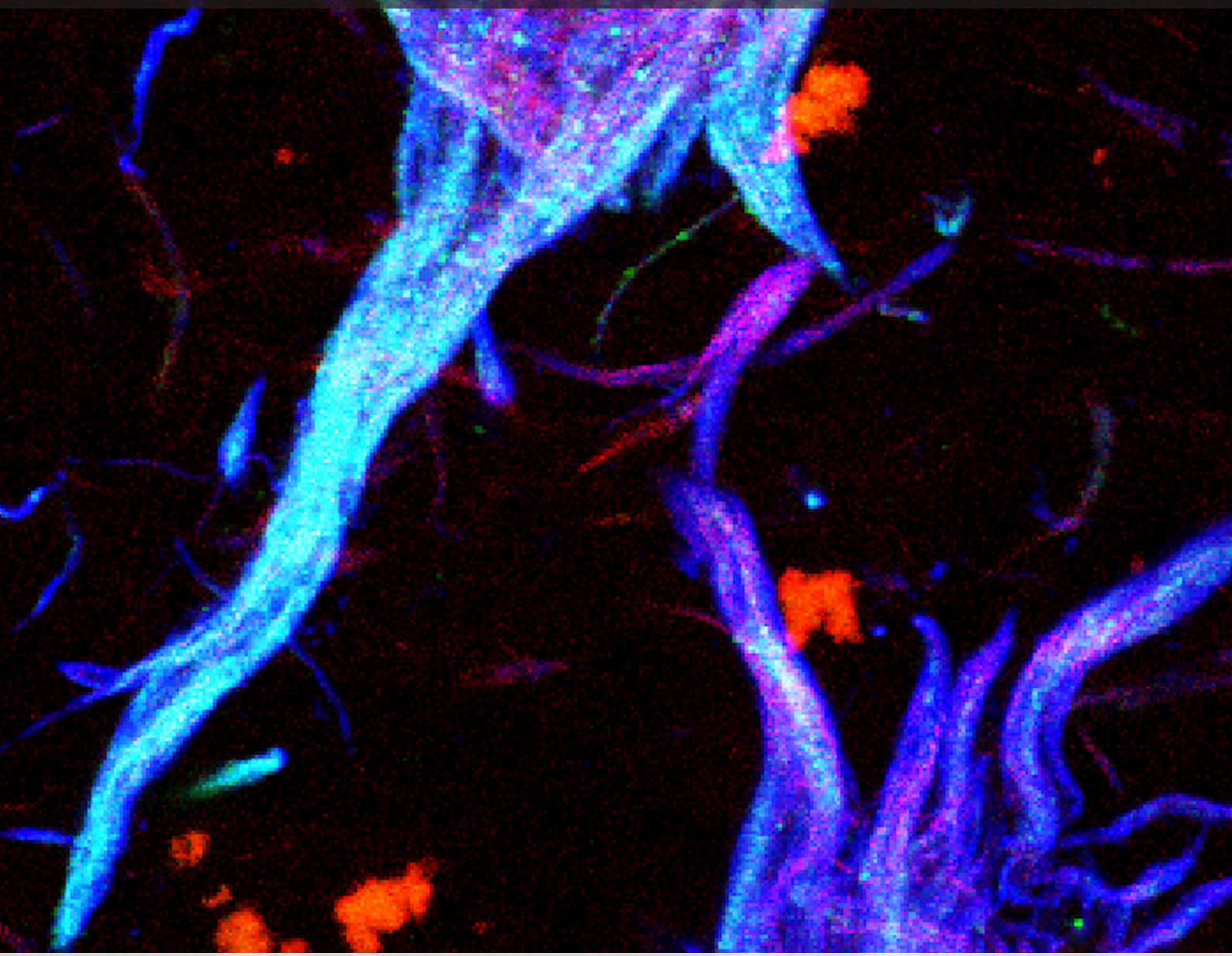

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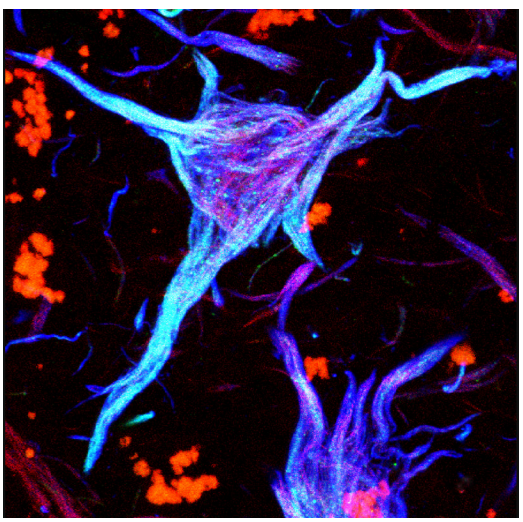
MECHANISMS OF INNATE NEUROPROTECTION

Topic Editor:
Giuseppe Pignataro, Federico II University of Naples, Italy



Double immunolabeling of tangles with antibodies. Intracellular neurofibrillar tangles (purple) were detected by both pS396 (green) and AD2 (blue) antibodies.

Figure taken from: Flores-Rodríguez P, Ontiveros-Torres MA, Cárdenas-Aguayo MC, Luna-Arias JP, Meraz-Ríos MA, Viramontes-Pintos A, Harrington CR, Wischik CM, Mena R, Florán-Garduño B and Luna-Muñoz J (2015) The relationship between truncation and phosphorylation at the C-terminus of tau protein in the paired helical filaments of Alzheimer's disease. Front. Neurosci. 9:33. doi: 10.3389/fnins.2015.00033

As clinical trials of pharmacological neuroprotective strategies in stroke have been disappointing, attention has turned to the brain's own endogenous strategies for neuroprotection. Two endogenous mechanisms have been recently characterized, ischemic preconditioning and ischemic postconditioning. In the present topic newly characterized mechanisms involved in preconditioning- and postconditioning- neuroprotection will be discussed. The understanding of the mechanisms involved in the neuroprotective pathways induced by preconditioning and postconditioning will be clinically relevant for identifying new druggable target for neurodegenerative disorder therapy. Furthermore, the importance of these neuroprotective strategies resides in that it might be easily translatable into clinical practice. Therefore, the data presented here will highlight the capacity of ischemic preconditioning and postconditioning to be of benefit to humans.

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Table of Contents

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EDITORIAL
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Editorial: Mechanisms of Innate Neuroprotection

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Keywords: preconditioning/postconditioning, epigenetic, ionic homeostasis, microRNA, stroke, seizures, Alzheimer's disease

The Editorial on the Research Topic

Mechanisms of Innate Neuroprotection

Two endogenous mechanisms of neuroprotection have been recently described: ischemic preconditioning and ischemic postconditioning. In fact, the concept of tolerance has been introduced long time ago and, remaining in the modern era of medicine, in 1927, the Austrian physician Julius Wagner-Juaregg was awarded the Nobel Prize in medicine for his research on pyrotherapy to treat psychiatric disorders. At that time, patients affected by dementia paralytica were preconditioned by the inoculation of malaria parasites (1).

In the present Research Topic, newly characterized mechanisms involved in preconditioning and postconditioning neuroprotection will be discussed in order to provide tools to plan strategies which induce, mimic, or boost these endogenous protective responses.

In particular, this Research Topic discusses the most important mechanisms involved in preconditioning and postconditioning, presenting a series of papers that provide up-to-date, state-of-the-art information on molecular and cellular mechanisms involved in the neuroprotective process elicited by these endogenous neuroprotectant strategies during brain ischemia and other neurological disorders, such as seizure and Alzheimer. In particular, these aspects are faced by tackling multiple angles of this complex phenomenon. In the first part of the book, three chapters are dedicated to the innate immune system and inflammation, giving special attention to the role of microglia (Fumagalli et al.), macrophages (Amantea et al.), and to some important modulators such as NF-kB (Lanzillotta et al.). The core of the Research Topic is dedicated to posttranscriptional modifications occurring during pre- and postconditioning. These aspects are discussed in seven chapters, including reviews (Saugstad; Jimenez-Mateos; Aune et al.), hypothesis and theory paper (Gidday), and original research articles (Reynolds et al.; Meller et al.; Flores-Rodríguez et al.), that highlight the importance of epigenetic modifications and their roles in mediating pre- and post-conditioning neuroprotection. The role of non-coding RNA, with particular regards to microRNA, in innate neuroprotection is summarized in two up-to-date papers (Saugstad; Jimenez-Mateos). Experimental manipulation of miRNAs and/or their targets to induce pre- or post-stroke protection is also presented, as well as discussion on miRNA responses to current post-stroke therapies. Finally, in the last two chapters are described cellular mechanisms for neuroprotection, giving a special attention to those proteins involved in ionic homeostasis maintenance. In fact, although the mechanisms through which these two endogenous protective strategies exert their effects are not yet fully understood, recent evidence suggest that the maintenance of ionic homeostasis plays a key role in propagating these neuroprotective phenomena. In this last part of the book, it will be reviewed the role of plasmamembrane transporters and ionic channels involved in the control of ionic homeostasis and taking part to the neuroprotection induced by ischemic preconditioning and postconditioning, with particular regards to the Na^+/Ca^{2+} exchangers (NCX), the plasma

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membrane Ca^{2+}-ATPase (PMCA), the Na^+/H^+ exchange (NHE), the $Na^+/K^+/2Cl^-$ cotransport (NKCC), and the acid-sensing cation channels (ASICs) (Cuomo et al.). An interesting view from inside organelles is provided in the review by Sisalli et al.

We hope that this Research Topic will stimulate the continuing efforts to understand the cell and physiological mechanisms underlying the origin of endogenous neuroprotective mechanisms. The understanding of the mechanisms involved in the neuroprotection elicited by preconditioning and postconditioning will be clinically relevant for it will contribute to discover new druggable targets in neurodegenerative disorder intervention. Therefore, the data presented here will highlight the capacity of ischemic preconditioning and postconditioning to be of benefit to patients.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW ARTICLE
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The ischemic environment drives microglia and macrophage function

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Cells of myeloid origin, such as microglia and macrophages, act at the crossroads of several inflammatory mechanisms during pathophysiology. Besides pro-inflammatory activity (M1 polarization), myeloid cells acquire protective functions (M2) and participate in the neuroprotective innate mechanisms after brain injury. Experimental research is making considerable efforts to understand the rules that regulate the balance between toxic and protective brain innate immunity. Environmental changes affect microglia/macrophage functions. Hypoxia can affect myeloid cell distribution, activity, and phenotype. With their intrinsic differences, microglia and macrophages respond differently to hypoxia, the former depending on ATP to activate and the latter switching to anaerobic metabolism and adapting to hypoxia. Myeloid cell functions include homeostasis control, damage-sensing activity, chemotaxis, and phagocytosis, all distinctive features of these cells. Specific markers and morphologies enable to recognize each functional state. To ensure homeostasis and activate when needed, microglia/macrophage physiology is finely tuned. Microglia are controlled by several neuron-derived components, including contact-dependent inhibitory signals and soluble molecules. Changes in this control can cause chronic activation or priming with specific functional consequences. Strategies, such as stem cell treatment, may enhance microglia protective polarization. This review presents data from the literature that has greatly advanced our understanding of myeloid cell action in brain injury. We discuss the selective responses of microglia and macrophages to hypoxia after stroke and review relevant markers with the aim of defining the different subpopulations of myeloid cells that are recruited to the injured site. We also cover the functional consequences of chronically active microglia and review pivotal works on microglia regulation that offer new therapeutic possibilities for acute brain injury.

Keywords: neuroinflammation, microglia, macrophages, acute brain injury, phenotypical polarization, cell morphology

CLASSICAL VIEW OF NEUROINFLAMMATION

In the late 19th century, Paul Ehrlich observed that a water-soluble viable dye injected into the peripheral circulation stained all organs except the central nervous system (CNS), providing the first indication that the CNS was anatomically separated from the rest of the body. The idea that the brain was a unique anatomical compartment was further confirmed by Edwin Goldman who showed that a dye injected into the spinal fluid did not stain peripheral tissues. This is of course due to the blood–brain barrier (BBB) that restricts access of soluble factors to the brain, including 98% of antibodies and immune cells. This feature together with the lack of a lymphatic system, low constitutive levels of major histocompatibility complex (MHC) class I and II molecules, local production of suppressive factors, and limited numbers of

Abbreviations: AD, Alzheimer's disease; ADAM, a disintegrin and metalloproteinase domain-containing protein; ADP, adenosine diphosphate; AIM2, absent in melanoma 2; ASC2, apoptosis-associated speck-like protein containing a CARD 2; ATP, adenosine triphosphate; BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; CC, corpus callosum; CNS, central nervous system; CX, cortex; DAMPs, danger-associated molecular patterns; DAP12; DNAX activation protein of 12kDa; EMR1, EGF-like module-containing mucin-like hormone receptor-like 1; GDNF, glial cell-derived neurotrophic factor; GFP, green fluorescent protein; HIF-1α, hypoxia-inducible factor-1α; HMGB1, high mobility group box 1; Hsp, heat shock protein; Iba1; ionized calcium-binding adaptor 1; ICAM; intercellular adhesion molecule; IFN-γ, interferon-γ; IGF-1, insulin-like growth factor-1; IL, interleukin; iNOS, inducible nitric oxide synthase; LAMP; lysosomal/endosomal-associated membrane glycoprotein; LPS, lipopolysaccharide; LV, lateral ventricle; μ, microglia; M, macrophage; MBL, mannose-binding lectin; MCA, middle cerebral artery; MCP-1, monocyte chemoattractant protein 1 (also known as CCL2); MCSF, macrophage colony stimulating factor (MCSF); MerTK, Mer receptor tyrosine kinase; MFG-E8, milk fat globule EGF-like factor-8; MHC, major histocompatibility complex; MSC, mesenchymal stem cells; NCX, sodium calcium exchanger; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP, NOD-like receptor protein; NO, nitric oxide; OGD, oxygen-glucose deprivation; PCR, polymerase chain reaction; PD, Parkinson's disease; POP1, pyrin-only protein 1; PRR, pattern recognition receptor; PTP, protein tyrosine phosphatase; RANTES, regulated upon activation normal T cell expressed and presumably secreted; rμ, rod microglia; ROS, reactive oxygen species; SCI, spinal cord injury; STR, striatum; TBI, traumatic brain injury; TLR, toll-like receptor; TNFα, tumor necrosis factor α; TGF-β, transforming growth factor β; TREM2, triggering receptor expressed on myeloid cells 2; WBC, white blood cells; WT, wild type.

professional antigen-presenting cells drove the concept of the CNS as an immuno-privileged site (1).

In normal conditions, the presence and trafficking of immune cells in the brain are negligible. However, we now know that the brain is far from being an inactive immune organ. It has actually its own resident immune population, microglia, in addition to the fact that the BBB can allow active import of immune molecules and cells. After injury, brain immunity includes a variety of events that develop non-linearly in response to multiple factors and involve complex interactions between cells and environmental signals. As a consequence, in CNS disorders, activation of distinct inflammatory pathways may affect the course of an injury in different and possibly opposing ways (2).

Cells of myeloid origin, such as microglia and macrophages, are major actors in brain inflammation, a hallmark of acute brain injury. Microglia reside in the CNS and actively monitor the surrounding microenvironment (3). Under physiological conditions, these cells play a variety of roles that go beyond classical inflammatory activity, including support to synaptic wiring during development and monitoring of neuronal firing in the mature brain, thus contributing to brain homeostasis (4, 5). After an acute CNS injury, microglia act mainly as a player of the immune system. Virtually all CNS disorders involve reactive microglia and the progression and resolution of many diseases also depend on microglial activity (6).

Microglia share the theater of their action with blood-borne macrophages, which infiltrate into the inflamed CNS. Both cell populations show a range of functional states, with a specific pattern of receptor expression, molecule production, and morphological feature acquisition. The inflamed environment plays a major role in the definition of their overall function by providing stimuli that induce specific phenotypical/functional states.

An open challenge is to properly characterize the roles of microglia and macrophages in brain injury progression and resolution. This would help define the milestones for effective manipulation of brain inflammation, with the specific aim of favoring its protective arm and boosting innate neuroprotective mechanisms. This review will look at the latest findings on inflammation in acute brain injury, specifically addressing the impact of environmental signals on the function of microglia and macrophages in injury and neuroprotection. We will specifically discuss (1) the latest view of neuroinflammatory mechanisms, depicting the rationale for focusing on myeloid cell protective modulation; (2) the ability of the inflamed environment to drive myeloid cell behavior, particularly their distribution, expression markers, and morphology; (3) the impact of selectively primed/modulated states of microglia on the exacerbation or resolution of neurological disorders.

CHANGING THE ANGLE: A NEW VIEW OF NEUROINFLAMMATION

Data accumulated over the last decade have deeply changed the general view of inflammation in the CNS. A striking new concept has been that brain immunity, in addition to the well-known pro-inflammatory actions, can initiate protective mechanisms by exploiting the bivalent nature of microglia and macrophages. *In vitro* experiments indicate that these cells may develop either a classic pro-inflammatory M1 or an alternative anti-inflammatory and pro-healing M2 polarization (7). *In vivo,* microglia and macrophages appear to acquire intermediate phenotypes, whose ultimate functions rely on the combination of different polarization markers ranging from M1 to M2 (2, 8). Multiple factors concur to determine myeloid cell functional states and may offer a way to therapeutically manipulate myeloid cell activation. From this viewpoint, microglia and macrophages seem at the crossroads of several inflammatory mechanisms, throughout the entire course of brain pathophysiological events. They can be viewed as *in situ* expert operators whose timely actions may contain and resolve brain injury. Experimental research is now dedicating considerable effort to understanding the rules that govern brain innate immunity, to implement strategies for manipulating the innate immune response to favor its protective functions (9).

In the inflamed CNS, activated microglia and recruited macrophages present some common features and some distinct characteristics. Common features include the expression of common phenotypic markers, the ability to polarize toward M1/M2 phenotypes, the phagocytic behavior, and the ameboid shape that activated microglia may acquire. Microglia and macrophages, however, differ in several aspects and recent work has attributed exclusive features to each of these cell populations. Microglia originate from primitive hematopoiesis in the fetal yolk sac, take up residence in the brain during early fetal development, and retain the ability to proliferate. By contrast, macrophages derive from granulocyte–monocyte progenitors during both development and adulthood (6, 10). Microglia have a lower turnover rate than macrophages: respectively 6 months and 17 h in mice (11). The activation of microglia depends on ATP/ADP signaling, whereas macrophages are equipped to maintain viability and function in hypoxia/ATP loss (3, 10). Finally, only microglia have a ramified morphology, with branches that emerge from the cell body and communicate with surrounding neurons and other glial cells (12, 13).

Whether these differences imply different roles in brain injury progression and repair has yet to be fully determined, though there is increasing evidence that microglia should be considered functionally distinct from macrophages (14, 15).

THE HYPOXIC ENVIRONMENT, A MAJOR CUE TO MICROGLIA AND MACROPHAGE ACTIVATION

After acute brain injury such as stroke, traumatic brain injury (TBI), subarachnoid, or intracerebral hemorrhage, a series of neurochemical processes is unleashed and gives rise to a complex pathophysiological cascade that can be viewed as cellular bioenergetic failure triggered by hypoperfusion (16). Hypoperfusion leads to hypoxia, which precedes and causes detrimental events, such as excitotoxicity, oxidative stress, BBB dysfunction, microvascular injury, hemostatic activation, post-ischemic inflammation and, finally, cell death (17, 18). All these events contribute to changing the ischemic environment over time and, consequently, the behavior of microglia and macrophages. Because hypoxia immediately follows hypoperfusion, it affects the brain myeloid cell response to injury early. Here, we discuss the effects of hypoxia on microglia and macrophage behavior and the different

activations and recruitments of these two populations in an ischemic environment.

MICROGLIA AND MACROPHAGE BEHAVIOR IN HYPOXIC CONDITIONS

Microglia consume energy in an ATP-dependent manner for their broad range of activities, including inflammatory mediator production (19) and cytoskeleton reorganization (20, 21). Microglia are thus highly susceptible to energy deficits and local changes in blood perfusion after acute injury probably affects microglia reactivity and survival.

Hypoxia induces a time-dependent autophagic cell death in microglia cultures with increased release of pro-inflammatory cytokines (IL-8 and TNFα) through the hypoxia-inducible factor-1α (HIF-1α) dependent pathway (22). HIF-1α is a transcription factor responsible for the adaptation of cells to low oxygen tension, for the regulation of glucose metabolism and for cell proliferation and survival (23, 24). Its expression is induced in the ischemic brain (25). However, its exact role still needs clarification, as it has been seen to display either protective or detrimental functions (26–29).

Unlike microglia, macrophages can switch their metabolism to anaerobiosis and remain viable in hypoxic/ischemic conditions (10, 30). Many pathological processes, such as tumors, atherosclerosis, and ischemia, involve a low oxygen concentration and the concomitant presence of macrophages (31). Efforts have been made to clarify how macrophages adapt to low oxygen concentrations. The HIF-1α and nuclear factor κB (NF-κB) families of transcription factors are major regulators of this adaptation (31, 32). Myeloid cell-mediated inflammatory response requires HIF-1α and involves a decrease in the expression of inducible nitric oxide synthase (iNOS) and reduced production of ATP by glycolysis (33, 34). Hypoxia can mediate NF-κB activation, favoring the production of inflammatory cytokines (32), and hypoxia-induced CXCL12 expression regulates mobilization and homing of hematopoietic stem and progenitor cells to the ischemic tissue (35–37).

MICROGLIA AND MACROPHAGE BEHAVIOR IN THE ISCHEMIC BRAIN LESION

The ischemic environment drives macrophage recruitment, and this results in the co-presence of infiltrating blood-borne macrophages and resident reactive microglia in the lesioned site (12). In experimental brain ischemia/reperfusion injury, green fluorescent protein (GFP)-expressing microglia studied by *in vivo* two-photon microscopy show prolonged resilience to ischemic conditions by becoming stalled, with reduced dynamic behavior. As blood perfusion is re-established, microglia recover their behavior and rearrange the cytoskeleton, acquiring either a bushy morphology, with multiple short processes around enlarged cell bodies (38, 39), or a reactive ameboid shape (21, 40).

When there is no reperfusion, e.g., after permanent ischemia or TBI, hypoxia may persist beyond the resilience limit of microglia, causing microglia irreversible damage and death (3). Accordingly, compared to ischemia/reperfusion, injuries with no reperfusion cause a larger lesion area depleted of microglia. The microglia-empty territory is rapidly replenished by round-shaped CX3CR1-/CD11b+/CD45^{high}+ cells, which are likely to be the infiltrating macrophage population (8, 41).

As discussed above, macrophages can switch to an anaerobic metabolism and adapt to hypoxic/ischemic conditions. In experimental stroke models, immune cell infiltration is more evident after permanent than transient occlusion of the middle cerebral artery (MCA) (42, 43). A schematic representation of differential distribution of microglia and macrophages at early stages after permanent or transient ischemia or TBI is depicted in **Figure 1**.

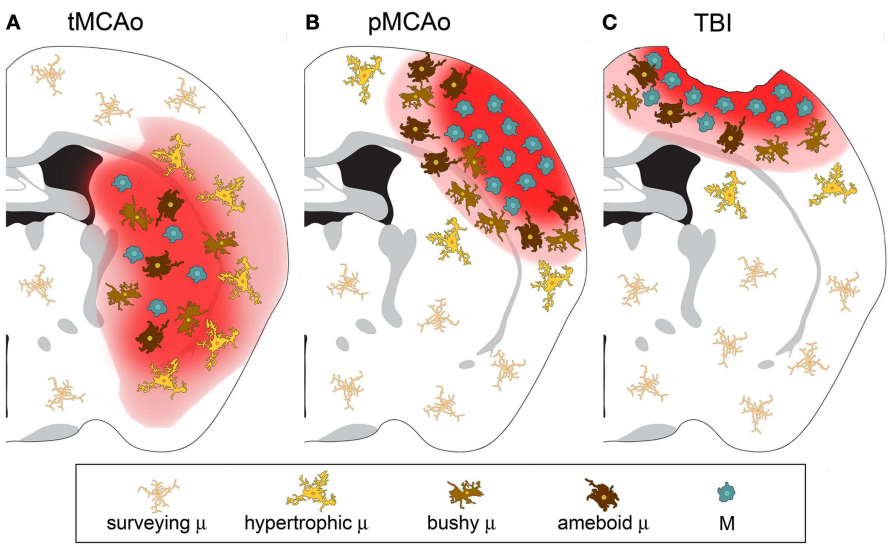

FIGURE 1 | Microglia and macrophage distribution over the lesion area 24 h after injury in three different models of acute brain injury. **(A)** After transient middle cerebral artery occlusion (tMCAo), the lesion core is populated by infiltrated macrophages (M) and bushy/ameboid microglia (μ). The penumbra contains hypertrophic μ. **(B)** After permanent middle cerebral artery occlusion (pMCAo) no μ cells are present in the lesion core where there is high M infiltration. Bushy μ cells surround the lesion core. **(C)** The distribution is similar after traumatic brain injury (TBI). M are recruited to the lesioned tissue close to its lesion edge, while bushy and hypertrophic μ cells populate more distant areas.

The metabolic status of the lesioned environment is thus a major determinant in the recruitment/activation of myeloid cells in the CNS and in the balance between microglia and macrophages. The consequences of the specific composition of the myeloid population still need clarification. Yamasaki et al. recently compared the transcription profiles of microglia or monocyte-derived macrophages in a model of experimental autoimmune encephalopathy. They showed that microglia downregulated metabolic pathways, whereas macrophages displayed active phagocytic and pro-inflammatory behavior (44). Although this observation cannot be extended to other injury models, these findings reinforce the idea that microglia and macrophages have different intrinsic properties that govern their specific responses to environmental signals.

Macrophages engrafted microglia-depleted brain regions in *CD11b-HSVTK* mice (45), a model of selective microglia depletion obtained by intracerebroventricular valganciclovir (46, 47). In this model, macrophages infiltrated within 2 weeks after depletion and showed microglia-like behavior, extending processes toward an ATP source. This might indicate that macrophages could populate the adult brain and replace microglia in sustain cerebral homeostasis (45). After a severe acute injury with impairment of the cerebral blood flow and no reperfusion, brain homeostasis is disrupted and metabolic crisis occurs, leading to massive death of cerebral cells, including microglia. Macrophages invade these lesioned regions and become activated. In these conditions, their role is likely to be different from that of surveying microglia. At early times, infiltrated round-shaped CD11b/CD45^{high} cells express M2 polarization markers, while microglia are mostly located at lesion boundaries with lower expression of polarization markers (8).

The picture in acute phase of brain injury may not apply at longer times when other events, such as microglial proliferation, might define a different balance between microglia and macrophages. Microglia proliferate starting 72 h after focal brain ischemia induced with the filament model in mice (48). Microglial proliferation in the lesioned brain areas is affected by the severity of injury, being clearly observable after 30 min of transient ischemia, but only weakly after more severe 60 min of ischemia. This latter caused wide areas of microglia loss where subsequent replenishment with fresh microglia was limited (48).

Local proliferation may also be promoted by pericytes, as recently shown after permanent ischemia (49). In response to ischemia, pericytes may leave the vessel wall and settle in the brain parenchyma. Pericyte infiltration occurs specifically in the lesioned area depleted of microglia cells, reaches its peak 7 days after injury and is still detectable at 21 days. These infiltrated pericytes express typical microglia markers, such as Iba1, CD11b, and GAL-3, but interestingly, they are negative for CD68 and CD45^{high}, these latter associated with leukocytes (49). Thus pericytes may function as a local source of microglia, and this may be a potential mechanism of microglial repopulation of severely injured areas.

The ischemic milieu changes over time, possibly also as a consequence of changes in blood perfusion. Areas with preserved blood flow soon after ischemia may have defective perfusion at later times because of the formation of secondary clots. This is generally referred to as "no-reflow" (50, 51) and may depend on different mechanisms, such as fibrin deposition and platelet activation (52–54), or clotting of immune cells in small capillaries (55). Delayed perfusion deficits can potentially affect microglial activity and proliferative ability in selected brain areas and shifting the balance between resident microglia and recruited macrophages.

SURFACE ANTIGENS: THE INTERFACE BETWEEN MYELOID CELLS AND ENVIRONMENT

The differential behavior of microglia and macrophages in response to the hypoxic environment suggests intrinsic differences in the nature of these populations. However, in experimental research, microglia and macrophages are often referred to as an unique population, because their study relies on labeling non-selective surface antigens, which are expressed constitutively or after activation by either cell types. For some markers different patterns of expression may help distinguish resident from infiltrated myeloid cells. Here, we discuss the most widely used markers, particularly murine markers of myeloid cells, providing information on their interaction with the environment and on the biology of myeloid cell subtypes.

CONSTITUTIVE MARKERS

CD11b is one of the most commonly used surface markers for immunostaining microglia/macrophages (8, 56). It belongs to the integrin family of surface receptors and is covalently bound to a β2 subunit to form integrin aMb2 (Mac-1, CD11b/CD18), which is implicated in diverse responses including cell-mediated killing, phagocytosis, chemotaxis, and cellular activation. CD11b is upregulated after microglia/macrophage activation and recognizes several ligands including C3 fragments, resulting from complement activation, fibrinogen, intercellular adhesion molecule-1 (ICAM-1), denatured products, and blood coagulation factor X. With its presence on the membrane surface and its constitutive expression, CD11b is particularly suitable for studying myeloid cell morphology in either physiological or pathological conditions (**Figure 2**).

Myeloid cells are often labeled by another constitutive marker, ionized calcium-binding adaptor 1 (Iba1) that provides information on their activation and morphology comparable to that with CD11b (57, 58). Iba1 is a calcium-binding protein specific for myeloid cells and has a role in bundling actin, in membrane ruffling and phagocytosis (59).

CD45 is a transmembrane glycoprotein expressed by cells of hematopoietic origin, except erythrocytes. It is a member of the protein tyrosine phosphatase (PTP) family. Its intracellular carboxy-terminal region contains two PTP catalytic domains, whereas the extracellular region varies widely due to alternative splicing of exons 4, 5, and 6 (designated as A, B, and C, respectively). CD45 has a role in T- and B-cell signal transduction and in the adult mouse the expression of selective isoforms depends on the cell type and activation state. Microglia express low constitutive levels of CD45 and even after activation they maintain lower levels of CD45 than circulating/infiltrating leukocytes. CD45 labeling, therefore, gives a weak signal in microglia that can be exploited to distinguish resident microglia from infiltrated immune cells by either immunohistochemistry or flowmetry (8, 40, 60, 61) (**Figure 2**).

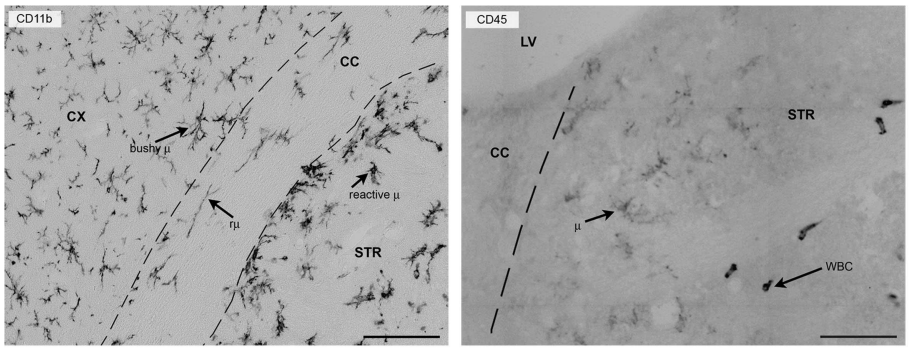

FIGURE 2 | CD11b or CD45 label myeloid cells in the mouse brain 24 h after focal transient ischemia. Left panel: CD11b is commonly used to label myeloid cells in the mouse brain and provides detailed information on morphology because it is expressed uniformly on the cell membrane. Twenty-four hours after focal transient ischemia, different microglia cell types can be found in the hemisphere ipsi-lateral to the lesion. Bushy microglia (μ) populate the cortex (penumbra) and rod microglia (rμ) with elongated morphology are present in the white matter (corpus callosum) and hypertrophic reactive microglia (reactive μ) populate the striatum, the area that corresponds to the lesion core. Right panel: CD45 labeling helps distinguish resident microglia from recruited leukocytes. Microglia have ramified morphology and pale staining ($CD45^{low}$), whereas infiltrated leukocytes (white blood cells, WBC) can be identified by their round-shaped morphology and strong, well-contrasted staining ($CD45^{high}$). The elongated shape of $CD45^{high}$ cells may depend on crawling along vessel walls or their perivascular location. Bars = 100 μm, microphotograms modified from Ref. (40). Neuro-anatomical structures are indicated by CX (cortex), STR (striatum), CC (corpus callosum), and LV (lateral ventricle).

Isolectin B4 binds α-D-galactosyl residues, which are expressed on microglia surface and on activated endothelial cells. This marker labels myeloid cells non-homogeneously and it also binds to endothelial cells (62). So it cannot be used to label microglia/macrophages clearly in tissue specimens. Thus, isolectin B4 is mainly employed to label *in vitro* microglial cells, giving a homogeneous staining and a quick immunostaining protocol (63, 64).

The receptor for fractalkine (CX3CR1) is constitutively present on microglia and is inducible in specific subsets of macrophages. Its main function in the brain is to support the communication between microglia and neurons. We discuss below the role of CX3CR1 and its deletion in different pathological conditions. Transgenic mice expressing GFP under the promoter of *cx3cr1* (*cx3cr1*$^{GFP/+}$) are commonly used to visualize microglia in *in vivo* (40) and in *ex vivo* experimental settings (65). Our group has shown that the expression of GFP in *cx3cr1*$^{GFP/+}$ mice helps distinguish microglia and infiltrated immune cells at early times (24 h) after ischemia (40) or TBI (41). Only ramified CD11b positive cells express GFP, whereas round-shaped cells, positive for CD11b and $CD45^{high}$, or CD3 (a marker for lymphocytes) do not. The first subset of infiltrating macrophages is recruited through CCR receptors and they do not express CX3CR1. Circulating monocytes have been divided into two subsets, the classical one expressing the CCR family receptors and prone to tissue invasion upon recruitment (66–68) and the non-classical subset, positive for CX3CR1. This latter subset patrols along blood vessel walls and accumulates in peripheral tissues, such as spleen, lung, and liver (68–70). Non-classical monocytes are recruited in the brain at later stages after acute injury, so they are not present at early times, so CX3CR1 positivity can be used as a selective marker for resident microglia early after injury.

The identification of a unique microglial molecular fingerprint would facilitate the study of microglia biology and help develop their therapeutic modulation. Recent gene profiling of murine CD11b+/$CD45^{low}$ microglia compared to that of $Ly6C^{high}$ or $Ly6C^{low}$ macrophages showed that there were a number of genes highly or uniquely expressed by microglia, such as *P2ry12*, *Gpr34*, *Mertk*, *C1qa*, *Gas6*, and *Fcrls* (71). FACS analysis indicated that antibodies against P2ry12 or FCRLS stained adult microglia, but not CD11b-gated myeloid cells isolated from spleen, bone marrow, and peripheral blood (71). P2ry12 and FCRLS need further validation, for instance in brain pathologies, but they offer promise as selective molecular markers for resident microglia.

The genetic profiles of microglia and macrophages have been recently associated with environmental signals that drive these cell identities (72). Gene profiling of mouse microglia and peritoneal macrophages revealed that the cerebral and peritoneal environments drive different programs of gene expression by differentially activating a common repertoire of enhancers. The activated enhancer repertoire in turn promotes the expression of secondary transcription factors that collaborate with PU.1 – the critical transcription factor initiating myeloid cell differentiation – to establish tissue-specific enhancers, ultimately defining the myeloid cell identity (72). This study indicates that microglia express high levels of *Cx3cr1* and *Nav3*, whereas peritoneal macrophages express *Lyz2*, *Fn1*, *H2-Eb1*, *Cebpb*, *Gata6*, and *Ciita* in a subset-specific manner. Whether brain-infiltrating macrophages have similar genetic profile to peritoneal ones is still to be determined; however, this study further demonstrates that microglia and peripheral macrophages have intrinsic genetic differences and propose the environment as a major determinant of these differences.

POLARIZATION MARKERS

As mentioned above, after activation the commitment of microglia and macrophages can be either toxic or protective. Microglia can affect neuronal function and promote neurotoxicity (73) through the release of several pro-inflammatory molecules, such

as IL-1β, TNF-α, proteases, and reactive oxygen species (ROS) (74). However, under certain circumstances microglia can be neuroprotective and promote adult neurogenesis and lesion repair (75, 76). Microglia can be neurosupportive through several mechanisms including glutamate uptake (77), removal of cell debris (78), and production of neurotrophic factors, such as insulin-like growth factor-1 (IGF-1) (79), glial cell-derived neurotrophic factor (GDNF) (80), and brain-derived neurotrophic factor (BDNF) (81). The dual nature of microglial polarization is similar to that of macrophages generally referred to as M1 or M2 (82). According to this classification, macrophages can develop a classic pro-inflammatory (M1) or an alternative anti-inflammatory (M2) polarization. Specific environmental signals induce these different polarization states (7). In particular, stimulation through toll-like receptor (TLR) ligands and INF-γ induces classical M1 activation, while IL-4/IL-13 stimulation favors the alternative M2 activation (83).

M1 and M2 canonical definitions represent the extreme of macrophage-polarized states documented *in vitro* (84). A generally accepted classification of the microglia/macrophage repertoire of polarized states indicates one M1 toxic polarization and three M2 polarization subtypes, M2a, M2b, and M2c, each with a specific function and pattern of marker expression (84). M1 is associated with phagocytosis, ability to kill pathogens by iron restriction, phagosome acidification, and ROS release (85–88). M1 phenotype markers include CD16, CD32, CD86, MHC II, and iNOS. M2a-polarized state is associated with immunity against parasites, recruitment of Th2 cells, tissue repair, and growth stimulation. This state is marked mainly by the expression of arginase-1, Ym1, and Fizz (85–95). M2b has either pro- or anti-inflammatory functions and is associated with memory immune response (B-cell class switch and recruitment of regulatory T cells). High IL-10 expression, MHC II, and co-stimulatory CD86 define the M2b pattern (85–87, 96–100). M2c is involved mainly in scavenging cell debris, has healing functions, and expresses arginase-1, CD163, and CD206 (85–87, 89, 90, 95, 100–102). An M0 phenotype has been recently introduced to indicate a non-polarized state. Adult microglia cultured with macrophage colony stimulating factor (MCSF) and TGF-β have a phenotype similar to fresh-cultured adult microglia and therefore have been defined to as M0 (71).

These antigenic fingerprints for polarization are well mirrored in some pathological conditions *in vivo*, including parasite infections, allergy, and cancer (83). In neurodegenerative disorders, myeloid cells often present mixed phenotypes indicating their plastic nature and their ability to acquire multiple phenotypes in response to environmental signals and time-dependent changes in the inflammatory milieu (83). As a further element of complexity, some markers are clearly assigned to a specific polarization state, while others do not clearly belong to one or the other. As an example MHCII and CD86 may be found in either the M1 or the M2 phenotype (84). MHCII is involved in antigen presentation to immune cells and CD86 functions as a co-stimulatory signal for T-cell activation. It is intriguing that microglia/macrophages acting as antigen-presenting cells are not easily classified as toxic or protective. Quite possibly T-cell activation needs to be finely tuned to yield a tolerization state, associated with protective action (103, 104), rather than an aggressive (auto)-immune response. For a satisfactory definition of myeloid cell polarization in CNS diseased states, further studies in models of pathology are needed. Events such as infiltration of leukocytes are important in orchestrating polarization, making the *in vivo* studies far more instructive than *in vitro* models, which cannot reproduce the complete network of recruited/activated cells.

REACTIVE MARKERS

The activation of microglia/macrophages is driven by many factors, including cytokines, chemokines, released degradation products, and extravasated molecules. Specific responses to these factors have been thoroughly reviewed elsewhere (105–108). Here, we provide information on the reactive markers that tag specific microglia/macrophage functions. In addition to the upregulation of constitutive molecules and the expression of polarization markers, activated microglia/macrophages also express novel antigens associated with specific functions. Reactive antigens include, among others, CD68, CD200, F4/80, CD14, HLADR, TLRs, heat shock protein (Hsp)-70, C3b/iC3b, CR3, and sodium calcium exchanger (NCX)1 (109–114). These markers indicate a reactive status, but they cannot be used to define whether myeloid cells have toxic or protective functions. The activation of microglia and macrophages is linked to different functions, including damage-sensing activity, chemotaxis, and phagocytosis. These events are among the most distinctive features of these cells, and specific markers, discussed below, apply to each reactive phase.

Damage-sensing activity

Microglia are distributed uniformly throughout the CNS. Specific microglial populations may exist in selective CNS regions (115), depending on the white or gray matter localization, proximity to vasculature, interaction with BBB components, biochemical features of the microenvironment, and substances released by neurons (116–118). Functionally, these adaptations guarantee tissue homeostasis under physiological conditions and are readily activated in case of threats to the CNS. Like macrophages, microglia are well equipped to sense any change in tissue homeostasis thanks to the expression of different surface receptors with damage-sensing functions. These receptors, generally referred to as pattern recognition receptors (PRR), bind ligands of different types: high mobility group box 1 (HMGB1), Hsp, hyaluronic acid and fibronectin produced by extracellular matrix degradation, apoptotic or injured cells, nucleic acids, immune complexes, mannose residues, and proteolytic enzymes (119). These ligands are called danger-associated molecular patterns (DAMPs) and are released as a consequence of insults to the CNS.

Toll-like receptors are a family of PRR that recognize a wide variety of danger signals and consequently activate different inflammatory cascades. These receptors, originally identified as initiators of innate immunity in response to exogenous microorganisms, have a role in the inflammatory response to ischemic injury in the absence of infection (120). TLRs are expressed by myeloid cells in response to activation and are involved in the switch toward M1 polarization (83) either by inducing NF-κB, which in turn induces pro-inflammatory cytokines (TNFα, IL-12, SOCS3) and HIF-1α to promote iNOS synthesis, or by inducing IRF-3, one of the main transcriptional regulators initiating M1

polarization (via STAT1) and M2 gene silencing (121). TLR4 has a pivotal role in the promotion of the M1 polarization (83). The induction of IRF-3 by brief activation of TLR4 or TLR9 can also have protective effects. Preconditioning with low doses of either the TLR4 ligand LPS or the TLR9 ligand CpG confers protection against ischemic injury through IRF-3 and IRF-7, and importantly, this effect is mediated by glial cells (122).

In the CNS, a few PRR are expressed by other cell types, such as NOD-like receptor proteins (NLRP) expressed in astrocytes (NLRP2) or neurons (NLRP1) and the-absent-in-melanoma-2 (AIM2)-like receptor, expressed in neurons. Along with caspases these receptors form the inflammasome and are involved in pyroptotic events (119). Non-surface-bound PRR exist too. These include the initiators of the complement cascade, such as serum proteins C1q, mannose-binding lectin (MBL), ficolins, and collectins, which circulate in the bloodstream and, on recognition of a wide array of target ligands expressed also by myeloid cells (123), trigger complement activation leading to injury progression (124).

Findings in animal models and in humans support the idea that there is a high degree of interaction between TLRs and complement. Complement receptors including C5aR, C3aR, and C5L2 crosstalk with TLRs, result in the regulation of innate immune and inflammatory responses in addition to regulation of adaptive immunity (125). This interaction may lead to the production of inflammatory molecules or dampening of excessive inflammation. The molecular mechanisms leading to this functional regulation are not completely clear but they include the possibility that complement receptor activation is needed for optimal TLR signaling or that complement fragments can directly activate TLRs (126). Crosstalk of complement components with other PRR is also described. C1q can inhibit NLRP3-dependent cleavage of caspase 3 and subsequent IL-1β cleavage, possibly by increasing the expression of negative regulators of the inflammasome activity, such as NLRP12 and/or POP1/ASC2 (127).

The complement system has a major role in the activation of microglia, cells that constitutively express the receptors for C1q and for cleavage products of C3, complement components that mediate phagocytosis and stimulate cytokine production by microglia. On binding, microglia become activated and in turn contribute to complement component production that feeds autocrine/paracrine signaling. Microglia respond to C1q with a marked shift toward pro-inflammatory activation in CNS diseases with BBB impairment (128). A role of C1q-mediated activation of microglia was identified in CNS development, when it is required to prune neuronal synapses (129). However, C1q influences a multiplicity of functions associated with macrophages, for instance upregulating IL-33, a cytokine that can amplify the M2 polarization induced by IL-13 (130, 131). Benoit et al reported that C1q induced the secretion of anti-inflammatory cytokines, such as type I interferon, IL-27, and IL-10 by human macrophages stimulated with low doses of LPS. These effects were abolished with higher doses of LPS, suggesting that this immunoregulatory activity occurs with limited inflammatory challenge (127).

There is also evidence suggesting the involvement of MBL, one of the activators of the lectin complement pathways, in the activation of microglia. MBL may indirectly induce the activation of microglia through enhancement of fibrin deposition (53), which drives microglial recruitment and activation (52).

The purinergic system mediates intimate crosstalk between microglia and neurons at synapses (132), based on extracellular ATP/ADP sensing (3). Microglia express P1 receptors – including all subclasses of adenosine receptors, e.g., adenosine A_1, A_2A, A_2B, and A_3 receptors (133) – and also P2 receptors, including ionotropic P2X ($P2X_4$, $P2X_7$) and metabotropic P2Y ($P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, and $P2Y_{14}$) receptors (134–136). Microglia show a certain degree of constitutive expression of these receptors, allowing scanning activity during physiological surveillance. Surveying microglia express high levels of $P2Y_{12}$ receptors (137) that are involved in branch extension. On activation, microglia can alter their set of purinergic receptors and respond to specific demands of the surrounding environment. Different purinergic receptors may be induced simultaneously to obtain cooperative effects, whereas others may function independently or be silent. For example, microglial activation and reactive transformation into their ameboid phagocytic phenotype require specific profiles of purinergic receptors: $P2Y_{12}$ and A_3 receptors cooperate to allow process extension (damage sensing), A_2A receptors induce retraction (ameboid transformation), and $P2Y_{12}$, $P2X_4$, and A_1 receptors interact to induce migration by ATP/ADP sensing (recruitment to lesion site). Finally, $P2Y_6$ receptors sense UDP to start phagocytosis (138).

$P2Y_{12}$ receptors have been proposed as pharmacological targets (137). As already mentioned, they take part in primary damage sensing through ADP-induced chemotaxis. Early inhibition of ADP sensing by ticagrelor induces protection from ischemia at 48 h and is associated with reduced microglia/macrophage recruitment to the lesion site and with decreased expression of pro-inflammatory mediators, such as iNOS, IL-1β, and MCP-1 (137). This suggests that microglia have an early toxic effect that can be counteracted by inhibiting their activation and homing to the lesion.

Chemotaxis

Following damage-sensing activity, microglia/macrophages become responsive to chemokines released by the host tissue and promote the invasion of the inflamed CNS site. The ability of infiltrating immune cells, such as macrophages, to migrate within the invaded tissue has long been known by immunologists. Macrophages abundantly express CC and CXC family surface receptors for chemokines, among which CCR2 plays a major role in driving macrophage invasion through monocyte chemoattractant protein 1 (MCP-1) sensing (66–68). Positivity to CCR2 can be exploited in mice to define a specific macrophagic subset, which expresses Ly6C, high levels of Gr1 and low levels of CX3CR1 (139). In humans, the homologous subset is $CCR2^{high}$/CD14+/$CX3CR1^{low}$. Macrophages belonging to this subset have been associated with the development of the M1 polarization state. A second subset, negative for CCR2, with a Ly6C-/$Gr1^{low}$/CX3CR1+ phenotype, has its human homolog in $CCR2^{low}$/CD14-/$CX3CR1^{high}$ cells and is associated with the M2 final commitment (139). However, sharp commitment of CCR2 positive or negative subsets is not easily determined because high levels of its ligand MCP-1 (also known as CCL2) have

also been described in a M2-polarized inflammatory environment (140).

Microglia express chemokine receptors of the CC and the CXC family that, with purinergic receptors, regulate the machinery for microglial movements. In the early metabolic crisis after acute brain injury, microglia may use chemotactic signals to extend/withdraw their branches so as to receive activatory stimuli and change morphology. Their dynamic proprieties have only been recently explored, using *in vivo* imaging by two-photon microscopy (3, 51). Microglia show constant movements of their branches to patrol the environment although microglial ability to displace within the inflamed tissue seems scarce. The fact that active microglia release a number of factors with paracrine effects including BDNF (141), IGF-1, and cytokines (142) implies that they need to reach their site of action. However, their ability to travel over brain tissue depends on the kind of danger signal received. In a model of spreading depression in organotypic slice cultures, causing synaptic activity impairment, microglia showed displacement by Lévy flight-like movements in response to diminished synaptic activity (143). In contrast, in models, such as TBI (induced by laser ablation) (3), photothrombotic stroke, or brain ischemia (3, 21, 40), microglia did not display any migratory behavior. Their lack of displacement after acute brain injury may be related to the rapid decline of ATP levels due to perfusion impairment, ATP being needed for the cytoskeletal modifications that microglia exploit for migration (3, 143).

Little is known about microglial dynamics at later times. Proliferating microglia might migrate and settle in specific lesioned areas where new ones are needed to re-establish tissue homeostasis. Further studies will help decipher microglia dynamics over time better, because the literature on this issue so far mainly focuses on acute points (few hours after insult).

Some markers may be used to detect microglia/macrophages responding to chemotactic stimuli. F4/80 is commonly used to stain these cells in tissue. Its expression is enhanced in recruited tissue macrophages compared to circulating monocytes, indicating that it is involved in adhesion and tissue migration. Microglia also express F4/80 at high levels after activation. Interestingly, the human homolog of F4/80, EMR1, is not present on mononuclear phagocytic cells, such as macrophages, and in human specimens, it preferentially stains eosinophils (144).

The NCX1 is involved in the calcium-mediated functions of microglia, including chemotaxis. Calcium influx through the action of the exchanger is a prerequisite for bradykinin-induced microglial motility (145). In a model of brain ischemia, microglia significantly upregulated NCX1, reaching a peak of expression 3 and 7 days after the insult. NCX1 expression was identified in round-shaped Iba1+ cells that progressively invaded the ischemic core. NCX1 knockout mice show impaired chemotaxis and microglia migration even in the heterozygous state. Impaired microglia chemotaxis in these mice dramatically increased the brain ischemic lesion, suggesting that NCX1 is needed to develop microglial protective functions (110).

Phagocytosis

Phagocytosis is a complex event, whose role in acute brain injury is still not clear. Phagocytic activity is not clearly linked to a specific M1 or M2 polarization state. Cells showing M1 toxic as well as M2c pro-healing polarization may have phagocytic functions (84), indicating that phagocytosis may serve either for killing cells (toxicity) or scavenging debris (protection).

There is *in vitro* evidence that M2-polarized cells enhance their phagocytic behavior (146). This is regarded as a protective function because phagocytosis is needed for removal of cell debris to limit the spread of toxic signals and potentially favor tissue healing. In organotypic slice cultures, microglia may be neuroprotective by engulfing neutrophils (147).

On the other hand viable neurons exposed to sub-lethal stimuli can also express "eat-me" signals that tag them for elimination (148), a process also referred to as primary phagocytosis or phagoptosis (149). Two key proteins involved in the elimination of viable neurons exposing phosphatidylserine have been identified, namely milk fat globule EGF-like factor-8 (MFG-E8) and Mer receptor tyrosine kinase (MerTK), both of which bridge phosphatidylserine-exposing neurons to vibronectin receptors on phagocytic cells (150–153). The fact that the lack of these proteins prevents neuronal loss and improves behavioral deficits demonstrates the detrimental function of primary phagocytosis.

In the CNS, myeloid cell phagocytic activity can also be labeled by CD68, or macrosialin, a scavenger receptor member of the lysosomal/endosomal-associated membrane glycoprotein (LAMP) family (8, 109), which is found mainly in phagocytic macrophages (154). *In vivo* observations suggest that the expression of CD68 is inversely related to that of M2 markers during the temporal evolution of the lesion (8) or in response to protective manipulations, such as LPS preconditioning (155), stem cell treatment (140, 156), and CX3CR1 deletion (40).

These observations confirm that the phagocytosis cannot be ascribed to either M1 or M2 and imply that, although phagocytosis is needed for limiting the propagation of danger signals, any excess has detrimental effects.

A major effector of phagocytosis is the complement system cascade (124, 157). All the complement activation pathways, including those activated by the danger signals released in response to acute brain injury, converge on the activation of C3 convertase that leads to C3 cleavage. Its products, C3b/iC3b, are specifically able to opsonize damaged or apoptotic cells and function as "eat-me" signals, driving phagocytosis. C3b/iC3b deposits are recognized by the CD11b/CD18 (or CR3) receptor and may be used to reveal neurons committed to die.

DOES MORPHOLOGY INDICATE FUNCTION?

Similar to phenotypic markers, morphological changes can provide information on microglia/macrophage functional commitment. A morphological description is thus a priority to clarify the specific functional significance of these cells in brain pathology. The literature has explored the morphology of myeloid cells in physiological or diseased conditions with the aim of defining specific shape indicators of a given active/functional state (40, 57, 158). This can be done by combining the morphological data from imaging with those from conventional molecular analysis – immunostaining, real time PCR, western blots – in different experimental settings.

Microglia/macrophage activation is usually measured by immunohistochemical assessment of the specific antigenic markers discussed above, but this does not take into consideration morphological changes and shape descriptors. Actin polymerization leading to microglia/macrophage shape changes is strongly related to ATP/ADP availability and morphological changes are therefore finely tuned by the injured microenvironment.

Microglia show a wide range of morphological transformations, from highly ramified with thin branches, to hypertrophic and ameboid with no branchings. In between these extremes, they can acquire a variety of shapes that mirror their functional state [**Figure 3** (57)]. Macrophages, in contrast, are less prone to morphological changes. However, a certain amount of shape change is possible for macrophages too, because due to their plastic nature they can change their contractility state and establish interactions with the extracellular matrix and with cell surface adhesion molecules (159). When macrophages are cultured on substrates that force their elongation, such as arrays of narrow fibronectin lines, they start upregulating the M2 markers arginase-1, CD206, and Ym1 and reduce the release of pro-inflammatory cytokines (160). The *in vitro* induction of M2 phenotype by IL-4/IL-13 treatment causes elongation of the cultured macrophages, indicating that this is needed to drive the M2 polarization and, more generally, that geometry plays a role in the phenotype definition (160). Whether elongation defines polarization *in vivo* needs investigating because infiltrating cells may elongate merely as a consequence of crawling along vessel walls or a perivascular location.

Elongation seems to be an important feature for the definition of microglial functions as well. The white matter is populated by a specific microglial subtype, which has a bipolar, elongated morphology, called rod microglia [**Figure 3** (161)]. These cells are close to neurons (162, 163) and take part in axonal damage and recovery after injury (118). In other brain areas, elongation may result as a consequence of directional ramification extending toward a specific site of injury. This process may be more evident at early times after injury, being necessary during the damage-sensing microglia re-orientation. As early as 24 h after ischemic injury, microglia are not elongated and display a reactive hypertrophic ameboid morphology with numerous short processes, symmetrically extending from the cell soma (40). This morphological state correlates with increased lysosomal activity and, interestingly, is decreased in CX3CR1-deficient mice that show increased expression of M2 markers and concomitant protection from ischemic injury (40). A protective role for ramified microglia was suggested by Vinet et al. who showed that these microglia not only survey their microenvironment but also contribute to protecting neurons during neurodegeneration induced by the glutamate receptor-agonist *N*-methyl-D-aspartic acid (158).

The complex morphological features of specific myeloid populations can be evaluated by measuring different shape

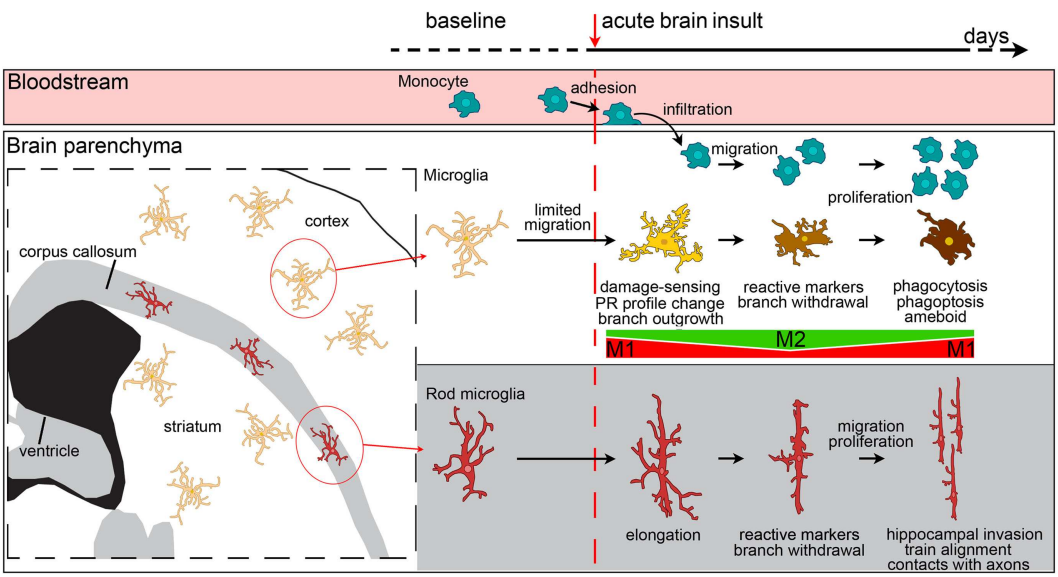

FIGURE 3 | Evolution of myeloid cell morphology and phenotype in the brain during the acute phases of brain injury. Under physiological conditions, monocytes, microglia and rod microglia populate specific compartments, respectively blood, gray matter and white matter. After an insult to the brain, monocytes, microglia, and rod microglia are activated. Monocytes migrate to lesioned areas, whereas gray matter microglia are less likely to displace. Microglia change morphology in a time-dependent fashion, sprouting new ramifications soon after injury, and then withdrawing branches to develop an ameboid phenotype. Both infiltrated monocytes and microglia change their phenotypical profile, acquire specific functional commitments, and proliferate with time. In the very early phases after injury, microglia have a M1 phenotype, then, with the recruitment of macrophages, both myeloid populations upregulate M2 markers. The peak of M2 marker expression soon vanishes and is followed by upregulation of M1 markers that lasts longer. *In vivo*, mixed polarization phenotypes are observed and the M1-M2 definition may only serve to set the extremes of a continuum of polarization states. Macrophages from blood-borne monocytes seem to be more able to express M2 markers than microglia. In the white matter, rod microglia activate after injury by enhancing their elongated morphology, expressing reactive markers (CD68, MHCII), and migrating to other areas, such as the hippocampus. Rod microglia also cluster into trains of cells that align to neuronal axons. Neither the exact phenotype profile nor the functions of activated rod microglia are fully understood, though a role of these cells in synaptic stripping due to their contacts with axons has been hypothesized.

indices, whose temporal variations are clearly indicative of functional states, providing a quantitative analysis of shape changes (**Figure 4**). As an example, the longest branches of surveying microglia are found within a distance of 20–25 μm from cell centroid when measured by Sholl analysis *in vivo* in mice (40). One hour after transient ischemia, the longest branches are found at 30–35 μm from cell centroid indicating ramification sprouting, a process involved in the sensing of danger signals. Other changes of myeloid cells after transient ischemia are associated with cell size. One day after transient ischemia, CD11b+ cells display a mean cell area of 85.8 μm^2, far higher than that in naïve mice (54.6 μm^2). Similarly, myeloid cells increase perimeter (75.3 μm) and cell caliper (20.1 μm) after ischemia compared to controls (61.5 and 17.1 μm, respectively) (41).

Microglial morphology at late times after acute injury or in conditions of chronic activation have been explored little. After TBI, microglia develop a primed phenotype, defined by increased Iba1 labeling and hypertrophic cell soma that persists up to 30 days after insult (166). Primed microglia respond to a secondary inflammatory challenge with quick activation, yielding depressive-like behavior.

As amply discussed, microglia modify their shape dynamically because they can extend or withdraw their branches. While the number and length of ramifications are indicative of morphological features associated with microglial function, it is not clear whether the rate of extension/withdrawal per ramification reveals a given state. The average speed of extension/retraction (micrometers per minute) declines during a vascular occlusion when ATP is not available, but soon recovers to the baseline values when new perfusion is allowed (21). This implies that after ischemia the intrinsic process dynamics of the activated microglia remain unaltered. Results are similar with LPS because the process motility does not change either 2 or 28 days after LPS (167). Hypothetically microglia may quickly rearrange the length and number of branches and change morphology without losing their exploratory ability. However, the residual availability of an energy source is important for their movements, so environmental changes can alter microglial exploratory behavior under specific metabolic circumstances. This point calls for further studies to understand the dynamic response of microglia during their morphological transformation.

FUNCTIONAL CONSEQUENCES OF CHRONICALLY ACTIVATED MICROGLIA

To ensure brain homeostasis, microglia need to finely tune their physiological activity. The key to this control is the neuron-mediated inhibitory activity which, under normal conditions, prevents microglia from becoming active (6). Neurons control microglia through several components, including contact-dependent inhibitory signals, such as CD200-CD200R (168), Hsp60–TREM2–DAP12 (169), CD22-CD45 (170), and CD47-CD172 (171), and soluble molecules such as ICAM-5 (172), fractalkine (CX3CL1) (173), IL-10 (174), and TGF-β (175). Any change in microglial activity control may lead to chronic activation or a primed state with specific functional consequences. Here, we discuss three cases in which microglia are chronically activated and their implications.

CX3CR1-DEFICIENT MICROGLIA ACQUIRE A CHRONIC ACTIVE STATE

CX3CR1, selectively expressed in the brain by microglia, is an important regulator of their activity. The main effect of CX3CR1 binding to its unique ligand CX3CL1 released by neurons is to control microglial activation. Under physiological conditions, the neuronal membrane-anchored CX3CL1 is cleaved by the disintegrin-like metalloproteinases ADAM10 and ADAM17 (176) and is released in its soluble form. Continuously released CX3CL1 keeps microglia in a non-reactive state. The generation of *cx3cr1*$^{GFP/GFP}$ mice, knockout for CX3CR1 and referred to here as *cx3cr1*$^{-/-}$ mice, has led to a number of studies investigating CX3CL1:CX3CR1 signaling either under physiological conditions (177) or in CNS diseases (178–181), showing the involvement of this pathway in several aspects of development, homeostasis and injury, including synaptic pruning, promotion of neuron survival, synaptic transmission and plasticity, enhancement of synaptic networking and facilitation of neuropathic pain circuits (173).

In *cx3cr1*$^{-/-}$ mice, the suppressive function of CX3CL1 is prevented and this causes a chronic microglial activation state, with a larger number of branches than in WT mice, as shown by

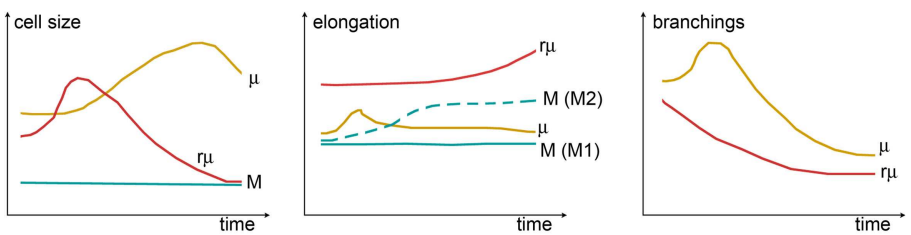

FIGURE 4 | Shape changes of different myeloid cell types over time. Cell size (left panel) can be measured by assessing cell area or cell width (caliper). While in monocytes (M), the size is constant, microglia (μ) grow larger (hypertrophic reactive morphology), and rod microglia (rμ) first grow and then shrink (elongated not ramified morphology). Elongation (center panel) can be evaluated from the ratio between the cell's major and minor axes. A certain degree of elongation is selectively associated with M switch to M2 polarization. Elongation of μ might occur during early damage-sensing as μ direct ramifications toward the site of injury. Then, along with ameboid transformation, μ recover their symmetric shape. In contrast, rμ progressively elongates so as to align with axons. The rate of branching (right panel) can be measured by counting the number of ramifications or by Sholl analysis. M does not have ramifications, while μ or rμ withdraw branchings over time to acquire, respectively, the ameboid or the elongated morphology: μ show a transient increase of ramifications early after injury that may be related to damage-sensing activity. Shape changes have been plotted according to the data and observations described in Ref. (40, 118, 160, 161, 164, 165).

in vivo two-photon microscopy (40). This state has been associated with less hippocampal neurogenesis and cognitive deficits (177, 182, 183).

After an acute insult, such as brain ischemia, affected neurons significantly reduce the release of CX3CL1, allowing microglial activation (184, 185). Chronically activated microglia in *cx3cr1*$^{-/-}$ mice respond to acute injury by enhancing their ramified morphology, downregulating the phagocytic activity and acquiring features of M2 polarization, together with protection from ischemic injury (40). There are also reports of early protective effects of CX3CR1 deletion in different models of acute brain injury, including ischemia, trauma, or spinal cord injury (SCI) (179, 181, 186, 187). However, this response may be transient and at longer times other events may take place, changing the overall impact of CX3CR1 deficiency. Recruitment of the CX3CR1+/Ly6C^{low}/CCR2- macrophage subset has been associated with beneficial effects as this population is capable of tissue healing (188). CX3CR1 deficiency would impair the recruitment of this protective population, resulting in a detrimental phenotype.

The deleterious consequences of CX3CR1 deficiency at late stages of pathology have been described in ischemia and SCI (189, 190). There is ample literature on *cx3cr1*$^{-/-}$ mice in chronic CNS diseases (67). In models of lateral amyotrophic sclerosis (178), Parkinson's disease (PS) (191) and Alzheimer's disease (192), the absence of CX3CR1 has been associated with a worse outcome possibly due to the chronic pro-inflammatory function of CX3CR1-deficient microglia (193, 194).

These findings indicate that specific microglia signaling pathways may give opposite outcomes depending on the temporal pattern of their action or on the disease stage. The longitudinal effects of CX3CR1 deficiency at different stages of pathology (**Table 1**) need to be carefully investigated to understand the exact role of the CX3CL1:CX3CR1 axis in brain injury.

MICROGLIA CHANGE THEIR MORPHOLOGY AND DYNAMICS IN THE AGING BRAIN

In aged mice (27–28 months), microglia increase their cell soma volume and shorten mean ramifications. This is accompanied by quicker soma movements and less baseline process motility than in young mice [3 months (200)]. Although the morphological features of microglia in aging brains are reminiscent of a reactive state, it is not clear whether the aged microglia are truly activated. It is likely that these cells enter a dysfunctional state that may affect the integrity of other structures with which they come into contact, such as neuronal or vascular networks. Possible consequences of the dysfunctional behavior of aged microglia include dysregulated response to injuries, changes in neuroprotective functions and increased toxic responses (200).

Table 1 | Effects of CX3CR1 deficiency in different models of CNS diseases.

Model	Time from injury	Deletion protective?	Effects of deletion	Reference
SCI	5–35 days	Yes	↑ hindlimb function, ↓ myelin and axon loss, ↑ CD45, ↓ IL-6, and iNOS	(181)
	42 days	No	Worsened locomotor function, ↓ myelin, ↑ infiltrating monocytes/macrophages	(189)
pMCAo	24 h	Yes	↓ ischemic volume	(187)
tMCAo	3 days	Yes	↓ infarct size	(186)
	1–3 days	Yes	↓ infarct size, ↓BBB breakdown, ↓ apoptosis, ↓microglia, and ↓IL-1β	(179)
	24 h	Yes	↓ infarct size, ↑ ramification in microglia, ↓ CD11b and CD68, ↑ CD45^{high}, ↑Ym1, and ↓iNOS	(40)
	72 h	Yes	↓ infarct size, ↓ neurological deficit, ↓ apoptosis, ↓ IBA1, and CD45^{high}, ↑ Ym1, ↓ iNOS, ↓ microglia proliferation, ↓ ROS, ↓ IL-1, IL-6, and TNF-α	(195)
	43–60 days	No	↑ microglia activation, ↑ IL-1β, and TNF-α, ↓ IL-4 and IL-10, worsens cognitive functions	(190)
LPS	4 days	No	↑ TUNEL, ↑ IL-1β	(178)
PD	7 days	No	↑ loss Nissl-stained cells, ↑ loss TH-IR	(178)
ALS	15–20 weeks	No	↓ neuronal cell density, ↓ motor function, ↓ survival	(178)
AD	28 days	No	↑ IL-6 and TNF-α, ↑Mac2 (activated microglia), ↓ cognitive and memory deficits	(192)
	28 days	Yes	↓ neuronal loss	(196)
	28 days	Yes	↓ Aβ deposition, = CD45, ↓GFAP, ↓ TNF-α and MCP-1/CCL2, ↑IL-1b, microglia more rounded, ↓ CD68, ↑ Aβ phagocytosis	(197)
	28 days	Yes	↓ Aβ deposition, ↑ Aβ phagocytosis, ↑ microglia proliferation, = neuronal injury	(198)
Tau pathology		No	↑ MAP phosphorylation, behavioral impairments, ↑ microglial activation, ↑IL-1b	(199)

The role of the fractalkine receptor (CX3CR1) in CNS pathologies has been studied using cx3cr1$^{-/-}$ *mice. In different disease models, including SCI, permanent ischemia (pMCAo), transient ischemia (pMCAo), lipopolysaccharide (LPS)-induced inflammation, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and Tau pathology. The results are often contrasting and depict a puzzling situation. A potential general hypothesis is that CX3CR1 deletion is protective in the early phases after acute injury, but becomes deleterious at later stages or in chronic conditions.*

Microglial modifications during aging may be related to changes in either central or peripheral inflammatory events over the lifespan. As already discussed, many molecules with paracrine effect are needed for controlling microglial activation under physiological conditions. However, aging may change the expression of these molecules, resulting in changes in microglial activation states. An interesting example of dysregulated microglia control involves TGFβ1. This cytokine has been defined as a determinant of microglia support of CNS homeostasis, favoring the microglia anti-inflammatory phenotype through IRF-7 signaling (71). However, chronic exposure to TGFβ1 has the opposite effect, inhibiting IRF-7 and consequently impairing the anti-inflammatory switch of microglia (201). This latter effect may be important during aging because this condition has been reported to induce chronic TGFβ1 upregulation (202) and can thus cause chronic pro-inflammatory microglial activation.

As for peripheral inflammatory changes, recent work demonstrates that mice injected systemically with blood from aged mice have decreases in neurogenesis, learning, and memory – all effects mediated by the chemokine system (203). The rules that govern the interaction between systemic inflammation and microglial behavioral changes throughout life are far from understood. However, a recent hypothesis attributed to a lifetime peripheral inflammatory state a fundamental role in degenerative pathologies, possibly through an impact on central inflammation.

The upregulation of MCP-1, a chemoattractant molecule for myeloid cells, in multiple models of neurodegenerative diseases, including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and prion disease (204–207), has been linked to the possibility that myeloid cell lineages play a major pathophysiological role. In chronic disease states, microglia appear to be primed by earlier pathology or genetic predisposition and respond with hyperactivation to inflammatory stimulation. This might lead to an adaptive CNS inflammatory response changing into one with deleterious outcomes (164). Other authors reported a major involvement of peripheral macrophages rather than microglia in driving chronic diseased states. In a model of Parkinson's disease induced by injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), peripheral M1 macrophages activation proceeds by days microglia activation and neuronal loss (208). This suggests that, at least in this model, innate peripheral inflammation is a critical player rather than a mere consequence.

MICROGLIA IN DEPRESSIVE BEHAVIOR

Individuals who suffered an acute brain injury have a higher incidence of depressive behavior (209). The etiology of increased depression is still not clear but it may be inflammatory related. Clinical and experimental data indicate a cause/effect relationship between inflammation and depression (210), which is mediated, at least in part, by high levels of circulating inflammatory cytokines (211). Experimental studies suggested a strong correlation between the volume of damaged brain tissue and the extent of systemic immune alterations, irrespective of the site of infarct (212). Patients with acute brain injury have high levels of circulating IL-6, and antidepressant therapies fail to reduce TNF-α (211). These cytokines are released soon after acute brain injuries and accumulate in cerebrospinal fluid and serum (213–215). Moreover, markers of neuroinflammation (e.g., CD68, CR3/43) persist in the brain parenchyma up to 16 years after acute CNS insults (216).

Recent work on experimental models helps attribute a specific role to microglia in driving neuroinflammation-induced depressive behavior. Fenn et al. reported that experimental TBI induces microglia priming that results in a hyperinflammatory response to an immune challenge, persisting weeks to months after injury, finally triggering the development of depressive-like behavior (166).

It is likely that increased microglial sensitivity to secondary inflammatory challenges (e.g., stressors, infection and injury) involves mediators of the complement system and PRRs expressed on the microglial cell surface. However, this has so far been studied little, and the exact role of primed microglia in late stages after initial brain insult remains unexplored.

STEM CELL TREATMENT AS A PROOF OF PRINCIPLE OF THE POSSIBILITY OF DRIVING THE MICROGLIA PHENOTYPE FOR THERAPEUTIC PURPOSES

Promoting a microglial neuroprotective phenotype is an emerging therapeutic goal for CNS conditions. One strategy of interest is the use of stem cells. There is increasing evidence of the efficacy of mesenchymal stromal cells (MSC) in acute brain injury (217). The paracrine effects of MSC-released bioactive factors modify the injured microenvironment favoring reparative and restorative processes. Among the cerebral populations affected by MSC, microglia are an important target. *In vitro* and *in vivo* evidences show that MSC can tune protective microglial polarization. A direct link between MSC infusion and changes in the polarization of microglia has been documented *in vitro*, exposing MSC/microglia co-culture to different toxic stimuli (140, 146, 218). Primary murine microglia co-cultured with MSC increase the mRNA and protein expression of the M2 markers Ym1 and CD206 (140). This is obtained when MSC/microglia are co-cultured in direct contact or in a transwell system, the latter allowing only a paracrine effect. More interestingly, MSC may reverse the M1 phenotype acquired by microglia after a pro-inflammatory challenge (by either TNFα + IL-17 or TNFα + IFN-γ stimulus), inducing M2 pro-regenerative traits, as indicated by the down-regulation of iNOS and upregulation of Ym1 and CD206 mRNA expression (140). Results are similar after microglia exposure to a LPS toxic stimulus and MSC co-culture. Again, in control and inflamed conditions, MSC upregulate mRNA and protein levels of the M2 marker arginase-1 (218) and can to reverse the microglial M1 phenotype increasing the expression of the M2 marker CD200R (146).

Besides the expression of specific markers, the M2 phenotype is associated with a specific profile of released cytokines. MSC may alter the ratio of IL-10 to TNF-α in favor of the anti-inflammatory cytokine IL-10 in the supernatants of LPS-stimulated microglia cultures (218). In addition, the secretome obtained even with unchallenged MSC reduces the production of TNFα, IL-1β, RANTES/CCL5, and MIP-2 by exposed microglia (219). Interpretation of the microglia cytokine profile after MSC/secretome exposure is not always easy. Rahmat et al. showed that together with a decrease in the production of TNF-α, MSC increase the production of IL-6, a cytokine usually

considered pro-inflammatory (220). Kim and Hematti found a similar pattern (221) after MSC/macrophage co-culture, revealing a new CD206+ M2 subtype with a IL-10^{high}/IL-12^{low}/IL-6^{high} phenotype and low secretion of TNF-α^{low}, potentially implicated in tissue repair. Increases in MCP-1/CCL2 and IL-1β are reported in MSC/microglia co-culture (146, 218). MCP-1/CCL2 is a potent chemoattractant for monocytes and may have a role in tissue repair and regeneration (222).

Although several aspects of MSC/microglia interaction are not completely clear yet, there are good reasons to use MSC *in vivo* as a driver of microglia post-conditioning after brain injury with the aim of pushing the M2 protecting phenotype. The first demonstration of MSC's ability to induce *in vivo* M2 protective modulation after acute brain injury was provided by Ohtaki et al. (223) after stroke in mice. After 15 min transient, common carotid artery occlusion followed by transplantation of 100,000 human MSC into the dentate gyrus the mice had improved neurological function and less neuronal cell death in the hippocampus, with increased protein expression of M2 markers (Ym1, IGF-1, and Gal-3). Microarray assays indicate that MSC downregulate more than 10% of the ischemia-induced genes, most of them involved in inflammatory and immune responses, showing that cell treatment has a potent immunomodulatory effect. M2 induction after MSC transplantation has also been seen after SCI (224) or TBI (156), and in both cases M2 polarization is associated with less scar tissue formation and improvement of behavioral deficits.

Our group has provided a detailed description of the effects on microglia/macrophage after MSC infusion in TBI mice, associating MSC-induced protection with increased activation of microglia/macrophages (156). This indicates that MSC *in vivo* do not reduce the inflammatory response, but rather pushes it toward a more protective phenotype. MSC enhance the expression of M2 markers (Ym1, Arg1, and CD206) over time, making the M2 phenotype last longer (up to 7 days) than in untreated animals (3 days). MSC induce general reprograming of the microenvironment, including increases of IL-10 and VEGF, reduction of astrogliosis and induction of the axonal regeneration marker GAP-43. These data indicate that the beneficial traits of microglia/macrophages induced by MSC skew the balance of the immune response toward protection and regenerative processes, further confirming that acting on the microglia/macrophage modulation would offer therapeutic benefit.

CONCLUSION

Recent evidence has helped define a new role for brain immune cells, highlighting their involvement in several stages of development, homeostasis, aging, or response to injury. Microglia and macrophages are the main players in neuroinflammation, and in view of their plastic nature, they may develop either toxic or protective functions. Specific commitments of these cells are closely associated with microenvironment signals, including metabolic crisis, exogenous/endogenous danger-associated molecules, and neuron-mediated activity control. Research in experimental settings has begun to lay a basis for understanding the behavior of microglia and macrophages in different physiological or pathological conditions, offering reasons for their therapeutic use. However, many aspects of their function, e.g., long-term response, crosstalk with other brain cells, and selective roles of microglia vs. macrophages, still need to be properly investigated if we are to acquire the ability to switch neuroinflammation protective functions.

AUTHOR CONTRIBUTIONS

MGDS, EZ, and CP drafted the manuscript; SF and FP drafted the manuscript and prepared the figures.

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REVIEW
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Rational modulation of the innate immune system for neuroprotection in ischemic stroke

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The innate immune system plays a dualistic role in the evolution of ischemic brain damage and has also been implicated in ischemic tolerance produced by different conditioning stimuli. Early after ischemia, perivascular astrocytes release cytokines and activate metalloproteases (MMPs) that contribute to blood–brain barrier (BBB) disruption and vasogenic oedema; whereas at later stages, they provide extracellular glutamate uptake, BBB regeneration and neurotrophic factors release. Similarly, early activation of microglia contributes to ischemic brain injury via the production of inflammatory cytokines, including tumor necrosis factor (TNF) and interleukin (IL)-1, reactive oxygen and nitrogen species and proteases. Nevertheless, microglia also contributes to the resolution of inflammation, by releasing IL-10 and tumor growth factor (TGF)-β, and to the late reparative processes by phagocytic activity and growth factors production. Indeed, after ischemia, microglia/macrophages differentiate toward several phenotypes: the M1 pro-inflammatory phenotype is classically activated via toll-like receptors or interferon-γ, whereas M2 phenotypes are alternatively activated by regulatory mediators, such as ILs 4, 10, 13, or TGF-β. Thus, immune cells exert a dualistic role on the evolution of ischemic brain damage, since the classic phenotypes promote injury, whereas alternatively activated M2 macrophages or N2 neutrophils prompt tissue remodeling and repair. Moreover, a subdued activation of the immune system has been involved in ischemic tolerance, since different preconditioning stimuli act via modulation of inflammatory mediators, including toll-like receptors and cytokine signaling pathways. This further underscores that the immuno-modulatory approach for the treatment of ischemic stroke should be aimed at blocking the detrimental effects, while promoting the beneficial responses of the immune reaction.

Keywords: cytokines, immune system, ischemic stroke, ischemic tolerance, macrophages, neutrophils, preconditioning

Introduction

As highlighted by recent expression profiling studies, the majority of the genes acutely modulated in the blood of stroke patients are implicated in the regulation of the innate immune system (Tang et al., 2006; Barr et al., 2010; Oh et al., 2012; Brooks et al., 2014). Moreover, serum levels of markers of acute inflammation correlate with the severity of brain damage and neurological deficit (Fassbender et al., 1994; Smith et al., 2004; Basic Kes et al., 2008; Whiteley et al., 2009; Chang et al., 2010).

Indeed, the innate immune system plays a pivotal role in the evolution of ischemic cerebral injury, as soluble mediators (i.e., cytokines and chemokines) and specialized cells, activated in the brain or recruited from the periphery, actively participate to the detrimental processes implicated in tissue damage, as well as to the repair and regeneration phases (Kamel and Iadecola, 2012; Amantea et al., 2014a). The dualistic role exerted by several mediators of the immune reaction may explain why most anti-inflammatory approaches, conceived disregarding the potential beneficial function of the target, have failed to reach the clinical setting.

In addition, a subdued activation of the immune system has been involved in ischemic tolerance, since different preconditioning stimuli act by reprogramming the immune response, through the modulation of inflammatory mediators, including toll-like receptors (TLRs) and cytokine signaling pathways (Garcia-Bonilla et al., 2014a). This further underscores that promoting the endogenous neuroprotective reactions of the innate immune system represents an attractive opportunity to develop novel effective stroke therapeutics.

This review has been conceived to describe the role played by the diverse mediators of the innate immune system in ischemic brain damage, also highlighting their beneficial role with the ambition to stimulate more extensive research aimed at selectively targeting these processes.

Cellular Mediators of the Immune Response

Resident Immune Cells

All the cellular components of the neurovascular unit participate to the inflammatory reaction involved in ischemic stroke injury (**Figure 1**). On the intravascular side, platelets and the complement system are rapidly activated after vessel occlusion, thus providing the first trigger for the inflammatory response (Atkinson et al., 2006; Nieswandt et al., 2011). Elevated endothelial expression of the adhesion molecules P-selectin and intercellular adhesion molecule (ICAM)-1 promotes polymorphonuclear leukocytes (PMN) recruitment that exacerbates microvessel obstruction, also due to the reduced bioavailability of nitric oxide (NO) (Granger et al., 1989; Mori et al., 1992; Wong and Crack, 2008). Recently, Sreeramkumar et al. (2014) have demonstrated that the interaction between PMN and platelets within the microvasculature of infarcted brains is inhibited by blocking P-selectin glycoprotein ligand-1 (PSGL-1), and this correlates with a significant decrease in the infarct volume after permanent occlusion of the middle cerebral artery. Moreover, the ischemia-induced release of pro-inflammatory cytokines [e.g., interleukin (IL)-1 and tumor necrosis factor (TNF)] further promotes adhesion molecules expression and, together with the activation of proteases [i.e., matrix metalloproteases (MMPs)], prompts blood-brain barrier (BBB) breakdown leading to leukocytes extravasation in the injured brain (Ishikawa et al., 2004; Amantea et al., 2007; Yemisci et al., 2009; Yilmaz and Granger, 2010).

Astrocytes are the most abundant glial cells of the human brain and form part of the BBB. Ultrastructural studies have shown that pericapillary astrocyte end-feet are the first cellular elements to swell during cerebral ischemia (Dodson et al., 1977). Cytokines and reactive oxygen species (ROS), released by neurons and glial cells few minutes after the ischemic insult, alter molecular expression patterns in astrocytes and induce cellular hypertrophy, proliferation and scar formation (Sofroniew, 2009). Moreover, stimulation of P2Y(1) receptors by adenosine 5′-triphosphate (ATP), released or leaked from injured cells, prompts production of pro-inflammatory cytokines and chemokines by astrocytes via activation of a phosphorylated-p65 subunit (RelA)-mediated NF-κB pathway (Kuboyama et al., 2011). IL-1β and MMPs produced by perivascular astrocytes participate to BBB disruption and vasogenic edema (Rosenberg et al., 1998; del Zoppo and Hallenbeck, 2000; Amantea et al., 2010). Nevertheless, astrocytes exposed to an ischemic insult may also participate to neuroprotective and reparative responses (**Table 1**) by extracellular glutamate uptake (Stanimirovic et al., 1997; Rossi et al., 2007), BBB rebuilding (Kinoshita et al., 1990; del Zoppo, 2009) and neurotrophic factors release (Shen et al., 2010; Barreto et al., 2011). Accordingly, impairment of astrocyte function amplifies ischemic neuronal death (Nakase et al., 2003; Ouyang et al., 2007) and ablation of their reactivity and proliferation delays neurovascular remodeling and disrupts scar formation, negatively affecting functional recovery after focal cerebral ischemia in rodents (Nawashiro et al., 2000; Li et al., 2008; Hayakawa et al., 2010). Alternatively, proliferation of astrocytes induced by environmental enrichment or by pharmacological induction of signal transducer and activator of transcription factor (STAT)-3 phosphorylation ameliorates histological and functional outcomes in stroke models (Keiner et al., 2008; Amantea et al., 2011). Thus, reactive astrogliosis may exert a dualistic role on the propagation of ischemic brain damage, depending on the polarization of astrocytes toward specific phenotypes (Zamanian et al., 2012; Rusnakova et al., 2013) and on their interaction with surrounding neurons and microglia (Bezzi et al., 2001; Kang et al., 2012).

After an ischemic insult, microglia, the resident immune cells of the central nervous system, is rapidly activated by ATP released by damaged neurons and other glial cells, acting on P2X7 receptors to prompt production and release of pro-inflammatory mediators (Melani et al., 2006; Dénes et al., 2007). Stimulation of microglia also relies on TLR4 stimulation, fractalkine receptor (CX3CR1) modulation and/or reduced CD200 receptor stimulation evoked by ischemia-induced disturbance of neuron-microglia cross-talk (Lehnardt et al., 2003; Dénes et al., 2008; Dentesano et al., 2012). Moreover, the increased release of specific

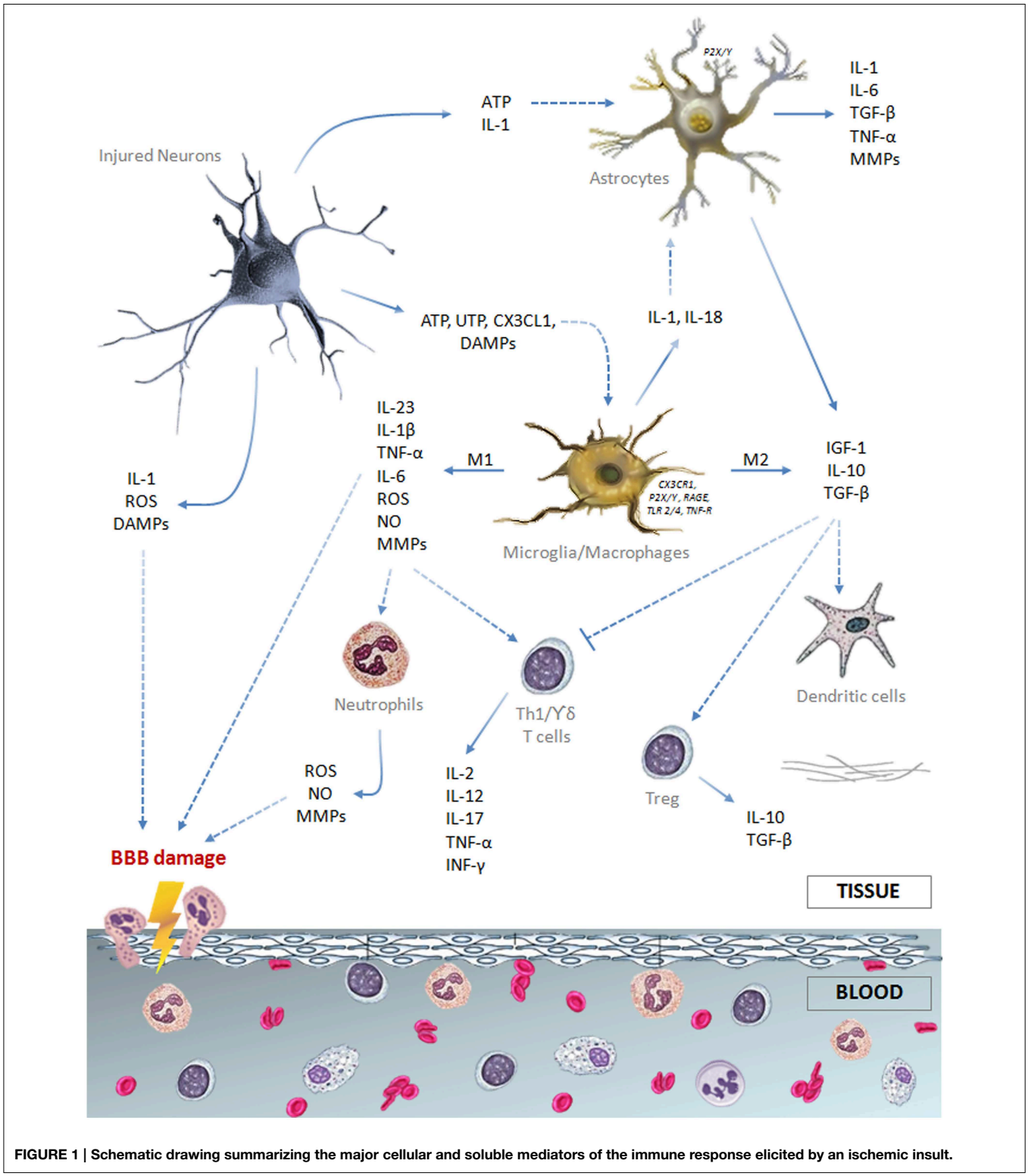

FIGURE 1 | Schematic drawing summarizing the major cellular and soluble mediators of the immune response elicited by an ischemic insult.

neurotransmitters, such as glutamate and γ-aminobutyric acid, may elicit an inflammatory or a neuroprotective phenotype in microglia by signaling through Nox (Pocock and Kettenmann, 2007; Mead et al., 2012).

Microglial activation is accompanied by a substantial morphological transformation characterized in the early stages by retraction of cellular processes and enlargement of cell bodies, to ultimately acquire an amoeboid macrophage-like phenotype

TABLE 1 | Dualistic effects of innate immune cells activated after ischemic brain injury.

Cell type	Detrimental effects	Beneficial effects
Astrocytes	Production of inflammatory mediators (e.g., TNF-α, IL-1 and MMPs). Edema formation, inhibition of axon regeneration and BBB disruption.	Extracellular glutamate uptake, synthesis and release of neurotrophic factors. Glial scar formation, BBB rebuilding and neurovascular remodeling.
Microglia/macrophages	M-1 phenotype: production of pro-inflammatory cytokines, including TNF and IL-1, reactive oxygen and nitrogen species and proteases, such as MMPs.	M-2 phenotype: resolution of inflammation (IL-10 and TGF-β release, production of arginase and phagocytic activity). Late reparative processes by producing growth factors (IGF-1, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor).
Neutrophils	Microvessel obstruction, ROS production and release of MMPs that contribute to BBB damage and exacerbate inflammation.	N2 phenotype: promote resolution of inflammation
Dendritic cells	Up-regulation of MHC-II and co-stimulatory molecules that prompt activation of lymphocytes.	

(Davis et al., 1994; Zhang et al., 1997; Schilling et al., 2003; Jung and Schwartz, 2012). As reported both in ischemia animal models (Zhang et al., 1997; Stoll et al., 1998; Dénes et al., 2007) and in stroke patients (Gerhard et al., 2005; Price et al., 2006), the reactivity and proliferation of microglia reach a peak few days after the insult and may persist for several weeks.

Reactive microglia may enhance inflammation and ischemic tissue injury via increased production and release of cytokines, such as IL-1 and TNF (Barone et al., 1997; Rothwell et al., 1997; Lambertsen et al., 2005; Amantea et al., 2010), reactive oxygen and nitrogen species (Green et al., 2001) and proteases (del Zoppo et al., 2007). These detrimental effects are underscored by the evidence that pharmacological- or microRNA-induced suppression of microglial activity limits ischemic cerebral injury (Hailer, 2008; Fagan et al., 2011; Zhang et al., 2012). However, other studies have demonstrated beneficial effects exerted by microglia in experimental stroke settings (Kitamura et al., 2004; Imai et al., 2007; Lalancette-Hébert et al., 2007). Indeed, by producing IL-10, transforming growth factor (TGF)-β and insulin-like growth factor (IGF)-1, microglia promotes resolution of the inflammatory reaction and reparative mechanisms involved in late tissue recovery (O'Donnell et al., 2002; Lalancette-Hébert et al., 2007; Neumann et al., 2008; Ransohoff and Cardona, 2010).

The reason for these apparently discrepant results can be found in the aptitude of microglia/macrophages to differentiate toward diverse phenotypes, depending on the dynamic evolution of the ischemic damage (Clausen et al., 2008; Perego et al., 2011). Indeed, the classic pro-inflammatory M1 phenotype is activated by interferon (INF)-γ or through TLRs modulation, whereas the alternatively activated M2 phenotype is induced by regulatory factors, including interleukins 4, 10, 13, or TGF-β (Italiani and Boraschi, 2014) (**Table 1**). Early after an ischemic insult, local microglia assumes the M2 "beneficial" phenotype, to then develop into a pro-inflammatory M1 phenotype prompted by the ischemic neurons (Hu et al., 2012). Thus, in addition to the classical approach aimed at suppressing detrimental M1 functions (i.e., production of TNF-α, IL-1β, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and IL-6), preservation of the alternatively activated M2 phenotype may represent an innovative strategy for stroke neuroprotection, as demonstrated in mice lacking the class-A scavenger receptor (Xu et al., 2012) or the myeloid-specific mineralcorticoid receptor (Frieler et al., 2011). Given the similarities between local microglia and macrophages, as well as the ability of microglia to develop active phenotypes indistinguishable from circulating macrophages, further insights into their polarization will be given in the following section.

Blood-Borne Cells

Genomic profile studies indicate a critical role of innate immunity in regulating stroke response and recovery. The genes identified in these profiles are involved in immune signaling at different levels, including the cerebral microenvironment, the vasculature and, most notably, the peripheral circulation (Brooks et al., 2014). In the first analysis performed in stroke patients, Moore et al. (2005) detected the expression of genes associated with the regulation of the cerebral microenvironment in peripheral blood mononuclear cells (PMBCs), thus supporting the important finding that peripheral blood is reflective of changes in the brain.

Interestingly, the majority of the genes identified in all the stroke-specific profiles published to date are related to the immune system (Moore et al., 2005; Tang et al., 2006; Barr et al., 2010; Oh et al., 2012). Among these studies, Brooks et al. (2014) recently identified a panel of overlapping genes, significantly expressed within 3 h from stroke onset, including arginase1 (ARG1), carbonic anhydrase 4 (CA4), lymphocyte antigen 96 (LY96), matrix metalloproteinase 9 (MMP9), and S100 calcium binding protein A12 (S100A12). Specifically, four of these genes (ARG1, LY96, MMP9, S100A12) are implicated in the innate immune response, the fifth (CA4) being highly expressed in the BBB (Brooks et al., 2014).

These findings strongly support the theory that the immune response to the ischemic insult engages specialized circulating cells, such as neutrophils, macrophages, dendritic cells and T

lymphocytes, that are recruited and migrate to the brain, upon activation triggered by soluble factors released by injured and dying cells (Price et al., 2004; Buck et al., 2008; Felger et al., 2010; Yilmaz and Granger, 2010; Kamel and Iadecola, 2012). Infiltrating leukocytes affect the evolution of tissue damage by releasing a series of mediators including purines, ROS and danger associated molecular patterns (DAMPs), such as high mobility group box (HMGB)-1 protein, heat shock protein 60, β-amyloid, DNA or RNA immune complexes (Amantea et al., 2014a).

Interestingly, the majority of the genes acutely regulated in the blood of stroke patients are expressed in neutrophils and, to a lesser extent, in macrophages (Tang et al., 2006). Accordingly, these are the first cells to infiltrate the ischemic brain, reaching a peak within 24–72 h after the insult (Clark et al., 1994; Garcia et al., 1994; Gelderblom et al., 2009). In patients, higher peripheral leukocyte and neutrophil counts, but not lymphocyte counts, are associated with larger infarct volumes (Buck et al., 2008), and brain accumulation of neutrophils correlates with poor neurological outcome and brain damage severity both in humans (Akopov et al., 1996) and in rodents (Matsuo et al., 1994a,b; Connolly et al., 1996; Atochin et al., 2000). In fact, neutrophils prompt microvessel obstruction/thrombosis (del Zoppo et al., 1991; Ritter et al., 2005), production of ROS and release of MMPs (Justicia et al., 2003; Gidday et al., 2005; Bao Dang et al., 2013); thus, their modulation may represent a useful strategy to ameliorate stroke outcome. This may be achieved by pharmacological approaches, as well as by remote ischemic preconditioning, as documented by the evidence that forearm transient ischemia reduces neutrophil function, including adhesion, exocytosis, phagocytosis and cytokine secretion (Shimizu et al., 2010).

Interestingly, recent findings have highlighted the ability of neutrophils to polarize toward beneficial N2 phenotypes. In the setting of stroke, neutrophil reprogramming can be induced by activation of the nuclear peroxisome proliferator-activated receptor (PPAR)-γ (Cuartero et al., 2013). Thus, despite promoting early neutrophil infiltration to the ischemic core, the PPAR-γ agonist rosiglitazone provides neuroprotection and resolution of inflammation after experimental stroke induced by permanent MCAo by promoting N2-polarization and increased neutrophil clearance (Cuartero et al., 2013).

Likewise neutrophils, hematogenous macrophages infiltrating the ischemic brain (Schilling et al., 2003; Jander et al., 2007), exert a dualistic role on the evolution of tissue damage (Frieler et al., 2011; Hu et al., 2012; Xu et al., 2012). In fact, they possess the ability to switch between the classically activated M1 phenotype and alternatively activated M2 phenotypes (Ballesteros et al., 2014a) (**Table 1**). The M1 cells initiate and sustain inflammation by releasing neurotoxic factors and ROS that underlie macrophage/microglia-mediated neurotoxicity after stroke; whereas, M2-polarized cells are involved in beneficial responses by clearing debris and by promoting angiogenesis, tissue remodeling and repair (Gliem et al., 2012; Shechter and Schwartz, 2013). Up-regulation of M2 markers observed in the ischemic brain (Frieler et al., 2011; Perego et al., 2011; Hu et al., 2012; Zarruk et al., 2012; Ballesteros et al., 2014a) is due to an increased cerebral infiltration of alternatively activated blood-borne monocytes (Perego et al., 2011), as well as to the ability of local microglia/macrophages to assume an M2 phenotype (Hu et al., 2012). Indeed, local microglia and newly recruited macrophages assume the M2 phenotype at early stages of ischemic stroke but, upon priming by ischemic neurons, gradually transform into the M1 phenotype (Hu et al., 2012). The exact mechanisms that control macrophage polarization in the setting of stroke have not been fully elucidated, as well as it is not clear whether the acquisition of a specific phenotype involves recruitment of circulating precursors or *in situ* cell re-instruction. Endogenous production of the M2-polarizing cytokine IL-4, triggered by MCAo in mice, has been shown to promote Th2 polarization and, thus, beneficial effects on stroke outcome (Xiong et al., 2011). Further studies have shown that a subpopulation of bone marrow-derived monocytes/macrophages, recruited via CCR2 and acting through TGF-β1, maintains the integrity of the neurovascular unit in murine stroke models (Gliem et al., 2012).

To date, only few studies have assessed the therapeutic benefits of reducing the M1/M2 ratio in stroke setting. Frieler et al. (2011) showed that deficiency of the mineralcorticoid receptor (MR) decreases the expression of M1 markers, while preserving the ischemia-induced expression of M2 markers. The resulting elevation of M2 polarized myeloid cells in the ischemic brain was correlated with a better stroke outcome in $MR^{-/-}$ mice (Frieler et al., 2011). Similarly, deficiency of the fractalkine receptor CX3CR1 has been associated with a protective inflammatory milieu, characterized by the promotion of M2 polarization markers (Fumagalli et al., 2013). PPAR-γ-mediated CD36 up-regulation has been involved in the modulation of microglia phenotype, promoting phagocytosis of apoptotic neutrophils, and thus contributing to the resolution of inflammation after stroke (Ballesteros et al., 2014b). By contrast, elevation of M1/M2 ratio promoted by the class A scavenger receptor expressed in microglia/macrophages has been associated with exacerbation of ischemic brain injury (Xu et al., 2012). At variance with the latter, the selective cannabionoid receptor 2 agonist, JWH-133, provides neuroprotection in the acute phase of ischemic stroke by reducing microglia activation, without affecting M2 polarization (Zarruk et al., 2012).

Although preclinical findings strongly suggest the therapeutic usefulness of M2-polarizing agents, the exact mechanisms modulating M1/M2 ratio need further investigation and their relevance for stroke outcome in human stroke has to be validated.

At later stages after the ischemic insult, a significant elevation of dendritic cells occurs in the injured brain hemisphere, reaching a peak 72 h after the insult (Kostulas et al., 2002; Reichmann et al., 2002; Gelderblom et al., 2009). Both peripheral and brain resident dendritic cells are pivotally involved in bridging innate and adaptive immunity by up-regulating major histocompatibility complex (MHC)-II and co-stimulatory molecules that contribute to the activation of lymphocytes (Gelderblom et al., 2009; Felger et al., 2010). Moreover, resident dendritic cells participate in orchestrating the early local immune response and in the late recruitment of lymphocytes (Felger et al., 2010) following activation by INF-γ (Gottfried-Blackmore et al., 2009). Brain infiltration of T lymphocytes occurs relatively late (i.e., 3–7

days) after ischemia (Jander et al., 1995; Schwab et al., 2001; Gelderblom et al., 2009); nevertheless, these cells contribute to the progression of brain damage (Yilmaz et al., 2006; Hurn et al., 2007; Jin et al., 2010), exerting distinct effects depending on the specific cell subset recruited (Amantea et al., 2014a). Recent evidence demonstrates that the functional sphingosine-1-phosphate receptor agonist FTY720 (fingolimod), minimizes brain damage and functional deficits in experimental stroke (Shichita et al., 2009; Hasegawa et al., 2010; Wei et al., 2011). Interestingly, the putative elevation of the incidence of bacterial pneumonia caused by the inhibition of the adaptive immunity by fingolimod does not seem to be actually relevant for the neuroprotective effects of the drug (Pfeilschifter et al., 2011).

Thus, the ischemic insult is associated to a relevant activation of the innate immune system, involving both local and blood-borne specialized cells that, upon recruitment, modulate the cerebral inflammatory response to stroke and set the stage for the activation of adaptive immunity (**Figure 1**).

Molecular Mediators of the Immune Response

Receptors

As stated above, activation of P2X7 receptors on microglia prompts the processing and release of pro-inflammatory cytokines (Brough et al., 2002; Melani et al., 2006). Moreover, overactivation of P2X7 receptors is involved in excitotoxic neuronal death (Arbeloa et al., 2012) and participates to ischemia-induced damage to olygodendrocytes and myelin (Domercq et al., 2010).

Activation of TLRs by HMGB1, peroxiredoxin (Prx) proteins and other DAMPS, plays an important role in ischemic brain injury (Fossati and Chiarugi, 2007; Shichita et al., 2012a,b; Pradillo et al., 2014). In particular, TLR2 and TLR4 crucially contribute to the induction of the inflammatory response and to the evolution of brain damage, as documented by the evidence that TLR2- or TLR4-deficiency is associated to reduced ischemic brain damage and to suppression of ischemia-induced expression and release of inflammatory cytokines (Tang et al., 2007; Hyakkoku et al., 2010). Indeed, TLR4 deficient mice display significant suppression of IκB phosphorylation, NFκB activity, pro-inflammatory mediators, including TNF-α and IL-6 (Cao et al., 2007; Hyakkoku et al., 2010) and the enzymes inducible nitric oxide synthase (NOS) and cyclooxygenase (COX)-2 (Caso et al., 2007, 2008).

The relevance of TLR2 and TLR4 has also been demonstrated in ischemic stroke patients, since up-regulation of these receptors is associated with greater inflammatory responses and with poor functional outcome (Brea et al., 2011). The stimulation of macrophages and T cells by TLRs-associated pathways induces strong inflammatory responses (Shichita et al., 2012a). Following cerebral ischemia, the activation of TLR4 by HMGB-1 induces MMP-9 up-regulation in neurons and astrocytes (Qiu et al., 2010) and promotes detrimental effects by macrophages infiltrating the injured brain (Yang et al., 2011). In fact, cerebral microinjection of HMGB-1 increases the transcript levels of pro-inflammatory mediators and sensitizes the tissue to ischemic injury (Faraco et al., 2007). In addition, deficiency of TLR4 in young animals subjected to focal cerebral ischemia, promotes subventricular zone cell proliferation, increasing the number of the transit-amplifying cells (type C cells; prominin-1+/EGFR+/nestin-cells) at 24 and 48 h, of proliferating immature (BrdU+) cells at 7d and of neuroblast cells (type A cells; doublecortin+ cells) at 14d (Moraga et al., 2014). Despite a negative effect on SVZ cell proliferation, TLR4 plays an important role in stroke-induced neurogenesis by promoting neuroblasts migration and increasing the number of new cortical neurons after stroke (Moraga et al., 2014).

Although the exact mechanisms by which TLRs modulate the evolution of ischemic brain injury have not been fully elucidated, pharmacological inhibition of TLR2 and TLR4 and/or blockade of some of their endogenous ligands (i.e., cellular fibronectin or heat shock protein 60), represent promising therapeutic options, effective in reducing the inflammatory response to stroke injury (Brea et al., 2011).

Paradoxically, by reprogramming TLRs signaling, stimulation of TLRs before ischemia leads to suppression of pro-inflammatory responses and to enhanced expression of numerous anti-inflammatory mediators that collectively contribute to neuroprotection (Pradillo et al., 2009; Vartanian et al., 2011). Activation of TLR4 by low doses of LPS reduces synthesis and release of some pro-inflammatory cytokines, and inhibits microglial activation and neutrophil infiltration, thus reducing ischemic brain injury (Rosenzweig et al., 2004; Pradillo et al., 2009). Tolerance to brain ischemia induced by low doses of the major TLR4 ligand, LPS, administered 1–3 days before the insult, has been demonstrated in several experimental stroke models (Tasaki et al., 1997; Hickey et al., 2007; Yu et al., 2010). Recent work has also demonstrated that ischemic preconditioning reduces brain damage from permanent middle cerebral artery occlusion in mice by increasing expression of TLR3 in cortical astrocytes (Pan et al., 2014). Therefore, induction of ischemic tolerance by subdued TLRs activation represents an interesting opportunity to exploit these receptors for stroke therapy.

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin family of cell surface receptors and has been implicated in the development and progression of stroke. The full-length, membrane-bound RAGE isoform (fl-RAGE) is mainly expressed in neurons and in microglia/macrophages. Up-regulation of this receptor has been documented after both permanent and transient focal cerebral ischemia in rodents (Qiu et al., 2008; Zhai et al., 2008; Hassid et al., 2009) and in the ischemic hemisphere of stroke patients (Hassid et al., 2009). More specifically, ischemia-induced modifications of the expression of RAGE and its isoforms in the brain strongly depends on the intensity and on the propagation of the insult; showing a distinct modulation in the core and penumbra regions (Greco et al., 2012, 2014). By activating fl-RAGE on microglia/macrophages, HMGB-1 protein released from dying neurons, contributes to the development of ischemic brain damage (Muhammad et al., 2008; Qiu et al., 2008). Conversely, blockade of fl-RAGE signaling promotes cell survival

and reduces stroke infarct volume in animal models (Kim et al., 2006; Liu et al., 2007; Muhammad et al., 2008).

A reduced plasma level of soluble RAGE isoforms (sRAGE) has been reported in rats subjected to either transient or permanent focal brain ischemia (Greco et al., 2012, 2014). This is of significance since sRAGE, generated either by alternative splicing or by proteolysis of the full-length form, effectively bind AGEs, thereby competing with the cell surface fl-RAGE, thus providing a "decoy" function that may counteract the detrimental effects of receptor signaling in neurons (Koyama et al., 2007; Muhammad et al., 2008; Tang et al., 2013). Interestingly, the reduction of sRAGE levels induced by transient MCAo in rats, is minimized by pre-treatment with a neuroprotective dose of the poly(ADP-ribose) polymerase (PARP) inhibitor PJ34 (Greco et al., 2014), suggesting that sRAGE may represent a useful biomarker of stroke severity and of effective neuroprotective treatment. In patients, some studies have reported an association between circulating sRAGE levels and brain infarct volume, stroke severity (Park et al., 2004; Yokota et al., 2009) and inflammatory status (Cui et al., 2013), others have suggested that sRAGE levels at onset may predict cognitive impairment after cerebral ischemia (Qian et al., 2012).

Inflammatory Cytokines

The inflammatory response to ischemic brain injury is orchestrated by a variety of cytokines released by resident brain cells, such as neurons and glia, and by blood-borne immune cells. TNF, IL-1 and IL-6 strongly affect the development of ischemic brain damage in animal models, and their levels are increased in the blood and in the cerebrospinal fluid of stroke patients (Lambertsen et al., 2012), thus attracting considerable interest as putative markers of stroke severity and neurologic outcome (Emsley et al., 2007; Jickling and Sharp, 2011). By contrast, other cytokines, including IL-10 and TGF-β exert immunoregulatory and anti-inflammatory effects, thus promoting reparative processes. In addition, mild systemic inflammation induced by remote preconditioning is neuroprotective in stroke models (Petcu et al., 2008) and several soluble mediators of the innate immune system have been involved in ischemic tolerance produced by diverse preconditioning stimuli (Garcia-Bonilla et al., 2014a).

A significant and rapid up-regulation of TNF-α occurs following focal cerebral ischemia both in animal models and in stroke patients. In fact, expression of this cytokine is elevated in neurons during the first hours after the insult; whereas, at later stages, it is increased in microglia/macrophages and in blood-borne immune cells (Gregersen et al., 2000; Dziewulska and Mossakowski, 2003). Accordingly, recent flow cytometry experiments have demonstrated that the major source of this cytokine in the stroke-lesioned mouse brain is microglia and macrophages (Clausen et al., 2008; Lambertsen et al., 2012).

TNF-α is believed to play a detrimental role in ischemic injury, since its neutralization with specific monoclonal antibodies or binding proteins provides neuroprotection in experimental stroke models (Barone et al., 1997; Nawashiro et al., 1997; Lavine et al., 1998; Lambertsen et al., 2012). Nevertheless, studies from transgenic animals demonstrate that the cytokine may also exert beneficial effects through the activation of p55 TNF receptor (TNF-RI) (Bruce et al., 1996; Gary et al., 1998; Taoufik et al., 2007; Lambertsen et al., 2009). In rodents, expression of TNF-RI is elevated in neurons and in non-neuronal cells few hours after MCAo, whereas up-regulation of TNF-RII occurs from 24 h after injury in resident microglia and infiltrating macrophages (Botchkina et al., 1997; Dziewulska and Mossakowski, 2003; Yin et al., 2004; Pradillo et al., 2005; Lambertsen et al., 2007). Despite its predominant inflammatory role, TNF-RII has also been implicated in neuroprotection (Marchetti et al., 2004), and this further complicates the interpretation of the pleiotropic effects of TNF in ischemic neuronal damage (Hallenbeck, 2002; McCoy and Tansey, 2008).

Moreover, the TNF pathway has been involved in ischemic, hypoxic, endotoxic and exercise-induced preconditioning (Garcia-Bonilla et al., 2014a). In fact, LPS-preconditioned TNF-α null mice are not protected from ischemic brain injury (Rosenzweig et al., 2007) and administration of a neutralizing TNF binding protein nullifies the beneficial effect of LPS pre-treatment in spontaneously hypertensive rats subjected to permanent MCAo (Tasaki et al., 1997). Paradoxically, TNF-α has a dualistic effect in stroke, since it's up-regulation has been shown to underlie LPS-induced tolerance in mice subjected to focal cerebral ischemia, whereas suppression of TNF-α signaling during ischemia confers neuroprotection after LPS preconditioning (Rosenzweig et al., 2007). Up-regulation of TNF-α converting enzyme (TACE) and increased serum levels of TNF-α have also been involved in preconditioning induced by prolonged and intermittent normobaric hyperoxia in rats subjected to MCAo (Bigdeli and Khoshbaten, 2008; Bigdeli et al., 2008). In rats, the increased expression of TNF-α lasting for 2–3 weeks of physical activity underlies amelioration of downstream inflammatory events, reduced BBB disruption and the consequent ischemic neuroprotection induced by exercise preconditioning (Ding et al., 2005, 2006; Guo et al., 2008b).

IL-1 is a proinflammatory cytokine that plays a pivotal role in the neurodegenerative processes triggered by the ischemic insult (Olsson et al., 2012; Dénes et al., 2013). Cerebral levels of both IL-1α and IL-1β are elevated within hours of reperfusion after focal ischemia (Hara et al., 1997; Amantea et al., 2007, 2010; Luheshi et al., 2011). IL-1α is mainly induced in microglia, whereas IL-1β can be released by all the elements of the neurovascular unit, including neurons, astrocytes, microglia/macrophages and endothelial cells, being its cellular source strongly dependent on the spatio-temporal evolution of the damage (Amantea et al., 2010; Luheshi et al., 2011; Giles et al., 2015). In astrocytes and microglia, ischemia-induced production of IL-1β involves activation of TLR4 (Simi et al., 2007) and p38 mitogen-activated protein kinase (MAPK) (Walton et al., 1998; Irving et al., 2000). Interestingly, under ischemic conditions, caspase-1-independent pathways seem to contribute to IL-1β maturation. Accordingly, it has been recently demonstrated that early elevation of IL-1β in the ischemic cortex of rats subjected to transient MCAo is not associated with caspase-1 activation, whereas production of the mature cytokine is strongly dependent on gelatinases, i.e., MMP2 and MMP-9 activity (Amantea et al., 2007, 2014b).

It is intriguing to observe that IL-1 is not directly toxic to healthy neurons, whereas it may become harmful via the modulation of other elements of the neurovascular unit, such as astrocytes and the endothelium. IL-1 stimulates astrogliosis (Herx and Yong, 2001) and prompts the release of cytokines, chemokines (Andre et al., 2005) and the activation of MMP-9 in astrocytes (Thornton et al., 2008). Furthermore, this cytokine induces endothelial expression of adhesion molecules, including ICAM-1 and vascular cell adhesion molecule (VCAM)-1, that together with the local stimulation of the release of chemokines, promotes neutrophil adhesion and infiltration in the injured hemisphere (Thornton et al., 2011; Allen et al., 2012).

Despite their established detrimental roles, both IL-1α and IL-1β have been shown to underlie tolerance to global ischemia induced by common carotid artery occlusion in gerbil (Ohtsuki et al., 1996). Moreover, ischemic preconditioning produced by bilateral common carotid artery occlusion protects mice against subsequent MCAo injury by differentially regulating cortical IL-1β and IL-1 receptor antagonist (IL-1ra) expression in order to promote a shift toward an anti-inflammatory state that contributes to neuroprotection (Shin et al., 2009). The regulation of the expression and of the effects of IL-1 has also been implicated in LPS preconditioning via the TLR4 signaling pathway (Gong et al., 2014).

Other cytokines involved in the evolution of brain infarction and contributing to aggravate neurological functions are IL-17 and IL-23 (Shichita et al., 2009; Konoeda et al., 2010; Ma et al., 2013; Swardfager et al., 2013). IL-17A secreted by γδ T cells promotes neutrophil recruitment and its blockade with specific antibodies exerts neuroprotection (Gelderblom et al., 2012). Despite the presence of IL-17A-positive lymphocytes in autoptic brain of stroke patients, IL-17 blood level does not seem to be a good predictor of stroke outcome as compared to other cytokines such as IL-6 (Zeng et al., 2013). In fact, plasma and cerebrospinal fluid levels of IL-6 correlate with stroke severity and poor clinical outcome in patients (Smith et al., 2004; Waje-Andreassen et al., 2005; Whiteley et al., 2009) and reduced blood concentrations of this cytokine have been correlated to the improved outcome induced by treatment with IL-1ra (Emsley et al., 2005). By contrast, in stroke animal models, IL-6 appears to play a neuroprotective role (Matsuda et al., 1996; Loddick et al., 1998; Herrmann et al., 2003), suggesting that its rapid and persistent elevation in the ischemic brain (Suzuki et al., 1999; Ali et al., 2000) may represent a compensatory mechanism to counteract the damaging effects of the insult. In fact, by activating its receptor and the downstream phosphorylation of STAT-3, IL-6 may induce neuroprotection (Yamashita et al., 2005; Jung et al., 2011). Moreover, IL-6 enhances the effectiveness of cell transplantation therapy in ischemic stroke, by reprogramming neural stem cells to tolerate oxidative stress and to induce angiogenesis through STAT-3 activation (Perini et al., 2001).

Anti-Inflammatory Cytokines

While plasma concentrations of detrimental cytokines are elevated, levels of IL-10, associated with better outcome, are decreased in patients (Perini et al., 2001; Vila et al., 2003; Basic Kes et al., 2008). Results from animal models demonstrate that IL-10 represents a major downregulator of the detrimental effects of proinflammatory mediators during stroke and modulates neuronal vulnerability to excitotoxic ischemic damage (Spera et al., 1998; Grilli et al., 2000; Frenkel et al., 2005).

Another cytokine playing beneficial effects in cerebral ischemia is TGF-β (Gliem et al., 2012). Levels of this cytokine are elevated in the blood of patients 1 day after ischemic stroke (Yan et al., 2012) and in activated astrocytes and microglia/macrophages of the ischemic brain for at least 1 week in animal models (Lehrmann et al., 1998; Yamashita et al., 1999; Doyle et al., 2010). In rats, administration of a TGF-β antagonist aggravates brain damage caused by focal cerebral ischemia (Ruocco et al., 1999); whereas, in mice, intranasal delivery of this cytokine after stroke reduces infarct volume and increases neurogenesis in the subventricular zone (Ma et al., 2008). The beneficial effects of TGF-β involve both anti-inflammatory effects, including inhibition of brain elevation of MCP-1 and MIP-1α (Pang et al., 2001), but also induction of glial scar formation (Logan et al., 1994) and anti-apoptotic effects (Zhu et al., 2002).

In mice, preconditioning with low dose LPS is associated with upregulation of anti-inflammatory cytokines, including TGF-β in brain and IL-10 in blood (Vartanian et al., 2011).

Chemokines

In patients with acute ischemic stroke, serum levels of stromal cell-derived factor (SDF)-1α have been correlated with favorable long-term outcome (Kim et al., 2012) and this chemokine has been suggested to be a predictor of future stroke (Schutt et al., 2012). SDF-1α promotes bone marrow-derived cell targeting to the ischemic brain and improves local cerebral blood flow (Cui et al., 2007; Shyu et al., 2008), thus representing a promising target to implement stem cell therapy in patients. In fact, in the injured hemisphere, it activates CXCR4 on neural progenitor cells guiding their specific migration to the site of damage (Robin et al., 2006). Moreover, SDF-1α participates to the regulation of post-ischemic inflammation and it is involved in neurovascular repair (Wang et al., 2012). Despite these documented beneficial effects, some detrimental roles have also been suggested to be mediated by this chemokine, since pharmacological blockade of CXCR4 is neuroprotective in stroke by reducing BBB damage and inflammatory processes (Huang et al., 2013). Soluble fractalkine (CX3CL1) released by neurons upon an ischemic insult controls leukocyte trafficking and participates to the activation and chemoattraction of microglia to the injured brain via the activation of CX3CR1 receptors (Tarozzo et al., 2002; Dénes et al., 2008; Zhu et al., 2009). The detrimental effects of this chemokine are mediated by IL-1β and TNF-α in stroke mouse models (Soriano et al., 2002; Dénes et al., 2008). By contrast, in ischemic stroke patients, higher plasma concentrations of fractalkine are associated with better outcome and with low levels of systemic markers of inflammation (Donohue et al., 2012). In fact, by inhibiting caspase-3 and by activating adenosine receptors, exogenous administration of fractalkine is neuroprotective in wild-type rodents undergone permanent ischemia (Cipriani et al., 2011; Rosito et al., 2014). The apparent discrepancies between this latter finding and those produced in transgenic

animals may be explained by the altered microglia responsiveness to fractalkine in the absence of constitutive fractalkine-CX3CR1 signaling (Cipriani et al., 2011).

Among the chemokines implicated in cerebral ischemia, MCP-1 (also known as CCL2) and MIP-1α have been shown to play an important role in promoting tissue damage via recruitment of inflammatory cells (Wang et al., 1995; Che et al., 2001; Takami et al., 2001; Minami and Satoh, 2003). The mRNA levels of both chemokines are elevated in the ischemic brain of rodents and MCP-1 levels are also increased in the cerebrospinal fluid of stroke patients (Losy and Zaremba, 2001). Mice lacking MCP-1 or its receptor, CCR2, display reduced infarct volume along with impaired leukocyte recruitment and reduced expression of inflammatory mediators in the injured brain (Hughes et al., 2002; Dimitrijevic et al., 2007; Strecker et al., 2011; Schuette-Nuetgen et al., 2012). Conversely, overexpression of MCP-1 prompts exacerbation of brain injury and increased cerebral recruitment of inflammatory cells (Chen et al., 2003). Moreover, MCP-1, as well as SDF-1α, promotes migration of newly formed neuroblasts from neurogenic regions to ischemic damaged areas (Robin et al., 2006; Yan et al., 2007).

Up-regulation of MCP-1 has been shown to underlie hypoxic preconditioning-induced stroke tolerance in mice (Stowe et al., 2012; Wacker et al., 2012); whereas, activation of CCR2 is involved in the neuroprotective effects exerted by both ischemic preconditioning and post-conditioning in mice subjected to global cerebral ischemia (Rehni and Singh, 2012).

Monocyte chemotactic protein-induced protein 1 (MCPIP1) deficiency exacerbates ischemic brain damage by upregulation of proinflammatory cytokines and this Zn finger-containing immunoregulatory protein also participates in LPS- and electroacupuncture-induced ischemic stroke tolerance (Liang et al., 2001; Jin et al., 2013).

Enzymes: MMPs, COX, and NOS

MMPs play a crucial role in the evolution of the inflammatory response to ischemic brain injury (Cunningham et al., 2005). Stroke-induced damage to the BBB and hemorrhagic transformation are both induced by the activation of the gelatinases, MMP-2 and MMP-9, as demonstrated in animal models (Romanic et al., 1998; Rosenberg et al., 1998; Asahi et al., 2000) and in patients (Horstmann et al., 2003; Rosell et al., 2006, 2008). RNA-expression levels of MMP-9 in circulating monocytes have been correlated with the brain infarct lesion in stroke patients (Ulrich et al., 2013); while, serum levels of this enzyme have been associated with clinical diffusion mismatch (Rodríguez-Yáñez et al., 2011).

Cerebral expression and activity of gelatinases increase very early after an ischemic insult, with a specific cellular expression pattern dependent on the spatio-temporal evolution of the damage (Rosenberg et al., 1998; Planas et al., 2001; Yang et al., 2007; Amantea et al., 2008). Pharmacological inhibition of gelatinases, as well as gene deletion of MMP-9, reduces infarct volume caused by focal cerebral ischemia in rodents (Romanic et al., 1998; Asahi et al., 2000; Gasche et al., 2001; Amantea et al., 2007, 2014b). The mechanisms by which gelatinases contribute to ischemic brain damage include disruption of BBB integrity, hemorrhagic transformation and white matter myelin degradation (Cunningham et al., 2005). Moreover, MMPs and their endogenous inhibitors (TIMPs) regulate neuronal cell death through modulation of excitotoxicity (Jourquin et al., 2003), DNA repairing enzymes (Hill et al., 2012), anoikis (Gu et al., 2002), calpain activity (Copin et al., 2005) and production of neurotoxic products (Gu et al., 2002; Zhang et al., 2003), including pro-inflammatory cytokines (Amantea et al., 2007, 2014b). Moreover, the degradation of tight junction proteins claudin-5 and occludin by MMPs prompts hemorrhagic transformation, suggesting that these enzymes may represent a promising target for reducing the hemorrhagic complications associated with thrombolytic therapy (Yang and Rosenberg, 2011; Liu et al., 2012).

The up-regulation of the extracellular MMP inducer (EMMPRIN) occurring in peri-ischemic regions 2–7 days after focal ischemia in mice is coincident with the delayed increase of MMP-9, suggesting its involvement in neurovascular remodeling (Zhu et al., 2008). In fact, the late activation of MMP-9 promotes vascular endothelial growth factor (VEGF) signaling, contributing to neuronal survival and new vessels formation (Zhao et al., 2006). Thus, MMPs play a dual role in stroke injury, including early detrimental effects and beneficial roles at later stages after the insult. Moreover, MMPs participate to the protective response evoked by several preconditioning stimuli. In fact, tolerance produced by ischemic preconditioning or pre-ischemic exercise has been shown to be associated with downregulation of MMP-9 and subsequent amelioration of brain oedema and BBB disruption in rats undergone focal cerebral ischemia (Zhang et al., 2006; Davis et al., 2007; Guo et al., 2008a). Similarly, the neuroprotective action of TNF-α induced by pre-ischemic physical exercise has been demonstrated to occur via reduced MMP-9 activity and amelioration of BBB dysfunction in rats subjected to transient MCAo, through the involvement of extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation (Guo et al., 2008b; Chaudhry et al., 2010). Reduced activity of gelatinases was also associated with the neuroprotective effects exerted by hyperbaric oxygen preconditioning in hyperglycemic rats subjected to MCAo (Soejima et al., 2013).

After an ischemic insult, the expression of COX-2 is elevated in neurons, vascular cells and neutrophils, as demonstrated both in stroke patients and in animal models (Nogawa et al., 1997; Iadecola et al., 1999, 2001; Chakraborti et al., 2010). This enzyme contributes to post-ischemic inflammation through the production of toxic prostanoids and superoxide, and its deficiency or pharmacological inhibition leads to reduce BBB damage and to lower cerebral infiltration of leukocytes in rodent models of ischemic stroke (Iadecola et al., 2001; Candelario-Jalil et al., 2007). COX-2-derived prostaglandin E2 may also contribute to ischemic cell damage by disrupting Ca^{2+} homeostasis in neurons through activation of EP1 receptors (Kawano et al., 2006). Interestingly, an association between functional outcome and specific COX-2 variants has recently been demonstrated in ischemic stroke patients (Maguire et al., 2011).

TABLE 2 | Acute ischemic stroke trials for the clinical validation of anti-inflammatory and immunomodulatory drugs.

Trial	Drug	Mechanism	Phase	Status
Safety Study of Interferon Beta 1a for Acute Stroke	Recombinant human interferon beta-1a (IFN-β1a) (Rebif®)	Inhibition of pro-inflammatory cytokines production and prevention of blood brain barrier disruption	I	Completed
Intravenous immunoglobulin (IVIG) in acute ischemic stroke: a pilot study	Immunoglobulin	Scavenging active complement fragments	I	Withdrawn
Study of a neuroprotective drug to limit the extent of damage from an ischemic stroke (MINOS)	Minocycline	Anti-inflammatory/anti-apoptotic effects	I/II	Completed
Acute stroke therapy by inhibition of neutrophils (ASTIN)	Recombinant neutrophil inhibitory factor (UK-279, 276)	Blockade of neutrophil adhesion to endothelium	II	Terminated
E-selectin nasal spray to prevent stroke recurrence	E-selectin	Induction of mucosal tolerance to human E-selectin causing a shift of immune response from T(H)1 to T(H)2 type	II	Terminated
Study of interleukin-1 receptor antagonist in acute stroke patients	IL-1 receptor antagonist	IL-1β receptor blockade	II	Completed
Efficacy and safety of FTY720 for acute stroke	The sphingosine-1-phosphate receptor (S1PR) regulator Fingolimod (FTY720)	Reduced trafficking of T cells, B cells, NK cells, and other S1PR-expressing cells into the brain	II	Recruiting
Controlled study of ONO-2506 in patients with acute ischemic stroke	Arundic acid (ONO-2506)	Astrocyte modulating agent	II/III	Completed
Hu23F2G Phase 3 stroke trial (HALT)	Monoclonal antibody (humanized) against the neutrophil CD11/CD18 cell adhesion molecule, Hu23F2G (LeukArrest®)	Reduction of brain infiltration of neutrophils	Pilot III	Terminated
Enlimomab acute stroke trial (EAST)	Murine anti-ICAM-1	Blockade of leukocyte attachment and migration through cerebral endothelium	III	Completed
Neuroprotection with minocycline therapy for acute stroke recovery trial (NeuMAST)	Minocycline	Anti-inflammatory/anti-apoptotic effects	IV	Terminated

COX-2 has been implicated in hyperbaric oxygen preconditioning, since pharmacological inhibition of this enzyme abolishes the beneficial effects of the conditioning stimulus in a rat model of transient global cerebral ischemia (Cheng et al., 2011). Similarly, COX-2 induction participates to ischemic tolerance induced by cortical spreading depression (Horiguchi et al., 2006) or by a brief ischemic episode in rats (Choi et al., 2006; Pradillo et al., 2009), likely via the stimulation of the PGE2/PI3K/Akt pathway (Park et al., 2008). The elevated expression of COX-2 induced by ischemic preconditioning has been suggested to occur via a cascade involving epsilon protein kinase C and ERK1/2 activation, as well as NFkB nuclear translocation, as demonstrated *in vitro* (Kim et al., 2007, 2010).

While elevated expression of inducible NOS is associated with ischemic (Cho et al., 2005), TLR4-mediated (Pradillo et al., 2009) and anesthetic preconditioning (Kapinya et al., 2002), induction of this enzyme has also been implicated in the release of toxic amounts of NO by infiltrating neutrophils, microglia/macrophages and endothelial cells (Nakashima et al., 1995; Iadecola et al., 1996; Forster et al., 1999; Garcia-Bonilla et al., 2014b). NO released by endothelial cells early after the ischemic insult plays a beneficial role by inducing vasodilatation; whereas, at later stages, overactivation of neuronal NOS and, more importantly, *de novo* expression of inducible NOS contribute to ischemic tissue damage (Iadecola et al., 1996, 1997; Moro et al., 2004; Murphy and Gibson, 2007). In fact, excessive production of NO by inducible NOS is cytotoxic by promoting NFκB activation, by inhibiting ATP-producing enzymes, by producing peroxynitrite and by stimulating other pro-inflammatory enzymes such as COX-2 (Nogawa et al., 1998; Greco et al., 2011). Moreover, NO contributes to ischemic cell death via S-nitrosylation and, thereby, activation of glutamate receptor (GluR)-6 signaling (Yu et al., 2008) and MMP-9 (Gu et al., 2002).

NO plays a crucial role in cortical spreading depression-induced tolerance to transient focal cerebral ischemia in

rats (Horiguchi et al., 2005). Moreover, recent findings have demonstrated the involvement of endothelial NOS in remote ischemic preconditioning (Peng et al., 2012), while neuronal NOS has been involved in the neuroprotection exerted by remote post-conditioning (Pignataro et al., 2013).

Concluding Remarks

Although ischemic stroke is a major cause of mortality and long-term disability worldwide (Go et al., 2014), current therapeutic approaches for its acute treatment only rely on blood flow restoration by thrombus lysis or removal (Mangiafico and Consoli, 2014; Berkhemer et al., 2015; Hacke, 2015). The therapeutic window, intended as the temporal range during which the endovascular treatment may reach the target of a useful recanalization, is conventionally set at a threshold of 6 h; therefore, only less that 10% of patients may actually benefit from these procedures. Thus, the identification of novel targets that allow widening the time-window for pharmacological intervention, as well as the possibility of limiting the development of ischemic brain damage by promoting innate beneficial responses, is a urgent challenge. To date, the clinical translation of immunomodulatory drugs has been hampered by the fact that most strategies tested in humans were purely based on anti-inflammatory approaches (**Table 2**), disregarding the beneficial roles of some elements of the immune reaction to stroke injury (Amantea et al., 2014a). In this context, targeting immune responses that evolve during hours or days after the ischemic insult, by selectively promoting their beneficial components, represents a promising avenue for the development of more effective and safe stroke therapeutics.

Author Contributions

All the authors participated in the collection, review, and analysis of the relevant literature, as well as to drafting and revising of the manuscript.

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NF-κB in innate neuroprotection and age-related neurodegenerative diseases

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NF-κB factors are cardinal transcriptional regulators of inflammation and apoptosis, involved in the brain programing of systemic aging and in brain damage. The composition of NF-κB active dimers and epigenetic mechanisms modulating histone acetylation, finely condition neuronal resilience to brain insults. In stroke models, the activation of NF-κB/c-Rel promotes neuroprotective effects by transcription of specific anti-apoptotic genes. Conversely, aberrant activation of NF-κB/RelA showing reduced level of total acetylation, but site-specific acetylation on lysine 310, triggers the expression of pro-apoptotic genes. Constitutive knockout of c-Rel shatters the resilience of substantia nigra (SN) dopaminergic (DA) neurons to aging and induces a parkinsonian like pathology in mice. c-rel$^{-/-}$ mice show increased level of aberrantly acetylated RelA in the basal ganglia, neuroinflammation, accumulation of alpha-synuclein, and iron. Moreover, they develop motor deficits responsive to L-DOPA treatment and associated with loss of DA neurons in the SN. Here, we discuss the effect of unbalanced activation of RelA and c-Rel during aging and propose novel challenges for the development of therapeutic strategies in neurodegenerative diseases.

Keywords: NF-κB, epigenetic drugs, BDNF, c-Rel deficient mice, RelA (K310)

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Introduction

In the central nervous system, NF-κB transcription factor acts as a pleiotropic regulator of target genes controlling physiological function (1) as well as pathological processes associated with neurodegeneration (2, 3).

The NF-κB family of transcription factors is composed by five different members that are p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). The RelA subunit, composing the activated p50/RelA dimer, and its post-transcriptional modifications play a pivotal role in the onset of neurodegenerative processes triggered by ischemic insults as well as glutamate or beta-amyloid toxicity (4–8). The c-Rel subunit within activated NF-κB dimers counteracts the ischemic injury acting as an innate mechanism of neuroprotection (9). The c-Rel factor is reduced in neurons exposed to oxygen–glucose deprivation (OGD), and its over-expression can limit the cell death.

Abbreviations: 1B/DMT1, 1B isoform of divalent metal transporter-1; AMPK, AMP-activated kinase; BDNF, brain-derived neurotrophic factor; BIRC3, baculoviral IAP repeat-containing protein 3; DA, dopaminergic; HAT, histone acetyltransferase; HDAC, histone deacetylase; K310, lysine 310; MCAO, middle cerebral artery occlusion; MnSOD, superoxide dismutase; MPP^+, 1-methyl-4-phenylpyridinium; NAD^+, nicotinamide adenosine dinucleotide; OGD, oxygen–glucose deprivation; PD, Parkinson's disease; RIP1, receptor interacting protein; ROS, reactive oxygen species; SIRT1, sirtuin 1; SN, substantia nigra; SNc, SN pars compacta; TH, tyrosine hydroxylase; TNF, tumor necrosis factor; UCP4, mitochondrial uncoupling protein 4.

Moreover, the deficiency of c-Rel induces an age-related behavioral parkinsonism in mice, with degeneration of nigral dopaminergic (DA) neurons and development of a Parkinson's disease (PD)-like neuropathology (10). Recent evidence has shown that activation of NF-κB drives the systemic and brain aging process in mice (11, 12). Notably, Tilstra and colleagues demonstrated that RelA is the most contributing subunit in degenerative changes associated with senescence in a progeroid mice model (13).

We propose that while RelA activation accompanies normal brain aging, a misbalance between RelA and c-Rel might drive pathological aging by affecting the survival of substantia nigra (SN) DA cell and turning old mice into a parkinsonian phenotype.

RelA and c-Rel: Two Opposing Regulators of Neuronal Resilience to Brain Ischemia

In the central nervous system, NF-κB factors are key players of a number of physiological processes such as neurogenesis (14), neuritogenesis (15), synaptic plasticity, learning, and memory (16–18). In recent years, a body of data has shown that NF-κB dysregulation participates to neurodegenerative mechanisms that occur in brain exposed to trauma or ischemia (19, 20), as well as in the brain of patients affected by PD (21, 22) and Alzheimer's disease (23).

The neuronal response to external stimuli relies on a differential activation of NF-κB dimers. We found that targeting RelA or c-Rel expression by antisense oligonucleotides (5) or siRNAs (6, 9) produces opposite effects on neuron survival. While over-activated p50/RelA dimers contribute to the apoptotic program, the c-Rel containing dimers increase the resilience of injured neuronal cells (**Figure 1**). Neurotoxic stimuli, such as ischemia (4, 9), glutamate (5), β-amyloid (7, 24), or 1-methyl-4-phenylpyridinium (MPP^+) (25, 26), induce p50/RelA dimers activation and the transcription of a panel of pro-apoptotic genes (4). Conversely, c-Rel-containing dimers are responsible for anti-apoptotic gene expression by signals promoting neuroprotection in diverse neurotoxic settings, such as S100B in models of NMDA-mediated excitotoxicity (27), agonists at mGlu5 receptors against β-amyloid- (7) and MPP^+ toxicity (25) or adipocyte-derived hormone leptin in neurons exposed to OGD (28). Over-expression of c-Rel in cultured neurons promotes anti-apoptotic effects by inducing the transcription of manganese superoxide dismutase (MnSOD) and Bcl-xL (7, 29, 30). c-Rel overabundance also limits the generation

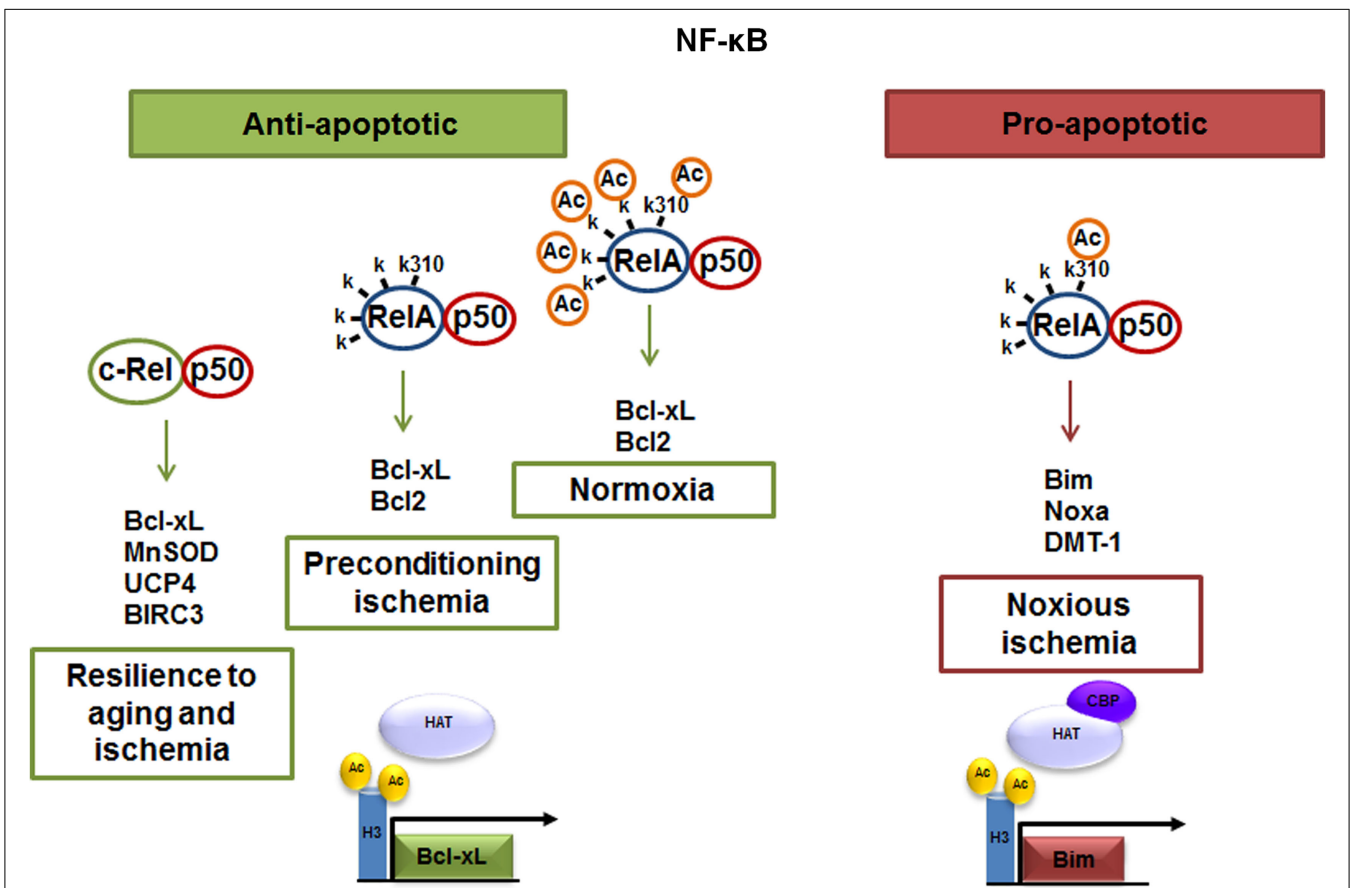

FIGURE 1 | The p50/RelA and p50/c-Rel dimers regulate neuronal survival. Anti-apoptotic effects of NF-κB can be mediated by c-Rel containing dimers, which enhance neuronal resilience to oxidative stress by inducing Bcl-xL, MnSOD, UCP4, and BIRC3 expression. NF-κB anti-apoptotic effects can also be mediated by p50/RelA during preconditioning ischemia through the transcription of Bcl-xL. The pro-apoptotic effect elicited by NF-κB p50/RelA dimer in noxious ischemia is dependent on changes in the RelA acetylation state. A lower grade of RelA general acetylation, respect to control condition, but a site-specific acetylation on Lys 310 residue addresses the NF-κB binding toward Bim, Noxa, and DMT1 promoter.

of reactive oxygen species (ROS) by inducing transcription of the mitochondrial uncoupling proteins 4 (UCP4) (31), a brain-specific mitochondrial ion channel producing mild reduction of mitochondrial membrane potential and neuroprotection (32). Moreover, c-Rel can control the expression of baculoviral IAP repeat-containing protein 3 (BIRC3), named also as cIAP2, an anti-apoptotic E3 ligase, which can modulate the receptor interacting protein 1 (RIP1) activity by ubiquitination (33). Depending on its ubiquitination status, RIP1 can dictates if tumor necrosis factor (TNF)-α induces cell survival (and inflammation), or cell death pathways (34). Ubiquitinated RIP1 can recruit other kinases and finally induce NF-κB-mediated transcription of pro-survival and pro-inflammatory genes (TNF-α-dependent NF-κB activation) (35).

The elucidation of the dual effects of NF-κB activation on neuron survival was more evident in studies of severe brain ischemia. The activation of p50/RelA rapidly occurs in neurons and glial cells and has been implicated in pathogenesis of post-ischemic injury (36–38). In brain ischemic tissue of mice subjected to permanent middle cerebral artery occlusion (MCAO) and in primary cortical neurons exposed to OGD, NF-κB followed a similar pattern of activation (8, 39) characterized by increased nuclear translocation of p50/RelA dimer (4, 36) and decreased translocation of c-Rel-containing dimers (9). In these conditions, NF-κB activity was associated with an unbalance expression of pro-apoptotic RelA target genes, i.e., an increased expression of the pro-apoptotic members of Bcl-2 family genes (4) and reduced level of the anti-apoptotic member Bcl-xL (9, 40). During brain ischemia, RelA induced the expression of the 1B isoform of the divalent metal transporter-1 (1B/DMT1), the membrane carrier responsible for iron accumulation and brain damage after injury (41). The RelA-induced 1B/DMT1 expression acted as an upstream mechanism responsible for iron accumulation and contributing to neuronal cell death. While the over-expression of RelA increased cell death, the over-expression of c-Rel prevented neuronal loss in cortical neurons exposed to OGD, by increasing the transcription of Bcl-xL gene (9, 39). Knocking-down c-Rel expression exacerbated neuronal susceptibility to OGD-mediated damage. Under brain ischemia, mice deficient for the c-Rel factor appeared insensitive to neuroprotective activity of leptin, a c-Rel inducer capable to limit cortical damage in wild-type mice (24, 28). These data strongly suggested that inhibition of c-Rel-containing dimers and activation of p50/RelA are key events in the pathogenesis of post-ischemic brain injury. In spite of these premises, p50/RelA activation *per se* appeared to be insufficient to drive pro-apoptotic transcription during brain ischemia. A similar pattern of p50/RelA nuclear translocation was found in mice exposed to a brief preconditioning ischemia (8) generating brain tolerance to a subsequent lethal ischemic injury (42). In neuronal cells, likewise in tumor cells, gene targeting by p50/RelA is finely regulated by post-transcriptional modification of RelA subunit, such as phosphorylation and acetylation (43). These modifications shape the strength and specificity of the NF-κB–DNA binding and final transcriptional responses. On this line, we investigated whether the activation of the p50/RelA dimer, in preconditioning or in lethal ischemia, differs in the RelA acetylation state (44).

RelA Acetylation is a Dynamic Process Which Tunes p50/RelA-Mediated Pro-Apoptotic Transcription in Brain Ischemia and is Modulated by Epigenetic Drugs

Acetylation is the key post-translational modification of histones that controls the accessibility of chromatin to the transcriptional machinery and plays an essential role in gene activation (45). Lysine acetylation is reversible and controlled by the opposing activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC).

Besides histones, diverse non-histone proteins, including transcription factors NF-κB, are modified by HAT co-activators and HDACs (46). Acetylation of RelA on specific lysine residues (K122, 123, 218, 221, and 310) is a dynamic process that differently affects the RelA interaction with IκBα, the DNA-binding ability and the transcriptional activity of the protein (43, 47).

Members of class I HDACs, particularly HDAC1, HDAC2, and HDAC3, inhibited by vorinostat and entinostat (MS-275) are the most responsible for the general deacetylation of RelA (43, 48). Conversely, sirtuin 1 (SIRT1), an atypical class III HDAC that requires nicotinamide adenosine dinucleotide (NAD^+) rather than zinc as a co-factor (49), activated by resveratrol, selectively deacetylates RelA at lysine 310 (K310) residue (8, 50).

Our studies have shown that mechanisms affecting the acetylation state of RelA might discriminate between protective and neurotoxic activation of NF-κB during ischemia (8). Protective ischemic preconditioning and harmful ischemia induced similar levels of p50/RelA activation, but only the ischemic injury induced an atypical RelA acetylation. The RelA that translocated to the nucleus in primary cortical neurons exposed to preconditioning OGD, or in cortices of mice subjected to preconditioning MCAO, showed a general deacetylation that paralleled the deacetylation on the K310 residue. Conversely, RelA activated in neurons exposed to lethal OGD or in cortices of mice subjected to noxious ischemia displayed a general deacetylation, but site-specific acetylation on the K310 residue. This suggested that a mismatch between general and "site-specific" (K310) acetylation of RelA – where total acetylation decreases and K310 acetylation increases – could be responsible for pro-apoptotic transcription in ischemic conditions (8). The relevance of K310 acetylation to RelA-mediated effects during ischemia was demonstrated by mutagenesis analysis. The substitution of lysine with arginine at the RelA 310 residue impaired the acetylation at this site. In cells expressing the mutated RelA subunit, the OGD-mediated DMT1 transcription and the cell damage were totally prevented (8, 41). By undergoing such aberrant acetylation, RelA detached from the anti-apoptotic *Bcl-xL* promoter to bind the pro-apoptotic *Bim* promoter (44). In addition to changing the acetylation state of RelA, lethal ischemia produced a significant reduction of H3 histone acetylation (44), in line with previous evidence (51).

Prompted by these findings and in order to correct altered acetylation of RelA and histones after brain ischemia, we studied the association of the specific class I HDAC inhibitor MS-275 (52), and resveratrol (53). MS-275 is a synthetic benzamide derivative

that currently is under clinical evaluation for cancer therapy (54). MS-275 has been shown to inhibit HDAC 1-3 with excellent pharmacokinetic properties (52).

Resveratrol is a polyphenol with multiple activities, including anti-oxidant, anti-tumorigenic, and neuroprotective activity (53, 55). In various models of brain ischemia, resveratrol delayed axonal degeneration after injury and mitigated the formation of free radical species as well as mitochondria-mediated apoptosis (56–59).

Widely known mechanisms of resveratrol action include the activation of the longevity factors SIRT1 (60) and AMP-activated kinase (AMPK), a serine–threonine kinase that acts as a key metabolic and stress sensor/effector (61). We found that treatments with either MS-275 or resveratrol in the post-ischemic period of mice subjected to MCAO decreased the infarct volume and displayed a significant neuroprotective activity in cortical neurons exposed to OGD (44). What's more, we showed that the combination of MS-275 and resveratrol at sub-threshold doses elicited a synergistic effect leading to maximal neuroprotection in both the animal and the cellular models of brain ischemia. MS-275 at the highest concentration tested, 1 μM, increased acetylation of H3 histones on K9/18 residues in neurons exposed to OGD. Resveratrol, unable to modify *per se* the H3 acetylation, produced a synergistic acetylation of H3 K9/18 when used in combination with MS-275.

Notably, the synergistic effect produced by co-administration of low doses of MS-275 (0, 1 μM) and resveratrol (3 μM) was sustained by AMPK activation by resveratrol. This could be ascribed to the fact that AMPK can activate many catabolic pathways to produce ATP and acetyl-CoA (62), the fundamental co-factor for HAT activity. AMPK has also been found to indirectly support the resveratrol-dependent SIRT1 induction by inducing NAD^+ generation (61). As a consequence of AMPK-mediated enhancement of HAT and SIRT1 activity, the combination of MS-275 and resveratrol reversed the mismatch of RelA acetylation state in neurons exposed to OGD by, respectively, enhancing the RelA general acetylation and by reducing the acetylation at the K310 residue. The neuroprotective effect and transcription of anti-apoptotic genes observed following the treatment with the drug combination appeared closely related to the restored optimal RelA acetylation state (8, 63). The protective and transcriptional effects produced by resveratrol and MS-275 in cortical neurons were entirely reproduced in the mouse MCAO model. The combination of sub-threshold doses of the drugs, administered during the reperfusion period, elicited a synergistic effect that limited the cerebral infarct volume and the subsequent neurological deficits. MS-275 and resveratrol in combination showed a long-lasting efficacy as the beneficial effects were still evident 72 h after the injury. Moreover, they displayed a wide therapeutic window as their efficacy was evident when administered within 7 h after the ischemic onset. The treatment induced a transcriptional switch from pro- to anti-apoptotic genes. The RelA binding shifted from the *Bim* to the *Bcl-xL* promoter and the acetylation of associated histones changed accordingly. H3 acetylation decreased at the *Bim* and increased at the *Bcl-xL* gene.

Recently, we evaluated the acetylation of histone residues at the brain-derived neurotrophic factor (BDNF) IV promoter in primary mouse cortical neurons exposed to OGD and treated with the synergistic combination of MS-275 and resveratrol (64).

We focused on promoter IV, which is known to be important for synaptic plasticity, both during neuronal development and in the adult brain (65). In the cortex, the promoter IV-dependent BDNF transcription accounts for the majority of the neuronal activity-induced BDNF expression (66, 67). Several studies have proposed BDNF as possible mediators of the beneficial effects of HDAC inhibitors in nervous system disorders (68–70). A ChIP analysis in cortical neurons showed that histones at the BDNF promoter IV were deacetylated after OGD exposure. Treatment in the post-OGD period with the combination of MS-275 and resveratrol significantly increased acetylation at H3K9/18 and H4K12 histones (64). These histone modifications may act cooperatively and possibly in parallel to other histone modifications to increase BDNF expression.

It can be proposed that neuroprotection elicited by MS-275 and resveratrol treatment is also closely related to modulation of BDNF expression and may improve neurologic function by enhancing neuronal plasticity.

All together, these data provide evidence that a pharmacological intervention targeting the epigenetic machinery can represents a promising strategy to limit post-ischemic injury with an extended therapeutic window.

c-Rel Deficiency Causes a Progressive Late-Onset Parkinsonism in Mice

Following the evidence that RelA and c-Rel play opposing effects on neuron survival, and prevalence of p50/RelA activation versus p50/c-Rel triggers apoptotic cell death in brain ischemia (9), we tested whether a constitutive defect in c-Rel protein might affect the brain aging. Behavioral and pathological analyses of c-Rel knockout mice were performed at 2, 12, and 18 months of age and c-rel$^{-/-}$ mice showed to develop a PD-like syndrome and pathology with aging (10). Besides c-Rel subunit, other NF-κB subunits have been previously investigated for possible correlation with the onset of a parkinsonian pathology. Increased levels of RelA have been detected in the brains of MPTP-intoxicated mice (21) as well as in the brain of subjects affected by PD (21, 22, 71). This increase was evident both in neuronal and glial cells of the SN, suggesting a role of RelA activation in neuronal cell loss and neuroinflammatory response associated with PD progression. The role of the other NF-κB subunits in PD remains unclear. The p50$^{-/-}$ mice treated with MPTP did not behave differently from wild-type mice (72), suggesting no, or only minor, role for p50 in the regulation of SN neuron resilience.

In 18-month-old c-rel$^{-/-}$ mice, the analysis of tyrosine hydroxylase (TH)-positive cells of the SN pars compacta (SNc) revealed a loss of DA neurons that paralleled the total loss of Nissl-stained cells. No significant change in the estimated number of DA cells was evident in 2- or 12-month-old c-rel$^{-/-}$ mice compared to age-matched controls. Notably, the loss of SNc DA neurons was associated with a decrease of TH-positive fibers and reduction of dopamine transporter (DAT) and dopamine content in the striatum. The 18-month-old c-rel$^{-/-}$ mice displayed no significant degeneration in the other brain areas examined, the nucleus

basalis magnocellularis and the medial septal area, or in the ventral tegmental area that is generally spared in PD. Additional examination of SNc in aged c-rel$^{-/-}$ mice revealed a marked immunoreactivity for α-synuclein, the main protein constituent of Lewy bodies and Lewy neuritis and the key pathological feature of PD (73). Of note, fibrillary aggregated α-synuclein, detected by Thioflavin-S labeling, was present in the spared DA neurons of the SNc. Accumulation of insoluble α-synuclein in the mesencephalon was confirmed by the presence of a monomeric α-synuclein in the urea/SDS extracts used to solubilize the insoluble fraction. Neuroinflammation characterized by chronic microglial reactivity, RelA activation, and iron accumulation are also important features of the PD neuropathology (74, 75). As observed in PD brain (76), SNc and striatum of aged c-rel$^{-/-}$ mice showed marked signs of microglia activation with increased number, swollen cell bodies, and thick processes of CD11b-positive cells. Both SNc and striatum of aged c-rel$^{-/-}$ mice displayed elevated levels of iron and increase of the iron transporter DMT1 that, as mentioned above, is a transcriptional target of aberrantly acetylated RelA. Preliminary investigation on NF-κB in striatal extracts of 18-month-old c-rel$^{-/-}$ mice confirmed, indeed, the presence of aberrantly acetylated RelA as reported in **Figure 2**. The immunoprecipitated RelA showed reduced level of general acetylation associated with increased site-specific acetylation at the K310 residue. These changes occurred without any significant variation in the cellular amount of RelA. This finding strongly suggests that a misbalance between c-Rel and RelA can evolve during aging in c-rel$^{-/-}$ mice to produce changes in RelA acetylation, microglia activation, and neuronal apoptosis.

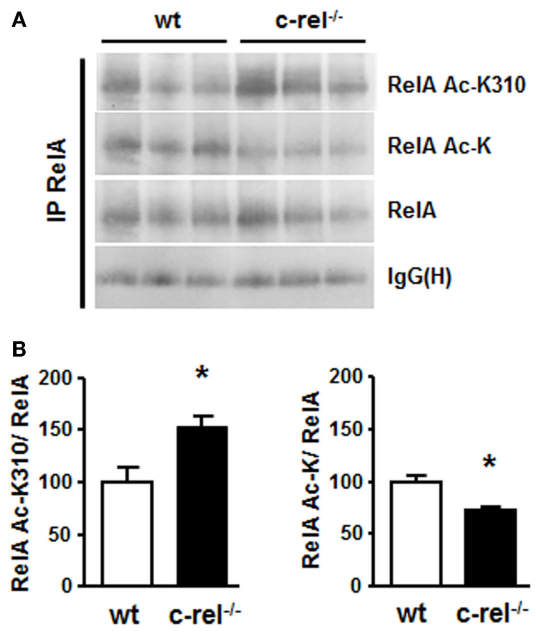

FIGURE 2 | RelA acetylation in the striatum of c-rel$^{-/-}$ and wt mice. **(A)** Representative picture of the immunoprecipitation analysis of RelA acetylation in total proteins of caudatus putamen. RelA acetylation at K310 residue increased, while total RelA acetylation was reduced in striatal total extracts of 18-month-old c-rel$^{-/-}$ mice. No significant change was detected in the total RelA content. **(B)** Values from densitometry analysis of immunoblots are expressed as a percentage of the wt value. The signal given by IgG(H) is used as a control for the quality of the immunoprecipitation. Bars depict the mean ± SEM ($n = 4$ animals per group), *$p < 0.01$ versus wt value.

Because inflammatory and neurotoxic activation of microglia have been also reported to be triggered by acetyl-RelA (K310) (77), it is feasible that microglia activation participate to the neurodegenerative process in c-rel$^{-/-}$ mice. The extensive analysis of the neuroinflammatory profile of c-rel$^{-/-}$ mice along with disease progression will reveal the exact entity of this inflammatory process and the specific participation of innate and adaptive immunity.

The neurochemical changes observed in aged c-rel$^{-/-}$ mice were also accompanied by the onset of motor deficits. A significant impairment in spontaneous motor activity was evident in c-rel$^{-/-}$ mice at 18 months, but not in younger mice as previously shown (17, 18). Indeed, either monitored for 1 h or six consecutive days to avoid stress-related bias, 18-month-old c-rel$^{-/-}$ mice displayed a lower locomotor activity. Furthermore, the gaiting analysis supported the presence of a locomotor dysfunction related to bradykinesia and rigidity. Noteworthy, the treatment with L-DOPA plus benserazide, a cocktail that is considered the gold standard for PD therapy, totally reversed the locomotor deficits and normalized most of the gaiting parameters.

Despite these findings, how the constitutive c-Rel deficiency can specifically affect DA neurons of SNc is still an open question (10). The selective vulnerability of SNc neurons in PD has been attributed to the peculiar "energy-demanding" physiology of these cells (78), which display enormous axonal field and impressive number of synapses for each axon (79). Moreover, during their pacemaking activity, SNc DA neurons, but not the ventral tegmental area neurons, generate autonomous action potentials by unusual engaging of L-type Ca^{2+} channels, which require subsequent activation of ATP-dependent Ca^{2+} pumps to maintain Ca^{2+} homeostasis (80). The energy production by mitochondria and endoplasmic reticulum in SNc DA neurons associates with the generation of large amounts of ROS that are constantly neutralized by anti-oxidant systems, including SODs catalases, glutathione peroxidase, and UCP4 and UCP5 (81). It can be inferred that in the absence of c-Rel a reduced expression of UCP4 (31) and MnSOD (7, 29) might enhance ROS accumulation during aging in SNc neurons (82), and synergize with reduced expression of anti-apoptotic Bcl-xL (9, 30) to affect neuronal resilience. Also, it is conceivable that mitochondria impairment associated with c-Rel deficiency may switch the acetylation state of RelA during aging to elevate Bim, DMT1, and iron (75) as well as the intracellular levels of α-synuclein (83). In turn, these events lead to α-synuclein aggregation (83), microglia activation, and neuronal damage (84). All together, these findings point to a role of c-Rel in the regulation of SNc susceptibility to aging.

Finally, latest results (unpublished results) indicate that at a premotor phase (7–12 months) when no loss of SNc DA neurons is evident yet, c-rel$^{-/-}$ mice display olfactory deficits, gut dysfunctions, and reduced DAT immunoreactivity in the striatum. This disease progression mimics the pathological and clinical progression observed in PD patients that at premotor stage of the disease show constipation, hyposmia (85), and reduced DAT imaging by PET or SPECT scan. These findings further strengthen

the notion that c-rel$^{-/-}$ mice represent an innovative disease model suitable both for studies aimed at dissecting the mechanisms of PD onset and to test novel therapeutic approaches for intervention at the premotor stages of the disorder.

Conclusion

Although NF-κB factors are transcriptional regulators of inflammation and apoptosis, their relevance in aging-related neurodegeneration is still underestimated.

Activation of RelA has been proposed to lead the systemic aging process in mice (86, 87), being negligible in the hypothalamus of young mice and progressively increasing, earlier in microglia and later in neuronal cells, as the mice become older (12). The genetic depletion of one allele of RelA attenuated the behavioral signs of neurodegeneration and extended the healthspan in a progeroid mouse model (13).

These stimulating results could be reread in the light of recent evidence showing that protective versus harmful p50/RelA activation strongly depends on the acetylation state of RelA. Activation of RelA subunit displaying reduced grade of general acetylation, but site-specific acetylation of K310, triggers apoptotic gene expression in brain ischemia. In the absence of such a mismatch, the activation of RelA is neuroprotective, as observed in preconditioning ischemia.

Whether a dysregulation of the RelA acetylation state is also involved in normal aging, or just in pathological aging, remains to be established. What seems promising is that aberrant RelA acetylation, more than RelA nuclear translocation, can be a suitable target. It is corrected by the synergistic association of HDAC inhibitors and resveratrol to produce neuroprotection.

While evidence suggests that RelA activation marks physiological elderly in mice, we show that the deficiency of c-Rel leads to a parkinsonian phenotype with aging (10). The degeneration of DA neurons in the SNc, the microglia activation and the α-synuclein pathology are associated with development of an L-DOPA-responsive parkinsonism. Intriguingly, in the basal ganglia of aged c-Rel deficient mice, but not in aged matched controls, the acetylation state of RelA was reminding the one observed in lethal ischemia.

This body of evidence supports the premise that the balance between c-Rel- and RelA-mediated transcription may be at the crossroad between normal and pathological aging of the mammal brain. A defect of c-Rel activity, associated with higher RelA activation, reduces SNc resilience to aging hereby leading to a late-onset form of parkinsonism. Validation of the impact of c-Rel activity in PD onset and progression is now the challenge of ongoing studies.

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Dynamic changes in DNA methylation in ischemic tolerance

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Epigenetic mediators of gene expression are hypothesized to regulate transcriptomic responses to preconditioning ischemia and ischemic tolerance. Here, we utilized a methyl-DNA enrichment protocol and sequencing (ChIP-seq) to identify patterns of DNA methylation in an established model of ischemic tolerance in neuronal cultures (oxygen and glucose deprivation: OGD). We observed an overall decrease in global DNA methylation at 4 h following preconditioning ischemia (30 min OGD), harmful ischemia (120 min OGD), and in ischemic tolerant neuronal cultures (30 min OGD, 24 h recovery, 120 min OGD). We detected a smaller cohort of hypermethylated regions following ischemic conditions, which were further analyzed revealing differential chromosomal localization of methylation, and a differential concentration of methylation on genomic regions. Together, these data show that the temporal profiles of DNA methylation with respect to chromatin hyper- and hypo-methylation following various ischemic conditions are highly dynamic, and may reveal novel targets for neuroprotection.

Keywords: DNA methylation, ischemia, preconditioning, stroke, ischemic tolerance, ChIP-seq

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Introduction

Pharmacological agents for neuroprotection have been disappointing in clinical trials. An understanding of the basic mechanisms of endogenous neuroprotection in brain, those induced by preconditioning ischemia and resulting in a tolerant phenotype, may yield novel approaches to reduce brain injury following ischemia (1, 2). We have previously described broad-based transcriptional suppression as operative in ischemic tolerance (3), and have discovered protein effectors of tolerance: upregulation of gene silencing polycomb proteins in tolerant brain (4). Polycomb protein's mechanism of action is via epigenetic chromatin binding, highlighting a putative biologic mechanism with neuroprotective potential. DNA methylation has been prominently featured as an epigenetic modulator in brain development as well as in brain injury (5, 6).

It has been proposed that a reduction in DNA methylation will confer protection against brain ischemia. Global DNA methylation increases in the brain following harmful focal ischemia, as measured by [^{3}H]-methyl incorporation into DNA (7), but does not change the expression of DNMT1 or 3 (DNA methyltransferase, DNMT). However, the chromatin sequences that are methylated by ischemia were not identified by Endres (7). Blocking the enzyme responsible for DNA methylation, DNMT, using 5′ azacytidine inhibits ischemic cell death (7). Heterozygous, but not homozygous knockouts of DNA methyltransferase1 also show a reduction in brain infarction following modeled ischemia (8). Therefore, reducing DNA methylation may protect the brain from subsequent harmful ischemia.

An increase in DNA methylation following ischemia would be consistent with a recent study which showed that MECP2 (methyl CpG binding protein 2), a transcription repressor, is increased following brief ischemia (9). In addition, the DNA methylating agent methylazoxymethanol (MAM) blocks ischemic tolerance induced neuroprotection (10). In that study, while the effect was attributed

to a blockade in progenitor cell proliferation, the ability of MAM to hypermethylate DNA such that preconditioning-induced changes in DNA methylation are blocked, cannot be ruled out. While it is unclear which genes are methylated in response to ischemia, ischemia can affect DNA methyltransferase activity in brain, and a reduction in DNA methylation may play a role in mediating the protective effects of ischemic tolerance.

The role of DNA methylation was recently studied in an analogous model of ischemic tolerance that of tolerance to seizure-induced brain injury (11). Following preconditioning seizures, brain injury following status epilepticus induced by an intra-amygdaloidal injection of kainic acid is reduced, specifically in the CA3 subfield of the hippocampus (12–14). Similar to ischemic tolerance, a reduction in gene expression has been reported in seizure tolerant brain, suggesting gene repression mediates tolerance to both seizure and ischemic brain injury (13). In the seizure tolerance study, DNA methylation patterns were profiled at 8 h post seizure, and revealed a dynamic pattern whereby both an increase and decrease in methylation were observed (11).

Thus, the role of DNA methylation in ischemic tolerance is not clear. Here, we offer an in-depth analysis of DNA methylation in a cell culture model of ischemic preconditioning-induced ischemic tolerance (15).

Materials and Methods

Cell Culture and Ischemic Tolerance Modeling

All animal use was approved by the Morehouse School of Medicine Institutional Animal Care and Use Committee. Primary neuronal cultures were prepared for mixed sex litters of rats (1 day post birth). Animals were euthanized with isoflurane, and cortices dissected in an ice cold buffer (dissociation media). Tissue was incubated with papain (37°C: 10 min) and triturated. Cells were seeded out a 5 million cells/6 cm dish. Cells were grown in a mixture of B27 (3.5%) and Neurocult (1.5%) supplemented NeuroBasal A media. Cells were used at 14 days *in vitro* (DIV).

An *in vitro* equivalent of ischemic tolerance is used, whereby primary neuronal cultures are subjected to oxygen and glucose deprivation (OGD) modeled ischemia (16–18). Cells are washed with phosphate buffered saline, supplemented with Mg Cl_2 and Ca Cl_2, and then incubated for 30 or 120 min in a Thermofisher anaerobic chamber. Cells were recovered in neurobasal A media. For modeling ischemic tolerance, we precondition cells with 30 min OGD, and injurious ischemia (120 min) is applied 24 h later. Cells were harvested 4 h following the final ischemic challenge.

DNA Preparation and Methylation Enrichment

Methylated DNA was enriched from cell lysates ($n = 2$/condition) using the Methylminer assay (Active Motif). DNA was extracted from frozen cell pellets using the Genelute DNA Kit (Sigma). DNA was quantified and 2.0 µg was sonicated in 200 µl Tris EDTA buffer (1 × 30% 10 s, 2 × 20% 10 s, 4°C). Sheared DNA (2000 ng) was used as starting material for the pull down reaction according to manufacturer's protocol. Following pull down, the DNA was size selected on a 1% agarose gel [200–500 base pair (bp) fragments] and extracted using a PCR (polymerase chain reaction) clean-up kit (Qiagen).

DNA Library Preparation

Samples were end-polished and ligated to barcode adapters using T4 Kinase. Libraries were amplified using Platinum taq for 12 cycles of PCR, size selected on a 2% agarose gel, and then subjected to an additional four cycles of PCR. Libraries were visualized on an agarose gel (see **Figure 1A**), excised, and cleaned up to remove primers (Qiagen). Samples were then subjected to qPCR to quantify the library against a known standard. Emulsion PCR was used to clone the libraries onto sequencing beads. The reaction was seeded at 1.0 pmol of equimolar library (all eight samples at the same concentration). A WFA run determined the final concentration of beads for deposition on the sequencing slide to obtain approximately 700 million beads deposited on the slide. Libraries were sequenced on an Applied Biosystems SOLiD 4 DNA sequencer using a F35 single end read. Data were stored as csfasta files, and uploaded to a penguin cluster running Lifescope™ for alignment to the RN5 genome (rat), using the RefSeq annotation guide (October 2014).

Data Analysis

Data were transferred to a Dell Dimension Desktop computer running Partek Genomic Studio v 6.6 for analysis. Data were analyzed using the Partek ChIP and the NGS Methylation workflows. Output bam files were combined according to experimental conditions for all analysis. Data were subjected to cross strand analysis to identify the fragment window size for genomic analysis (**Figure 1C**). Data were aligned to the rn5 genome, using the control sample as the reference, and a window bin size of 200 bp. Only fragments that show enrichment with a $p < 0.001$ false detection rate (FDR) were considered significant and identified in a "peaks file." These data were used for subsequent analysis (available upon request).

Results

Ischemic Stimuli Result in a Reduction in Global DNA Methylation

Methylation profiles of ischemia treated neuronal cell cultures were identified using methylated chromatin enrichment followed by DNA sequencing (ChIP-seq). We analyzed four treatment groups: sham-treated cells (Control: Cont), cells subjected to 30 min OGD (Preconditioning: PC) and recovered for 4 h, cell subjected to 120 min ischemia (Injurious Ischemia: Isch), and neurons subjected to 30 min OGD and recovered 24 h prior to harmful ischemia (Tolerance: PC + Isch). These conditions have been established in multiple studies as showing ischemic tolerance (3, 16, 18). We focused on a time point of 4 h following the last ischemic treatment to identify dynamic changes in methylation that may be responsible for subsequent gene expression changes.

Quantitative analysis of assembled libraries on an agarose gel suggests a global decrease in DNA methylation, detected using the Methylminer assay. Interestingly, methylation was reduced following all ischemia treatment protocols (**Figures 1A,B**). Libraries were assembled and DNA concentrations were normalized prior to sequencing. The sequenced data were aligned to the rn5 reference genome; there were similar numbers of alignments in each experimental group (mean of 20.2 million reads ± 1.8 million

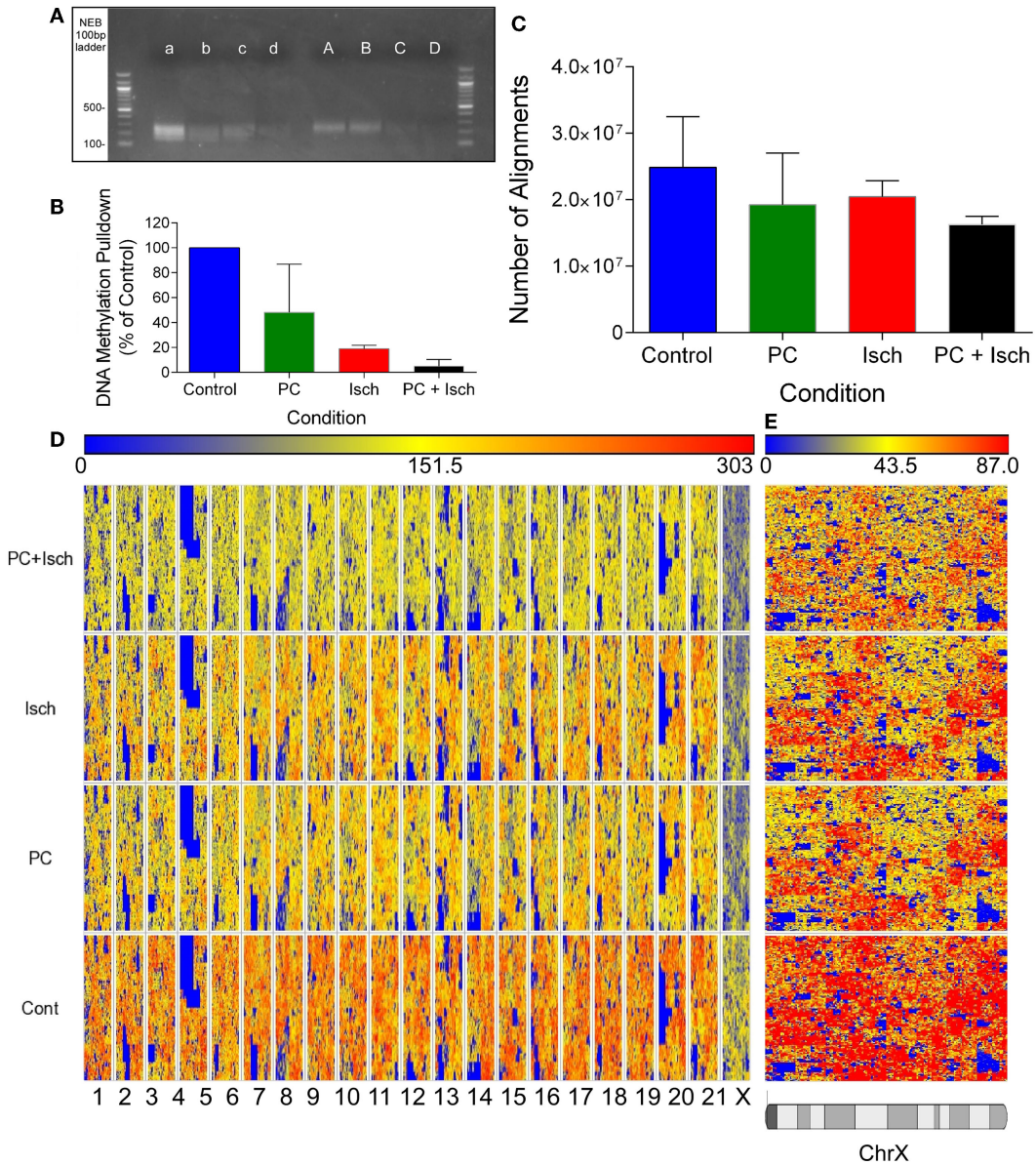

FIGURE 1 | Global decreases in DNA methylation revealed by DNA sequencing. **(A)** Agarose Gel showing approximate size of prepared libraries following enrichment of methyl-DNA using the methylminer assay. Libraries were amplified using PCR prior to running the gel. A–d refer to two independent experiments, A, a Cont, B, b-PC, C, c-Isch, D, d-PC + Isch. **(B)** Optical quantification of bands in **(A)** using Kodak Biostation 4000MM. **(C)** Quantification of number of reads aligned to the Rn5 reference genome using Lifescope software. Data are $n = 2$, mean ± SD. **(D)** Partek generated Hilbert graph showing methylation across the rat genome. Chromosome location is represented on the bottom of the figure and conditions are denoted on the left. Higher methylation is represented by warm colors (red) and hypomethylation by blue. **(E)** Expanded view of Chromosome X Hilbert Graph, showing representative decrease in methylation across all treatments compared to control.

reads; **Figure 1C**). The equivalent number of aligned reads is due to the concentration normalization step in the sequencing protocol. Therefore, all subsequent analysis reveals differences of methylation enrichment patterns rather than absolute DNA methylation levels.

In order to compare datasets, we combined the reads from each experimental condition (Cont, PC, Isch, PC + Isch), resulting in 30–40 million aligned reads per condition. Global methylation status was visualized with a Hilbert graph (19). Intensity of methylation is represented by red, and weaker intensity is represented as blue. Similar to previous published studies, the visualization of DNA methylation patterns showed a diffuse pattern of reads across the genome (**Figure 1D**). However, it is of note that the intensity of reads is lower globally across the genomes of the PC, Ischemia, and PC + Ischemia treated cells, consistent with the library analysis (**Figure 1A**). Of note, we also observed a lower global representation of methylation on the X chromosome (**Figure 1E**).

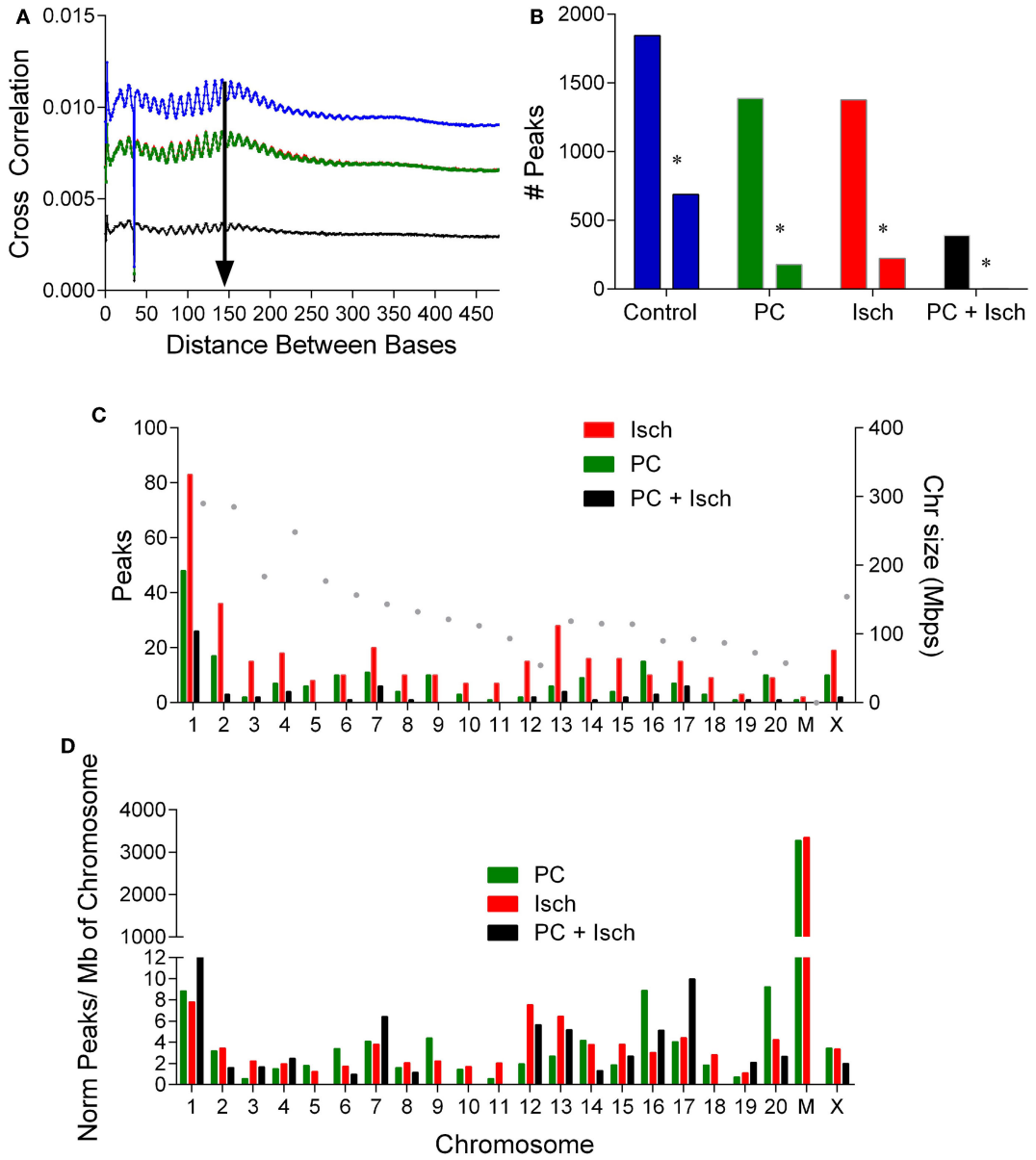

FIGURE 2 | Analysis of DNA methylation peaks reveals differential chromosomal methylation. **(A)** Cross strand analysis to determine library size for peak analysis. The PC and ischemia curves overlap (Cont: blue, PC: green, Isch: red, and PC + Isch: black). **(B)** Quantification of number of peaks in each treatment condition, and the number of unique peaks in each condition denoted by an asterisk. Data from the two sets were combined. **(C)**. Distribution of raw peak occurrence by chromosome for PC, Ischemia, and PC + Isch treated cells. The size of the chromosome is plotted on the right axis. **(D)**. Normalization of chromosome location data, by number of peaks and per Mbp of chromosome to reveal selective enrichment of chromosome patterns of DNA methylation. Note high relative methylation of mitochondria (discontinuous axis).

A peak analysis was performed on the DNA-seq data to identify clusters of methylation and relative differences in methylation compared to control. The window for the fragment size for analysis was determined by performing a cross strand analysis (20) (**Figure 2A**). Samples showed an approximate peak of around 150 bp (arrow **Figure 2A**). Reads were analyzed using a 150 bp fragment window, and using the control samples as a reference (to compare DNA methylation to control). The genome was divided into 100 bp windows, which could be combined. Data were fit to a negative binominal model with a peak cutoff false detection rate (FDR) of $p < 0.001$. From this analysis, 5035 peaks were identified in each of the four conditions. When we plot the number of methylation peaks (**Figure 2B**), we observed that the number of peaks decreases in the PC, Ischemia, and PC + Ischemia treated cells. The number of peaks unique to each condition are also plotted, and these have a similar distribution (peaks unique to each condition are marked with an asterisk; **Figure 2C**). Interestingly, although global methylation is reduced in ischemia-treated cells

compared to PC-treated cells (**Figure 1B**), the number of hyper methylated regions is the same (**Figure 2B**). Our interpretation of this result is that global levels of DNA methylation are reduced 4 h following harmful ischemia, but individual genes may show a strong increase in methylation, which we further investigated (below).

Dynamic Changes in Methylation Following Ischemia Show Chromosomal and Functional Bias

In order to identify genes whose methylation increased following ischemia treatments, we subjected the data to a one-tailed negative binominal test and selected a cut off FDR value of 0.05. Compared to the control sample, 618 regions show significant enrichment, which we interpreted as increased methylation. There were more enriched regions in the ischemia group (345) compared to those treated with preconditioning only (187), or in tolerant samples (65) (**Figure 2D**).

We separated the differentially methylated peaks by treatment, and identified which genomic feature they were close to and which chromosome they align with. Analysis of the enriched methylation peak distribution revealed an increase in chromosome 2, 12, and 13 methylation in the ischemia-treated cells (**Figure 2C**). In order to assess relative chromosomal distribution, we normalized the number of peaks to the total number of peaks/condition, and then scaled to the size of the chromosome (**Figure 2D**). Global methylation was reduced following PC + Ischemia/tolerance (**Figure 1B**); it is of note that chromosome 1, 7, and 17 showed relative enhancements in hypermethylation compared to control. Following PC, methylation of chromosome 6, 9, 16, and 20 showed enhanced hypermethylation compared to control. There was no chromosome selective enrichment of hypermethylated regions in ischemia-treated cells; rather, hypermethylated chromosomes were common preconditioned cells (chromosome 2 and chrX), or preconditioned cells subjected to harmful ischemia (chromosome 3, 12, and 13) (**Figure 2D**). This suggests that different chromosomes show predominant methylation in response to various ischemic treatments, which may result in differential activation of gene expression/repression in response to these stimuli.

The lists of significantly regulated genes were then analyzed, according to which genomic feature they correspond with using the RefSeq Transcripts Annotation guide (24 October, 2014) (**Figure 3A**). Promoters were defined as 5000-0 bases downstream of the transcription start site. Data from multiple introns and exons (CDS) were combined into one category. The most notable feature was the very high representation of intergenic regions in this data set; yet, only 1% of the genome encodes genes. It is not clear whether these intergenic associated regions of methylation are associated with non-transcribed DNA or novel transcripts of RNA, which are as yet unannotated (or not in the refSeq guide). Ischemia-treated and tolerant cells appear to have similar concordance with a control (total) distribution of methylation across genomic features. In contrast, following preconditioning ischemia, there was a relative increase in PC-mediated exon and intron-associated methylation, and a decrease in intergenic methylation (**Figures 3A,B**).

Finally, we investigated the location of reads relative to the transcription start site (TSS). This analysis is independent of the gene name; so, it only considers the relative location of peaks to the closest TSS. All samples had a similar profile in that we saw a relatively even distribution before and after the TSS (**Figure 3B**). A notable exception is at the actual TSS where the depth of reads is higher. When we expand this region (**Figure 3C**), we observed that control, PC, and ischemia treated samples showed a clustering around the TSS; in contrast, the largest peak from the tolerant cells was approximately 150 bp downstream of the TSS. Whether this was due to the loss of global methylation in these samples or a more selective methylation mark close to the TSS is not yet clear. Similarly, samples from PC and Ischemia treated cells also show a peak, but closer to 100 bp downstream of the TSS. The significance of these marks on gene expression awaits further investigation.

Pathway Analysis of Methylated Regions

For each treatment group, we created lists of genes closest to the methylation peaks, and genes unique to each treatment group. We performed a pathway analysis on the gene lists associated with methylated regions. Fisher's exact test was performed, and pathways were restricted to have at least two genes present. From this analysis, we first looked at enrichment scores and enrichment p-values, with a cut off of $p < 0.05$. (**Table 1A** depicts the pathways associated with the methylated genes in control, and 1B unique to control). In control samples, 13/19 of the pathways were associated with immune and inflammatory responses, or were identified as having genes associated with the immune system. Unique to control, we observe 19 pathways showing enrichment, again showing a high number of immune system associated pathways (**Table 1B**).

Following preconditioning ischemia, 17 pathways are identified, 10 of which are immune system, but 2 are associated with serotonin (**Table 2**). In the unique PC gene list, two-third of the pathways identified with synaptic pathways rather than the immune system. Following harmful ischemia (**Tables 3A,B**), 15 pathways showed enrichment, of which 13 are immune function associated. When we look at pathways unique to ischemia, three pathways associated with the synapse and receptor ligand interactions are identified. In ischemic tolerance, we do not observe any significant pathways identified, but one pathway, insulin secretion, was identified in the unique tolerance sample (**Table 4**). The low number of pathways identified in the tolerant sample is due to the low number of methylation associated peaks identified in these samples.

Discussion

Methylation of DNA is an important mechanism by which epigenetic modulation of gene expression occurs. Here, we show that both protection inducing ischemia (preconditioning) and harmful ischemia reduce global DNA methylation. The decrease in global methylation was more pronounced in ischemic tolerant cells. While global methylation was reduced, we observe an increase in methylation in chromosome 2, 12, and 13 in ischemia-treated cells and chromosome 1, 7, and 17 in tolerant cells, suggesting selective enhancement/targeting of methylation at these chromosomes.

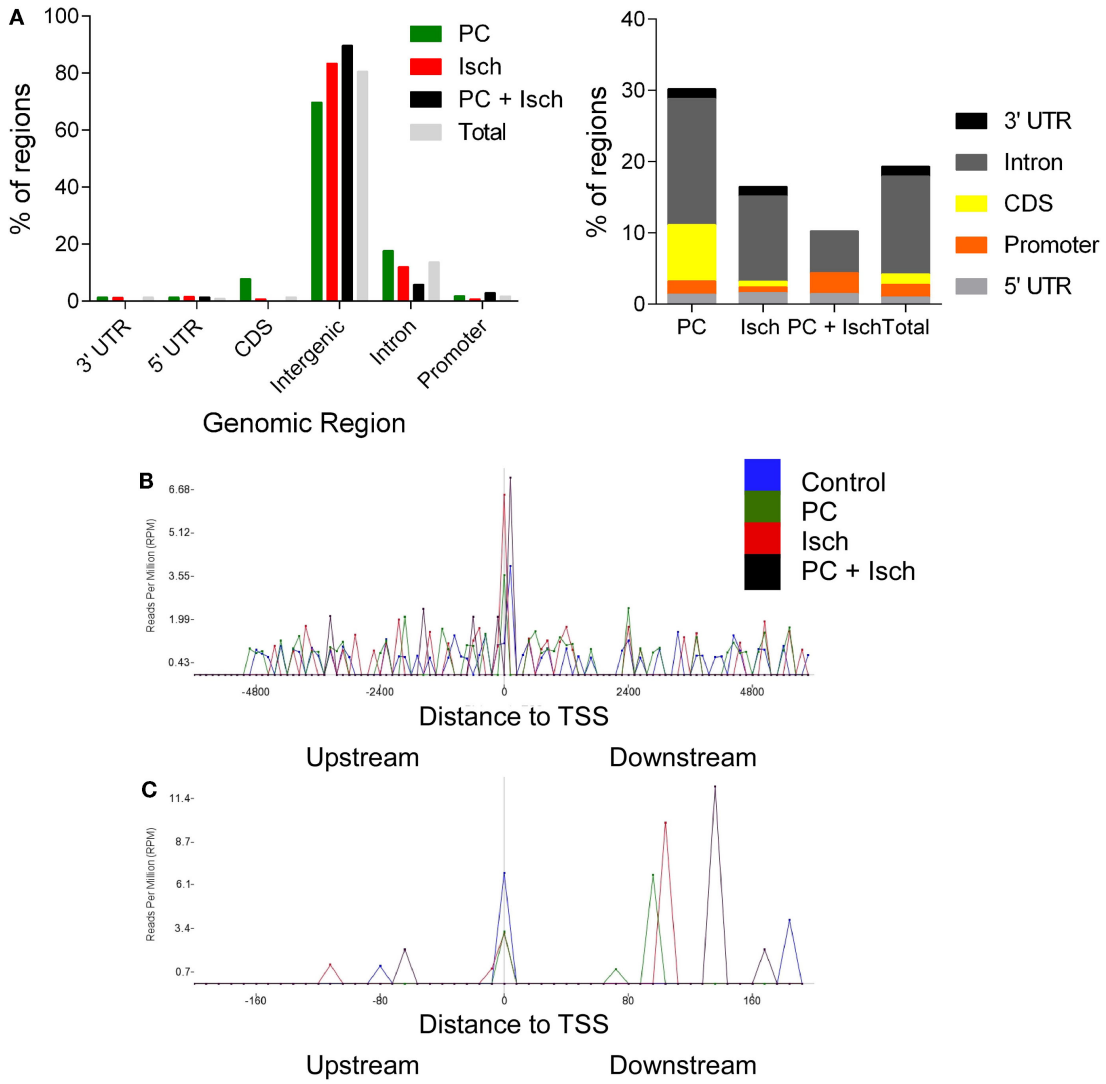

FIGURE 3 | Genomic location of DNA methylation following ischemia treatments. **(A)** Genomic locations of peaks as identified with RefSeq annotation guide. Most DNA methylation peaks are deemed intergenic, or between genes. Total represents a combination of all of the peaks and then analyzed for genomic location. Enlargement of non-intergenic associating reads, grouped by condition [N.B. re-draw of data in **(A)**]. **(B)** Analysis of reads relative to the transcription start site (+6000 to −6000 bases). Note the uniform distribution except at the TSS. The data are analyzed by determining the frequency of reads in 100 bins across the range of the *x* axis. **(C)** Enlargement of the −22 to +200 base region around the transcription start site.

While the distribution of methylation across ischemic and tolerant genomic regions was similar in incidence, in the preconditioned cells an exon associated methylation induction and attenuation in intergenic methylation were noted. A clustering of differential methylation at transcriptional start sites was observed in all cell treatment groups.

Similar global changes in methylation are reported in brain following injurious status epilepticus when compared to changes seen after induction of seizure tolerance (11). The hypothesis in both our ischemia studies and those of seizure was that the activation of neuroprotective mechanisms via previously described genomic reprograming and transcriptional suppression might occur via epigenetic processes, specifically by DNA methylation. However, in both our study of ischemia and the seizure study, a profound global hypomethylation event was observed in response to cell stress (ischemia or seizures). While this contrasts with studies showing increased methylation following harmful ischemia (7), it should be noted that the present tolerance and seizure studies used shorter recovery times following the stress 4 h (present study) and 8 h (11). It should also be noted that 4 h following preconditioning ischemia and preconditioning seizures, we have noted larger changes in gene expression compared to 24 h post preconditioning [(21) and Meller, unpublished].

Global demethylation was more pronounced in the ischemic tolerant group (cells subjected to preconditioning and harmful ischemia) compared to the ischemia or PC only-treated cells. It is not clear whether the reduction in methylation is due to the

TABLE 1 | (A) List of pathways identified from closest gene to methylation peaks in control MeDNA sample; (B) list of pathways identified from closest gene to methylation peaks unique to control MeDNA sample.

Pathway name	Database	Enrichment score	Enrichment *p*-value	% genes in pathway that are present	Sample ID vs. cont score	# genes in list, in pathway
(A)						
Allograft rejection	kegg	17.17	3.4889E-08	11.48	0.62	7
Autoimmune thyroid disease	kegg	16.61	6.1039E-08	10.61	0.62	7
Viral myocarditis	kegg	15.35	2.156E-07	8.86	0.62	7
Antigen processing and presentation	kegg	14.60	4.5532E-07	7.95	0.62	7
Graft-versus-host disease	kegg	14.09	7.6324E-07	10.17	0.62	6
Cell adhesion molecules (CAMs)	kegg	13.26	1.7457E-06	5.16	0.62	8
Type I diabetes mellitus	kegg	13.23	1.7874E-06	8.82	0.62	6
Phagosome	kegg	10.21	3.6978E-05	4.12	0.61	7
Epstein–Barr virus infection	kegg	8.86	0.00014	3.33	0.62	7
Endocytosis	kegg	8.69	0.00017	3.24	0.62	7
Herpes simplex infection	kegg	7.62	0.00049	3.28	0.62	6
HTLV-I infection	kegg	7.39	0.00062	2.62	0.62	7
Viral carcinogenesis	kegg	7.18	0.00076	3.02	0.62	6
Natural killer cell mediated cytotoxicity	kegg	4.37	0.01264	3.45	0.53	3
Linoleic acid metabolism	kegg	4.22	0.01464	6.06	0.34	2
Lysine degradation	kegg	3.77	0.02312	4.76	0.47	2
Serotonergic synapse	kegg	3.66	0.02585	2.63	0.33	3
Hippo signaling pathway	kegg	3.13	0.04357	2.14	11.40	3
Synthesis and degradation of ketone bodies	kegg	3.00	0.04965	11.11	0.57	1
(B)						
Allograft rejection	kegg	20.47	1.29E-09	11.48	0.26	7
Autoimmune thyroid disease	kegg	19.90	2.27E-09	10.61	0.26	7
Viral myocarditis	kegg	18.62	8.22E-09	8.86	0.26	7
Antigen processing and presentation	kegg	17.85	1.76E-08	7.95	0.26	7
Graft-versus-host disease	kegg	16.84	4.84E-08	10.17	0.26	6
Type I diabetes mellitus	kegg	15.98	1.15E-07	8.82	0.26	6
Cell adhesion molecules (CAMs)	kegg	13.94	8.85E-07	4.52	0.26	7
Epstein–Barr virus infection	kegg	11.90	6.77E-06	3.33	0.26	7
Endocytosis	kegg	11.72	8.16E-06	3.24	0.26	7
Phagosome	kegg	10.59	2.53E-05	3.53	0.26	6
HTLV-I infection	kegg	10.33	3.26E-05	2.62	0.26	7
Herpes simplex infection	kegg	10.17	3.84E-05	3.28	0.26	6
Viral carcinogenesis	kegg	9.70	6.15E-05	3.02	0.26	6
Linoleic acid metabolism	kegg	5.06	0.0063	6.06	0.27	2
Arachidonic acid metabolism	kegg	3.55	0.0288	2.74	0.27	2
Retinol metabolism	kegg	3.50	0.0302	2.67	0.27	2
Steroid hormone biosynthesis	kegg	3.47	0.0310	2.63	0.27	2
Chemical carcinogenesis	kegg	3.36	0.0348	2.47	0.27	2
Natural killer cell mediated cytotoxicity	kegg	3.23	0.0397	2.30	0.27	2

repeated exposure of the tissue to ischemic stress, resulting in an additive effect, or whether it is unique to the tolerance phenomenon. Hypermethylation was also reduced in this group compared to the other ischemia treatments. Since fewer "enriched" hypermethylation peaks were identified in the tolerance group, our mapping software only found one regulated pathway (insulin secretion). A reduction in hypermethylation in ischemic tolerance was, perhaps, unexpected given the established observations of gene silencing in ischemic tolerance. Indeed, on first appearance our data appear to contradict the hypothesized dogma that ischemic tolerance is associated with gene silencing (3, 4). However, care should be taken in interpreting these data sets together in this way, given that the current ischemic tolerance study identified chromatin methylation status 4 h following the final harmful insult, whereas gene expression and protein levels were reported 24 h following the final insult (3, 4). These discrepancies clearly support the need for further detailing the temporal profiles of both gene expression and chromatin methylation in response to such stimuli.

Most of the differential methylation is intergenic, which could associate with either non-transcribed DNA regions, or currently unannotated RNA species in the genome. RNA sequencing based transcriptome analysis shows approximately 30% of RNA is from such unannotated regions (22). It is interesting that there is a reduction in methylation in these regions of PC treated cells. When we remove the intergenic reads (**Figure 3B**), we observed that following PC there is a relative increase in methylation in coding regions compared to harmful ischemia in which methylation was focused more in intergenic regions. Whether this suggests changes in structural organization of chromatin or non-coding RNAs remains to be determined. In tolerant cells, we observed a reduction in coding region associated with methylated peaks. This may be due to the lower number of reads in this sample set, or a genuine decrease in exon associated methylation.

TABLE 2 | (A) List of pathways identified from closest gene to methylation peaks in PC MeDNA sample; (B) list of pathways identified from closest gene to methylation peaks unique to PC MeDNA sample.

Pathway name	Database	Enrichment score	Enrichment *p*-value	% genes in pathway that are present	Sample ID vs. cont score	# genes in list, in pathway
(A)						
Serotonergic synapse	kegg	8.08	0.00031	4.39	1.13	5
Graft-versus-host disease	kegg	5.60	0.003684	5.08	0.74	3
Allograft rejection	kegg	5.51	0.004049	4.92	0.74	3
Autoimmune thyroid disease	kegg	5.29	0.005055	4.55	0.74	3
Type I diabetes mellitus	kegg	5.20	0.005496	4.41	0.74	3
Viral myocarditis	kegg	4.79	0.008333	3.80	0.74	3
Antigen processing and presentation	kegg	4.49	0.011193	3.41	0.74	3
Phagosome	kegg	4.41	0.012174	2.35	0.71	4
HTLV-I infection	kegg	4.36	0.01282	1.87	0.87	5
Linoleic acid metabolism	kegg	4.33	0.013157	6.06	1.17	2
Retrograde endocannabinoid signaling	kegg	4.18	0.015373	3.03	1.20	3
Herpes simplex infection	kegg	4.16	0.015608	2.19	0.80	4
Tryptophan metabolism	kegg	3.83	0.021766	4.65	0.98	2
Endocytosis	kegg	3.62	0.026903	1.85	0.80	4
Ovarian steroidogenesis	kegg	3.55	0.028851	4.00	0.81	2
Synthesis and degradation of ketone bodies	kegg	3.06	0.04702	11.11	0.57	1
Cell adhesion molecules (CAMs)	kegg	3.02	0.048765	1.94	0.74	3
(B)						
Retrograde endocannabinoid signaling	kegg	3.633	0.026	2.020	2.506	2
HTLV-I infection	kegg	3.518	0.030	1.124	2.922	3
Serotonergic synapse	kegg	3.372	0.034	1.754	2.126	2
Dorso–ventral axis formation	kegg	2.936	0.053	4.762	5.644	1

TABLE 3 | (A) List of pathways identified from closest gene to methylation peaks in ischemia MeDNA sample; (B) List of pathways identified from closest gene to methylation peaks unique to ischemia MeDNA sample.

Pathway name	Database	Enrichment score	Enrichment *p*-value	% genes in pathway that are present	Sample ID vs. cont score	# genes in list, in pathway
(A)						
Cell adhesion molecules (CAMs)	kegg	6.42	0.0016	3.226	0.905	5
Serotonergic synapse	kegg	5.61	0.0037	3.509	2.831	4
Graft-versus-host disease	kegg	5.44	0.0043	5.085	0.785	3
Allograft rejection	kegg	5.35	0.0047	4.918	0.785	3
Autoimmune thyroid disease	kegg	5.13	0.0059	4.545	0.785	3
Type I diabetes mellitus	kegg	5.05	0.0064	4.412	0.785	3
Viral myocarditis	kegg	4.63	0.0097	3.797	0.785	3
Natural killer cell mediated cytotoxicity	kegg	4.37	0.0126	3.448	1.242	3
Antigen processing and presentation	kegg	4.34	0.0130	3.409	0.785	3
Linoleic acid metabolism	kegg	4.22	0.0146	6.061	1.969	2
Phagosome	kegg	4.22	0.0148	2.353	0.767	4
Neuroactive ligand-receptor interaction	kegg	3.94	0.0194	1.786	6.661	5
Glutamatergic synapse	kegg	3.80	0.0225	2.778	11.711	3
Endocytosis	kegg	3.43	0.0323	1.852	0.816	4
Ribosome	kegg	3.36	0.0348	2.344	3.650	3
(B)						
Neuroactive ligand-receptor interaction	kegg	6.94	0.000969	1.79	10.74	5
Glutamatergic synapse	kegg	5.66	0.003468	2.78	11.71	3
GABAergic synapse	kegg	3.68	0.025125	2.33	9.56	2

TABLE 4 | List of Pathways identified from closest gene to methylation peaks unique to tolerance MeDNA sample.

Pathway Name	Database	Enrichment score	Enrichment *p*-value	% genes in pathway that are present	Sample ID vs. cont score	# genes in list, in pathway
Insulin secretion	kegg	4.35823	0.012801	1.19048	18.4401	1

Additionally, methylation of intronic regions was increased following PC. This may be due to non-coding RNAs associated with intronic regions, or may represent regulation of alternative splicing of gene transcripts, which may occur following preconditioning (Meller, unpublished observation) or ischemia (23).

Following ischemia treatments, we observed a chromosomal difference in patterns of enhanced methylation compared to control cells. This suggests that chromosomal specific patterns of chromatin methylation may be regulated following various ischemic stimuli. How specific chromosomes are targeted for enhanced methylation is not clear, but studies of methylation following seizures support differential chromosomal location of methylation events following stress (11). Our analysis focused on specific increases in methylation clusters, because we observed global decreases in methylation events. Chromosomal specific gene expression effects of excitotoxicity have been reported previously (24). Chromosomal bias may be associated with movement of chromosomes into active nuclear territories (25). For example, chromosome X movement has been described following epilepsy (26). Changes in nuclear structure and epigenetic marks have been reported in response to synaptic and excitotoxic signaling (27).

Our study may upon first analysis appears to contradict the study of Endres; however, it should be noted that very different methodologies were utilized in these studies. In the Endres study, the incorporation of radiolabeled methyl groups into genomic DNA was measured. As such increased methylation may be due to dynamic DNA methylation or DNA methylation following DNA damage and repair, which has been reported following ischemia (28, 29). We also observed a global decrease in methylation following harmful ischemia, and DNA damage would be expected following harmful ischemia, hence re-methylation may occur. DNA methylation blockers are neuroprotective against ischemia (7). The agents were administered 10 min prior to harmful ischemia. However, the temporal profile of protection afforded by such blockade is not clear. In our study, we observed a reduction in global DNA methylation in tolerant neurons, suggesting that preventing the increased specific methylation observed following harmful ischemia could be protective.

In contrast, our study correlates with the observations of Miller-Delaney that DNA hypomethylation is associated with neuroprotection. In the current study, a methyl DNA binding protein related to the methyl binding domain of MeCP2 was used to enrich chromatin, in contrast to an anti-Me-cytosine antibody enrichment procedure (11). Increased MeCP2 has been reported in brain 24 h following preconditioning ischemia due to the loss in the levels of miR-132 (9). Thus, some of the differences in individual gene/chromatin region identification may be due to methods of enrichment. We also utilized DNA sequencing of the whole rat genome vs. mouse promoter arrays (11), which may also reveal differences between studies. Furthermore, sequencing libraries were normalized to approximate equimolar concentrations, prior to seeding the cloning PCR. The effect of this is to ensure even representation of the libraries in the sequencing reactions. However, as a result, some of the global decreases in methylation become less apparent. This was in part controlled for by comparing the methylation to the control sample, thereby revealing the dynamic changes in methylation. Clearly, more analysis of such studies will reveal additional information, especially when more advanced analysis methodologies and tools become available to incorporate such aspects of sequencing.

In summary, we performed a DNA methylation study using next generation sequencing which revealed dynamic changes in DNA methylation following preconditioning ischemia and in ischemic tolerance. Our study showed that 4 h following preconditioning ischemia, harmful ischemia, and in tolerant cells, DNA hypomethylation predominates as the response. However, selective and significant increases in hypermethylation events can also be observed, especially in response to harmful ischemia. The consequence of these events upon transcription awaits definition, but this study suggests that the temporal profile of epigenetic regulation of gene expression events needs further study to improve our understanding of this critical transcription control mechanism, and the identification of novel targets for neuroprotection from stroke induced brain injury.

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Extending injury- and disease-resistant CNS phenotypes by repetitive epigenetic conditioning

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Significant reductions in the extent of acute injury in the CNS can be achieved by exposure to different preconditioning stimuli, but the duration of the induced protective phenotype is typically short-lasting, and thus is deemed as limiting its clinical applicability. Extending the period over which such adaptive epigenetic changes persist – in effect, expanding conditioning's "therapeutic window" – would significantly broaden the potential applications of such a treatment approach in patients. The frequency of the conditioning stimulus may hold the key. While transient (1–3 days) protection against CNS ischemic injury is well established preclinically following a single preconditioning stimulus, repetitively presenting preconditioning stimuli extends the duration of ischemic tolerance by many weeks. Moreover, repetitive intermittent postconditioning enhances post-ischemic recovery metrics and improves long-term survival. Intermittent conditioning is also efficacious for preventing or delaying injury in preclinical models of chronic neurodegenerative disease, and for promoting long-lasting functional improvements in a number of other pathologies as well. Although the detailed mechanisms underlying these protracted kinds of neuroplasticity remain largely unstudied, accumulating empirical evidence supports the contention that all of these adaptive phenotypes are epigenetically mediated. Going forward, additional preclinical demonstrations of the ability to induce sustained beneficial phenotypes that reduce the burden of acute and chronic neurodegeneration, and experimental interrogations of the regulatory constructs responsible for these epigenetic responses, will accelerate the identification of not only efficacious but also practical, adaptive epigenetics-based treatments for individuals with neurological disease.

Keywords: stroke, neuroprotection, preconditioning, postconditioning, epigenetics, retina

STATE OF THE ART

It is now widely established that organisms, tissues, and cells respond to sublethal stressors by a transient augmentation in their innate capacity to resist injury. That low doses of a harmful stimulus can promote beneficial responses forms the basis of hormesis theory (1), but only relatively recently have the conceptual and mechanistic connections between conditioning-induced tolerance and the hormetic response been recognized (2–5). In any event, seeking to leverage conditioning- or hormesis-based adaptive responses in patients suffering from acute ischemia, we remain somewhat stymied by several features of our laboratory models that seem to preclude easy translation to the clinic.

Significant progress has been made in preclinical models that advance our understanding of the molecular and genetic mechanisms underlying ischemic tolerance in the CNS (6–9). Many unique subcategories of this general phenomenon have been identified and subject to experimental scrutiny (8). For example, we recognize two basic phases or therapeutic windows for conditioning-induced protection: an early "rapid preconditioning" phase that is manifested nearly immediately after the adaptive stimulus is presented, which is largely dependent on rapid post-translational protein modifications, and a delayed preconditioning phase that takes several hours, sometimes days, to fully develop, resulting from changes in gene expression. We know that improved outcomes can result if the conditioning stimulus is applied to another tissue ("remote conditioning"), although its neural/humoral mechanism remains elusive. We have also learned that, even when initiated after the injurious event ("postconditioning"), conditioning stimuli can still afford protection. However, in all of these conditioning paradigms, we have defaulted to the assumption that the duration of the resultant injury-tolerant state requires a defined period of time to become established, and, more importantly, that it cannot be sustained indefinitely. This latter supposition has hindered, directly or indirectly, the clinical applicability of this phenomenon, as physicians face the daunting challenge of how best to pre- or post-condition patients at a well-defined higher risk for an ischemic event, let alone post-condition those stroke patients that present in the absence of recognizable risk factors. Leveraging such innate adaptive responses to treat chronic neurodegenerative disease seems even more unlikely, if not impossible.

Against this backdrop, I explore herein the contention that, as a result of epigenetics-based changes in gene expression, the duration of injury resistance following a stress stimulus can be

increased quite significantly based on specific manipulations of the "dose" of the conditioning stimulus. Further interrogation of how best to establish protracted therapeutic windows of innate neuroprotection will provide some of the most valuable opportunities for multiplying the clinical potential of conditioning to prevent and treat both acute and chronic neurological disease.

REDEFINING STIMULUS-RESPONSE

Throughout most of its history at both the bench and in the clinic, the conditioning/tolerance field has evolved based on a fundamental, implicit definition of the very phenomenon we have come to understand: A transient, but robust, reduction in injury from acute ischemia can be realized if the tissue is preconditioned "X" hours prior to the lethal ischemic insult. For the aforementioned delayed phase of conditioning, the words "transient" and "X" in this definition have been understood to be ~8–48 h, essentially bookending the "therapeutic window" for treatment. Similarly, for postconditioning, preclinical protocols typically involve conditioning shortly after the ischemic event, as well as performing relatively early assessments of efficacy based largely on lesion quantification. Few studies have examined whether the duration of tolerance resulting from pre- or post-conditioning can be extended secondary to manipulating the "dose" of the conditioning treatment, or the related possibility that repetitive postconditioning during recovery can not only reduce injury but concomitantly enhance one or more long-term stroke recovery metrics; both of these advances would portend powerful clinical implications. In dose–response parlance, if the goal is to invoke a protracted response or prolonged period of tolerance to a given dose of conditioning, and if the given period of tolerance currently appears time-limited, then a closer examination of the conditioning dose is required to achieve this desired response. This has not really happened in any systematic sense; rather, we were quick to adopt the general assumption that the only type of tolerance is one defined by a short therapeutic window.

Every stimulus can be defined by at least three parameters: magnitude, duration, and frequency. Until the advent of postconditioning by instituting cycles of brief ischemia during early reperfusion, the vast majority of preconditioning paradigms for stroke have historically involved a single stimulus (i.e., frequency = 1) of a specific duration and magnitude that set into motion tolerance-signaling cascades; in the field of myocardial ischemia, repetitive preconditioning treatments have been explored (10), but not for the purpose of identifying a treatment that extends the duration of tolerance. Often overlooked is the fact that, like every stimulus, every response can also be defined by at least two of the same three parameters: its robustness and its duration. However, the preconditioning field settled quickly on a magnitude-focused, binary definition of tolerance as protection/no protection measured within ~24–72 h of conditioning; little attention has been focused on whether the duration of a given protective response – again, what is essentially the therapeutic window for this treatment – could be extended. The most obvious way to do so involves changes in stimulus frequency.

In fact, stimulus frequency is critical not only for determining the duration of the phenotypic change induced but also for its cumulative ability to promote an adaptive response, in contrast to the potential impotence of a single exposure or the potential injury resulting from multiple exposures combined as one or administered too closely together. Indeed, evidence emerging not only in the field of conditioning but across other dimensions of biology as well indicates that the frequency of the presented stimulus may be the key dose feature with respect to generating a beneficial, adaptive response instead of a harmful, maladaptive response (or no response at all). Exercise represents one example of benefit-inducing repetitive conditioning (11); moreover, recent clinical studies support the contention that increasing the number of short duration/low intensity bouts of exercise may provide more "advantages" to the cell, tissue, or organism than fewer exposures that are longer and/or more severe (and thus may afford no adaptive benefit or even cause injury) (12). As another example, repetitive intermittent hypoxia, initiated after incomplete spinal cord injury, promotes persistent functional recovery of respiratory and non-respiratory motor systems, as manifested by protracted improvements in gait performance in both rodents (13) and humans (14). In the field of psychology, the enhanced neuronal plasticity that promotes resilience – instead of vulnerability – to a number of different stress paradigms is becoming recognized as uniquely dependent on the frequency of exposure to the stressor (15–21). The long-term effects of intermittent fasting, and meal frequency and timing, on health and disease outcomes (22) represent additional facets of this same concept. Finally, repeated exposures to stress are also central to encoding a largely undefined but still well-recognized "resiliency" in medical, military, and law enforcement personnel and other individuals needing to perform well in adverse environments.

EXTENDING THE DURATION OF ADAPTIVE CHANGE

With respect to stimulus frequency influencing the duration of the resultant phenotype, examples can be found in the conditioning literature that either strongly hint at such a possibility or support it directly. For example, the therapeutic window for myocardial ischemic tolerance resulting from a single hypoxic challenge was extended significantly if intermittent mild hypoxia is used as the conditioning stimulus (23, 24). Similarly, long-term thalamic atrophy following transient focal stroke in mice was attenuated by an intermittent hypoxic postconditioning stimulus initiated 5 days after ictus (25). In neonatal (26) and adult rats (27), multi-day hypoxic postconditioning reduced cerebral injury when the first challenge was initiated an hour after the severe hypoxic-ischemic insult. We found that, relative to the <3-days duration of ischemic tolerance induced in the mouse neural retina by a single hypoxic preconditioning stimulus, tolerance lasting at least 4 weeks after the last hypoxic preconditioning treatment can be realized if the hypoxic preconditioning challenge is repeated six times over a 2-week period (28). A similar extension of the therapeutic window (>2 months) for protection against murine cerebral ischemia is also afforded by a 2-week intermittent hypoxia preconditioning regimen (29), but only with stochastic increases in the frequency, duration, and intensity of the hypoxic stimulus relative to that which was efficacious in protecting against retinal ischemia. This distinction, and the examples above, not only suggest that tissue-, cell, age-, and species-dependent hormetic dose–response relationships may be operative but also suggest that stimulus frequency

is not a simple concept in and of itself, and variations in the magnitude and duration of a given intermittently presented stimulus, as well as its temporal pattern, may make the difference between efficacy, impotence, and harm. The mixed preclinical track-record reported to date, wherein some repetitive conditioning protocols promote stroke tolerance, but not necessarily long-lasting stroke tolerance (30, 31), may be a reflection of this complexity. While the concept that extended periods of tolerance resulting from repetitive conditioning can be modeled in neuronal (32) and cerebral endothelial cell (33) cultures, the ultimate identification of a safe and efficacious, intermittent stimulus-based conditioning protocol – either physiologic or pharmacologic – that provides consistent, long-lasting protection in patients will prove extremely challenging in a diverse cross-section of human patients without reliable biomarker metrics to guide dosing regimens. Clearly, at this juncture, continued work in animal models is warranted.

Despite the above caveats, because most heart attacks and strokes are unpredictable, the inherent clinical advantage of postconditioning relative to preconditioning still generates considerable translational excitement. Progress might be accelerated if postconditioning protocols move beyond cyclical interruptions of early reperfusion to assessments of the benefits accruing from more protracted, repetitive treatments, and if long-term survival rates and functional metrics of post-stroke recovery become the more standard endpoints. Indeed, in cardiac ischemia, repeated remote postconditioning improved long-term survival relative to both a single preconditioning challenge and to an even lower frequency of repetitive remote postconditioning (34). Intentionally delaying the start of the repetitive postconditioning treatment volley may be prudent, given that, when initiated a week after stroke, moderate intermittent hypoxia rescued ischemia-induced impairments in learning and memory (35), whereas initiating the same treatment 1–2 days post-stroke increased mortality (36). Similarly, the aforementioned study of intermittent hypoxic postconditioning preventing post-ischemic thalamic atrophy was efficacious when the stimulus volley was initiated 5 days, but not 1 day, after ictus (25). Predictably, the optimal treatment window will likely vary depending on the age, species, injury type, and the postconditioning stimulus employed [including physical rehabilitation and exercise (37, 38)]. No matter what the tissue, rigorous, systematic investigations of multiple postconditioning "dose" regimens/protocols (34, 36, 39) – essentially time-consuming, iterative, titration-directed experiments – will be needed to identify both the dose and the optimal time after the acute injury to initiate treatment if this therapeutic approach is ever to be clinically implemented (40, 41). At present, we really know very little about the frequency of a given postconditioning stimulus needed to activate innate plasticity mechanisms capable of promoting and sustaining a neurorecovery-enhancing phenotype. We know less still about "preconditioning" treatments that effectively "post-condition" the stroked brain against a second stroke; repetitive stimuli may hold promise as the ideal treatment approach for this challenge. That said, some clinical trials of repetitive conditioning have moved forward empirically, and some have shown efficacy. As examples, hyperbaric oxygen treatments (5 days/week for 8 weeks) led to significant neurological improvements in both stroke (42) and head trauma (43) patients, even when the treatment was initiated many months after injury. And intermittent remote postconditioning by upper limb cuff inflation twice a day for 300 consecutive days proved effective in reducing the incidence of recurrent stroke in patients with symptomatic atherosclerotic intracranial arterial stenosis (44).

CONDITIONING FOR CHRONIC NEUROLOGIC DISEASE

That distinct patterns of "continuous" but intermittent conditioning treatments provide protracted periods of protection against stroke and other acute neurological disorders implies that the slow and progressive neuronal death that defines neurodegenerative disease might also be significantly impacted by a similar therapeutic strategy. Evidence from the hormesis field that repeated presentations of low-dose stressors extend beneficial, prevention-, and/or recovery-promoting effects in preclinical models of more chronic cardiovascular, immunological, metastatic, and other non-neurological and non-psychological pathologies, as well as in human studies (4), supports this contention. Not surprisingly then, when tested, the intermittent hypoxia protocol that we leveraged in mice to extend the duration of tolerance to acute retinal ischemia (28) also proved efficacious as a preconditioning stimulus against glaucoma, significantly reducing retinal ganglion cell somal and axonal loss at 3 and 10 weeks of disease in mice (45). Moreover, significant improvements in these same cell survival metrics were realized when we postconditioned mice with a repetitive intermittent hypoxia protocol initiated after disease onset (46). Repetitive postconditioning with brief bouts of retinal ischemia was also efficacious in reducing retinal neurodegeneration in rat models of glaucoma (47) and diabetic retinopathy (48). Suggestive of potential efficacy in Alzheimer's, intermittent hypoxic postconditioning reduced oxidative and nitrosative stress metrics and improved morphologic and functional (memory) outcomes following intracerebral injections of amyloid beta (49). Although not widely realized, an extensive number of preclinical and clinical studies in the Soviet Union, in which an estimated two million individuals were treated over long periods of time with intermittent hypoxia, document a variety of health-promoting and injury-resistant outcomes from such treatments, in the absence of adverse effects (50). Collectively, these findings provide strong support for the hypothesis that intermittent conditioning during the progression of chronic neurological disease may provide a means of both inducing and "holding on" to an adaptive phenotype that in turn is manifested not only as a reduction in the kinetics of disease progression but also as improvements in functional status and long-term survival.

LIFESPAN EFFECTS

As alluded to above, the idea that repetitive stress conditioning can trigger and possibly maintain long-lasting phenotypic change in the CNS actually derives strong historical support from the neuropsychology and behavior fields, which provide rich examples of sustained plasticity within and across age groups, and ideal opportunities for considering stress along a negative–positive continuum, with distress anchoring one end and "eustress" anchoring the other (51). For instance, when initiated during adulthood, repeated mild stress exposures lead to persistent decreases in behavioral disorders and improvements in memory (52, 53). And

the literature is actually replete with studies documenting adaptive phenotypes persisting into adulthood as a result of intermittent, mild stress challenges only experienced by the individual during intra-uterine or post-uterine development. This concept was introduced over 60 years ago based on the serendipitous findings of Levine (54), and is somewhat akin to the immunological concept of early childhood vaccinations for conferring life-long protection against disease. As it turns out, most research over the ensuing decades has focused on documenting illness phenotypes secondary to child or adolescent stress in an attempt to understand the origins of psychiatric disease (55). Nevertheless, reflecting yet another manifestation of "the dose makes the poison" and new ways of thinking about evolutionary fitness, there are still some investigations to be found of beneficial adult phenotypes resulting from exposing the same individual, when much younger, to a given stressor and/or exposing their caregiving mothers to stress (56, 57). Hypoxia-related examples of the former include the finding that postnatal mice exposed from birth to 4 weeks of age to mild intermittent hypoxia exhibit improved spatial learning and memory during their adult life (58), and the report that brief hypoxic challenges during neonate life confer resistance to senescence and better preservation of cognitive function in aged rats (59). Resilience to stress in adult mice raised by mothers with access to postpartum exercise (60) is an example of the latter. Intermittent separations of baby monkeys from their mother may represent a form of "stress inoculation" that leads to enhanced arousal regulation and resilience of these offspring in later life (15). There is also evidence that unpredictable, stochastic stress exhibits unique age-dependent effects, promoting future resilience when experienced by juvenile animals, but not by adults (61). Given these and other provocative findings, some have even proposed – for humans – intentional exposures to intermittent eustress (like mild hypoxia) during neonatal or adolescent periods as a way to prevent or lessen future disease burden (62).

EPIGENETICS AS PRIMARY MECHANISM

The molecular basis of the many adaptive responses to repeated stress highlighted thus far is predictably complex, given the relative permanence of the resultant phenotype. Many studies have sought to characterize the beneficial phenotypes, finding evidence for increased neurogenesis (53), changes in hormonal balance (15, 18), changes in modulators of synaptic plasticity (63, 64), changes in sodium–calcium exchangers (NCX) (65), and elevated levels of HIF gene target mRNAs/proteins (52) or other survival factors (31), to name a few. While identifying these phenotypes is interesting with respect to understanding how disease resistance is ultimately manifested, it is epigenetics that deserves attention as the fundamental mechanism responsible for the long-lasting responses to repetitive conditioning stimuli, and thus the likely target of future therapeutics. In brief, epigenetics involves the regulatory processes – DNA methylation and changes in chromatin structure secondary to post-translational histone modifications – that reside "above" the level of genes and control their readout. During development, epigenetics specifies cell fate determination and perpetuation, but we now know that these same mechanisms are engaged throughout the lifespan by "experience" or "environment," in all their different forms. Importantly, considerable evidence indicates that the changes in gene expression resulting from these stable covalent DNA modifications or epigenetic "marks" can be long-lasting, and, in some instances, endure throughout the lifetime of post-mitotic cells; some may even persist through cell division and be transmitted via the germline to future generations (see below). Thus, epigenetics is really the biochemically driven interface between nature (genes) and nurture (all manner of environmental and behavioral/psychological stresses or exposures). Proximal to this interface, so to speak, distinct features of an experience or a stressor (e.g., frequency, severity, etc.) that, as alluded to earlier we have only recently begun to dissect and define with respect to threshold and interactive effects, dynamically modify the epigenome. Moreover, epigenetic marks can accumulate over the course of multiple exposures and then act collectively to determine a new homeostatic phenotypic set-point. Distally, after encoding molecular memories of these experiences and exposures, CNS function and behavior is altered secondary to changes in gene expression, thus impacting one's vulnerability or resilience to future stressors/disease (66). In essence, epigenetics is really the "hard-wired" evolutionary response for successful adaptation to changing natural and social environments. With the realization that phenotype is fluid, and not rigidly defined by genotype, it is not an exaggeration to claim that broadening our understanding of "neuroepigenetics" (67) and its regulation at both cellular and organismal levels holds incredible promise for reducing CNS disease burden and enhancing neurological health.

The ischemia-tolerant phenotype resulting from a pre- or post-conditioning stimulus is, by definition, an epigenetic response, but only recently have we begun to directly recognize it as such (68–70). Genomic and proteomic analyses consistently reveal that the ischemia-tolerant CNS is defined by a broad transcriptional repression (6, 68, 71) – in effect, the manifestation of an epigenetically mediated response to the conditioning stimulus. Tests of the hypothesis that members of the evolutionarily conserved polycomb protein family – known repressors of gene transcription secondary to their ability to posttranslationally modify histones and thus maintain them in an inactive state (72) – may be causal epigenetic mediators ultimately responsible for establishing this metabolically downregulated phenotype confirmed that increases in polycomb group proteins define the "traditional" ischemia-tolerant CNS (68, 73). Certainly, other transcriptional regulators of gene repression – such as repressor element-1 silencing transcription factor (REST) (74) – may also be involved in orchestrating this response. Whether polycomb and/or REST proteins and/or others participate in mediating the longer periods of injury resistance induced by repetitive pre- or post-conditioning stress paradigms has yet to be examined. Additional studies uncovering other epigenetic features of conditioning-induced tolerance have been forthcoming (75).

A rapidly evolving subfield of epigenetics extends the aforementioned phenomenon of even life-long phenotypic change within an individual to the transmission of adaptive or maladaptive phenotypes from parents to their offspring. Epigenetic inheritance, or transgenerational epigenetics, provides a mechanistic foundation for Lamarck's notion that the effects of environment and/or experience could be inherited by, and thus benefit, one's immediate offspring – known as "soft inheritance" in Lamarck's day. This

phenomenon is not inconsistent with Darwinian evolutionary theory, given the selection pressure inherent in preserving adaptive phenotypic variation if it increases fitness when resources and/or other environmental stress levels change; but it does carry the important implication that random DNA mutations are not the sole driving force of evolution. In transgenerational epigenetics, gene expression is altered, not genetic inheritance; environment becomes heredity. At least in theory, this suggests the possibility of obtaining desired phenotypes in offspring that prevent or reduce disease burden and/or enhance vitality by therapeutically manipulating epigenetic regulatory systems in their parents. Conditioning across generations might seem at first blush to represent an implausible extension of tolerance's therapeutic window. However, while the vast majority of preclinical studies have focused on the germline linking of adverse or dysfunctional behavioral changes in an individual to life experiences of their parents or grandparents (76–78), a small number of reports to date provide convincing examples, in mammals, of the transgenerational persistence of beneficial, epigenetically acquired phenotypes (79–82). Moreover, there is very intriguing epidemiological evidence that this occurs in humans (80, 83–85). Obviously, understanding the mechanistic basis of both potential outcomes portends huge repercussions for evolutionary biology, and will likely change the face of our understanding of the genetic basis of neurological disease.

CONCLUSION

Despite the discovery of the robustness of endogenous cytoprotection almost 25 years ago, the thousands of published preclinical successes, and even the dozens of efficacious studies in humans, the time-limited therapeutic window that has inadvertently come to define conditioning-based responses continues to constrain its acceptance as a viable therapeutic strategy, even for acute tissue injury. However, given the accumulating evidence supporting long-lasting, epigenetics-mediated changes in phenotype in the CNS and other tissues secondary to repetitive stress, perhaps it is time to shelf the assumption that conditioning-induced tolerance is "transient," reevaluate our assumptions about the defining features of tolerance, and in so doing open new doors regarding its full clinical destiny. As scientists and physicians, capturing the ability to leverage sustained, beneficial phenotypic changes capable of providing protracted periods of resilience to acute and chronic neurological injury should be our next collective goal.

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Histone deacetylases exert class-specific roles in conditioning the brain and heart against acute ischemic injury

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Ischemia-reperfusion (IR) injury comprises a significant portion of morbidity and mortality from heart and brain diseases worldwide. This enduring clinical problem has inspired myriad reports in the scientific literature of experimental interventions seeking to elucidate the pathology of IR injury. Elective cardiac surgery presents perhaps the most viable scenario for protecting the heart and brain from IR injury due to the opportunity to condition the organs prior to insult. The physiological parameters for the preconditioning of vital organs prior to insult through mechanical and pharmacological maneuvers have been heavily examined. These investigations have revealed new insights into how preconditioning alters cellular responses to IR injury. However, the promise of preconditioning remains unfulfilled at the clinical level, and research seeking to implicate cell signals essential to this protection continues. Recent discoveries in molecular biology have revealed that gene expression can be controlled through posttranslational modifications, without altering the chemical structure of the genetic code. In this scenario, gene expression is repressed by enzymes that cause chromatin compaction through catalytic removal of acetyl moieties from lysine residues on histones. These enzymes, called histone deacetylases (HDACs), can be inhibited pharmacologically, leading to the de-repression of protective genes. The discovery that HDACs can also alter the function of non-histone proteins through posttranslational deacetylation has expanded the potential impact of HDAC inhibitors for the treatment of human disease. HDAC inhibitors have been applied in a very small number of experimental models of IR. However, the scientific literature contains an increasing number of reports demonstrating that HDACs converge on preconditioning signals in the cell. This review will describe the influence of HDACs on major preconditioning signaling pathways in the heart and brain.

Keywords: ischemia-reperfusion injury, histone deacetylase inhibitors, stroke, posttranslational modification, enzymatic crosstalk, preconditioning, postconditioning, reperfusion injury salvage kinase

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Introduction

Worldwide, 33 million people suffer a stroke each year (1). Ischemia, which can occur in all tissues, is defined as the stress that a tissue experiences when both oxygen and substrate are reduced (2). Stroke, defined as insufficient blood flow to the brain, is one major type of cerebral ischemia and may cause transient to permanent loss of brain function or death (3).

In a subset of patients (<15%), stroke occurs as a result of cardiac arrest and cardiac surgery; secondary stroke is, by definition, the result of embolic events originating in the heart (4). In spite of intense research effort directed toward reducing the impact of these conditions, world rates of morbidity and mortality as a result of brain and heart disease continue to rise, most strikingly in developing countries (5). The lack of broadly effective treatment continues to fuel the search for new molecular targets in ischemia-reperfusion injury (IRI). Intriguingly, studies of molecular pathology in the brain and heart are often informed by cancer research. Recent clinical advances have revealed the efficacy of using small molecule inhibitors of histone deacetylases (HDACs) to target malignancy (6). HDACs are enzymes which control signal transduction and gene expression in all cell types (7). Here we review the experimental evidence in support of applying HDAC inhibitors in settings of cerebral and cardiac ischemia, with emphasis on the roles that HDACs play in signaling events that occur as a result of IRI. Importantly, this nascent experimental work indicates that HDAC inhibitors show great promise for treating patients at high risk for stroke or cardiac arrest and for patients electing to receive brain and heart surgeries.

Endogenous tolerance to ischemia can be evoked in the heart and brain (4, 8). Classical ischemic conditioning in a tissue requires the mechanical application of several momentary, sublethal reductions in oxygen and substrate delivery, which reduce the injury caused by a more severe ischemic insult (9). Organs subjected to classical ischemic conditioning experience only transient stimulation of endogenous protective mechanisms, which limits application of the conditioning stimulus to shortly before prolonged insult, as in preconditioning a patient of elective surgery, or to shortly afterward, as in postconditioning a patient of out-of-hospital stroke or cardiac arrest. The idea that similar magnitudes of endogenous protection can be evoked pharmacologically is a considerable advancement over the barrier of time-dependence instituted by mechanical ischemic conditioning (10). It follows that drug-evoked conditioning is a promising treatment strategy for patients undergoing elective brain or heart surgery, or those at high risk of stroke or cardiac arrest (11).

Prevention of cell death is the primary effect of ischemic conditioning (12, 13). Many forms of ischemic conditioning (including classical, pharmacological, and remote conditioning) converge on signaling pathways that either inhibit cell death or activate endogenous cell survival maintenance programs. Once active, cell survival programs improve endoplasmic reticulum stabilization (14), increase expression of antioxidant enzymes (SOD2; catalase) (15), inhibit endogenous cell death programs (Bax/Bad/Bcl2; c-jun) (16), activate transcription of genes for repair enzymes (Hsp70; HIF-1-alpha) (17, 18), and stimulate autophagic flux (HDAC6) (19).

Acute adaptation to ischemia through conditioning stimuli is mediated by protein posttranslational modifications (PTMs) (20, 21). Historically, protein phosphorylation has been the most rigorously characterized of the PTMs in settings of experimental ischemic conditioning. These include the reperfusion injury salvage kinases (RISK) pathways of Raf/MEK/ERK1/2 and PI3K/Akt/eNOS, the JAK/STAT transcriptional pathways and the calcium-responsive PKC pathways (22–25). However, accumulating evidence shows that protein acetylation also plays major roles in regulating cell survival through ischemic conditioning (7).

Histone Deacetylases Regulate Cell Fate in Cerebral Ischemia

Histone deacetylases are a class of epigenetic enzymes that have come under recent intense scrutiny as pharmacological targets for patients suffering stroke. HDACs remove acetyl moieties from ε-amino groups of lysine residues on histones and non-histone proteins (25). Deacetylation of histones enhances chromatin compaction, which renders DNA less available for binding by regulatory factors leading to repression of gene expression (26, 27). In this function, HDACs exert classical transcriptional control. This process is reversible via enzymatic histone acetyltransferase (HAT) activity. The zinc-dependent HDACs have been divided into classes based on homology to yeast transcriptional repressors. Class I comprises HDACs 1, 2, 3, and 8; class IIa comprises 4, 5, 7, and 9; class IIb comprises 6 and 10; and class IV comprises HDAC11 (7). Class III, called sirtiuns, are NAD^+-dependent and will not be discussed in this review, but have been expertly reviewed elsewhere (28). HDAC enzymes are widely expressed in rodent brains, and are localized to specific cellular compartments in isoform-specific patterns (29, 30). Class I HDACs are generally restricted to the nucleus where they impose transcriptional control, whereas class IIa HDACs transit the nuclear membranes and enter the cytoplasm in a process mediated by phosphorylation (31). Furthermore, HDAC6 is primarily, though not constitutively, cytoplasmic (19).

As mentioned above, HDACs deacetylate both histone and non-histone proteins. When HDACs deacetylate histones and repress gene transcription, cell survival is impacted after several hours and days on time scales necessary for protein expression (7). Non-histone deacetylation is another element of this complex code of enzymatic crosstalk, which is distinct from direct inhibition of gene expression by histone deacetylation. Importantly, the acetylation state of a given metabolic signaling factor may mediate its phosphorylation, methylation, and ubiquitination state, thereby determining its subcellular location, activation, or degradation, with immediate implications for cell survival in the seconds to minutes following the insult (32). The acetylation state of transcription factors, co-activators, and co-repressors can regulate their activity (33). Decoding the complex patterns of HDAC enzymatic crosstalk will enhance our understanding of histone and non-histone protein lysine deacetylation and its impact on the survival of cells under ischemic stress (34).

Importantly, experimental cerebral ischemia causes upregulation of class I/IIb HDAC expression, which possibly implicates them in ischemic pathology (29). For example, HDAC1 must exist in complex with HDAC3 to promote apoptosis in cerebellar granule neurons; the toxic effects of HDAC1:HDAC3 association were mitigated by activation of PI3K/Akt signaling (35). Cortical neurons transfected with HDAC3 or HDAC6 shRNA each exhibited decreased apoptosis when exposed to prolonged oxygen–glucose deprivation (30). Furthermore, HDAC2 mutant mice exhibited reduced retinal degeneration following ischemia

(36). In response to these findings and many others, a variety of pharmacological HDAC inhibitors have been developed and applied in animal models of cerebral ischemia. Results from these studies have revealed that HDAC inhibitors can pharmacologically condition the neuron against ischemic injury through de-repression of transcription (37, 38). While these studies suggest that pharmacological inhibition of class I/IIb HDAC isoforms (so-called "pan-inhibitors") promote survival of stressed neurons, the evidence reviewed below indicates that the catalytically inactive IIa isoforms may be beneficial to cell survival from IRI.

Small Molecule HDAC Inhibitors Condition the Brain Against Ischemic Injury

Reduction in infarct volume and prevention of apoptosis are the most obvious physiological effects of class I/IIb HDAC inhibitors in experimental cerebral ischemia (see **Table 1**). The molecular mediators are numerous but consensus is forming around certain cellular processes: modulation of apoptotic intermediates (caspases, Bcl-2), stabilization of the cellular stress response (Hsp-70, EIF-2α, CHOP), transcription of oxygen-responsive enzymes (HIF-1α, Nrf2), regulation of calcium handling (BDNF, CREB), and activation of survival kinase cascades (Akt, ERK, AMPK, p21).

Administration of the mood stabilizer and weak HDAC inhibitor valproic acid (VPA) after permanent right carotid artery occlusion prevented neuronal apoptosis in a dose-dependent manner in neonatal rats (39). Rats treated with VPA during middle cerebral artery occlusion (MCAo) exhibited reduced infarct volume, enhanced angiogenesis (40), neurogenesis (41, 42), and reduction of monocyte infiltration (43). These effects were correlated with increased transcription of Hsp70 (43, 44), HIF1-alpha and MMP-2/9 (40), or GLT-1, a transporter protein, which accelerates clearance of glutamate in damaged gray and white matter (41). VPA protected retinal ganglion cells from ischemia-reperfusion (IR) through reduction of mitochondria-mediated apoptosis (45) and endoplasmic reticulum stress-induced apoptosis (46), and enhanced Hsp70 promoter acetylation in cortical neurons through inhibition of a class I HDAC (47). The potent hydroxamate trichostatin A (TSA) decreased infarct volume in rodents given MCAo, which depended on induction of Hsp70, Bcl-2, and Akt phosphorylation, or on gelsolin, an essential regulator of actin homeostasis (43, 48, 49). Mice treated with TSA at the onset of permanent MCAo exhibited reduced infarct volume through increased Nrf2-dependent transcription of antioxidant enzymes (50). TSA also reduced transcription of inflammatory proteins MMP-1 and MMP-3, and reduced caspase-3 activation up to 24 h after the onset of ischemia in the retina (51, 52). Sodium butyrate (a potent analog of VPA) treatment before permanent MCAo evoked a 50% reduction in infarct volume through increased expression of p21, a cyclin-dependent kinase inhibitor, which prevents pro-apoptotic gene transcription (53–55). Furthermore, mice treated with 4-phenylbutyrate before or after ischemia–hypoxia exhibited reduced ER-stress-mediated apoptosis through reduction of EIF2-alpha phosphorylation (56). Mice treated with the potent hydroxamate Vorinostat (FDA approved for treatment of T-cell lymphoma) at the onset of MCAo exhibited reduced infarct size and increased transcription of Hsp70 and Bcl2 (57, 58). Furthermore, neurons exposed to oxygen–glucose deprivation *in vitro* and mice subjected to MCAo *in vivo* exhibited increased acetylation at the Bcl-xL promoter when treated with Entinostat, a class I selective HDAC inhibitor; the effect was mediated by enhanced NF-kB p50 acetylation and decreased activation of the Bim promoter (59).

While class I HDACs seem to play pathological roles in cerebral ischemia, there is evidence that class IIa HDACs are required for cell survival following neuronal stress. Genetic heterogeneity surrounding the *HDAC9* gene is associated with large vessel ischemic stroke (60). By directly inhibiting the c-jun promoter, HDAC4 (61) and HDAC7 prevented neuronal cell death induced by low potassium (62). HDAC4 is required for the normal development of retinal neurons through the stabilization of HIF-1-alpha (63). HDAC4 and HDAC5 knock-in protected neuron-like pheochromocytoma cells from apoptosis induced by OGD, which was partly dependent on HMGB1 activity (64). Conversely, nuclear export of HDAC5 was required for regeneration after acute axonal injury, a condition that promotes rapid influx of calcium (65). In fact, nuclear calcium levels regulate the association of class IIa HDACs with a MEF2-SMRT corepressor complex (66–68). Given this, it is possible that class IIa HDACs may correct calcium-induced pathological gene expression in neuronal ischemia.

HDAC Enzymatic Crosstalk in Cerebral Ischemia

Evidence is accumulating that HDAC signal transduction pathways communicate in crosstalk with kinase signal cascades in cerebral ischemia. The ability of HDAC inhibitors to condition the neuron in the seconds to minutes following acute ischemic stress may be dependent on the concurrent activity of certain cell survival kinases. As mentioned above, TSA prevented oxidative cell death in cortical neurons through increased transcription of p21, which inactivates pro-apoptotic c-jun transcription by inhibiting the kinase ASK-1 (53–55). HDAC3 was phosphorylated by GSK3-beta and was required for cell death induced by low potassium in cultured cortical neurons; neuronal death was prevented by pharmacological inhibition of GSK3-beta, and with constitutively active Akt, a known inhibitor of GSK3-beta (69). Conversely, the class IIa HDAC4 protects neurons from cell death induced by low potassium by direct inhibition of cyclin-dependent kinase-1 activity, independent of PI3K/AKT, c-jun, or RAF/MEK/ERK signaling (61).

PI3K and AKT activities are both required for the neuronal conditioning achieved with VPA (47). Interestingly, induction of Hsp70 by VPA and other Class I HDAC inhibitors resulted in increased histone methylation in primary neurons and astrocytes (70). In particular, as confirmed by chromatin immunoprecipitation, HDAC inhibition caused increased methylation at the Hsp70 promoter, a histone landscape favoring transcriptional activation. This suggests an intricate interplay between histone acetylation

TABLE 1 | Physiological effects of HDAC inhibitors in experimental models of stroke.

Reference	Stroke model	Treatment	Treatment time	Molecular target	Acetylated protein	Physiological effect
(36)	Mouse retinal I/R	HDAC2$^{+/-}$			Ac-histone H3	↓Apoptosis
(39)	Rat pup RCAo + 1 h hypoxia	VPA 200 or 400 mg/kg/day	Post for 5 days			↓Neuronal apoptosis
(40)	Rat MCAo (1 h) with reperfusion	VPA 200 mg/kg/day	Post for 14 days	↑HIF-1α, VEGF, MMP-2/9	Ac-histone H3; Ac-histone H4	↓Brain infarction
(41)	Rat pMCAo	VPA 100 mg/kg/day	Post for 7 days	↑GLT-1	Ac-histone H4	↓Brain infarction; ↓neuronal apoptosis
(43)	Rat pMCAo	VPA 300 mg/kg/12 h × 2	Post for 1 or 2 days	↑HSP-70, p53; ↓iNOS	Ac-histone H3	↓Brain infarction
(43)	Rat pMCAo	SB 300 mg/kg/12 h × 2	Post for 1 or 2 days	↑HSP-70, COX-2, p-Akt	Ac-histone H3	↓Brain infarction
(43)	Rat pMCAo	TSA 0.5 mg/kg/12 h × 2	Post for 1 or 2 days	↑HSP-70, Bcl-2, p-Akt	Ac-histone H3	↓Brain infarction
(44)	Rat MCAo (1 h) with reperfusion	VPA 300 mg/kg/12 h × 2	Post for 1 or 2 days	↑HSP-70; ↓active caspase-3	Ac-histone H3	↓Brain infarction
(45)	Rat (optical nerve crush)	VPA 300 mg/kg/12 h × 2	Post for 5 or 8 days	↑CREB DNA binding, p-ERK; ↓active caspase-3		↓Retinal ganglion cell death; ↑axonal regeneration
(46)	Rat retinal I/R	VPA 300 mg/kg/day	Pre for 1 day and post for 7 days	↑GRP78; ↓active caspase-12, CHOP	Ac-histone H3	↓Retinal ganglion cell death; ↓ER stress-mediated apoptosis
(47)	Heat shock (42°C) 1 h in cultured rat cortical neurons	VPA 0.25–1.0 mM	Post for 1 day	↑HSP-70; ↓active caspase-3	Ac-histone H3K9/14; Ac-Sp1	
(53)	Rat pMCAo	SB 1200 mg/kg	Pre for 1 day and post for 30 min	↑p21	Ac-histone H4	↓Brain infarction
(42)	Rat pMCAo	SB 300 mg/kg day	Post for 14 days	↑BDNF, p-CREB, GFAP		↑Cell proliferation, migration, differentiation
(56)	Mouse MCAo + hypoxia	4-PBA 40 or 120 mg/kg/day	Pre for 3 days or post for 3 days	↓Active caspase-12, p-EIF-2α, CHOP		↓Brain infarction; ↓neuronal apoptosis; ↓ER stress-mediated apoptosis
(58)	Mouse pMCAo	Vorinostat 50 mg/kg x 2	Post at 0 h and 6 h	↑HSP-70, Bcl-2, p-Akt	Ac-histone H3 K18	↓Brain infarction
(59)	OGD (3 h) with reperfusion (2 h) in mouse cultured cortical neurons	Entinostat 0.1, 0.5, or 1 μM	Post for 2 h	↑p-AMPK, Bcl-xL promoter Ac; ↓Bim promoter Ac	Ac-NF-kB p50 K310; Ac-histone H3 K9/18	↓Neuronal apoptosis
(59)	Mouse MCAo (1 h) with reperfusion	Entinostat 20 or 200 μg/kg	Post at 1, 3, 5, or 7 h	↑Bcl-xL promoter Ac; ↓Bim promoter Ac	Ac-histone H3 K9/18	↓Brain infarction
(49)	Mouse MCAo (1 h) with reperfusion	TSA 1 or 5 mg/kg/day	Post for 14 days	Gelsolin	Ac-histone H4	↓Brain infarction
(49)	OGD for 90 or 150 min in mouse cultured cortical neurons	TSA 300 nM	Pre for 12 h			↓$[Ca2+]^{i}$, ↑$\Delta\Psi$
(50)	Mouse pMCAo	TSA 1 or 5 mg/kg/day	Post at 0 and 6 h			↓Brain infarction
(50)	OGD for 150 min in mouse cultured cortical neurons	TSA 3, 10, or 30 ng/mL	Pre for 1 h	↑Nrf2:ARE binding, NQO1, HO1		↓Neuronal apoptosis
(51, 52)	Rat retinal I/R	TSA 2.5 mg/kg/12 h	Post for 3 days	↑TNF-α	Ac-histone H3	↓Apoptosis

RCAo, right carotid artery occlusion; pMCAo, permanent carotid artery occlusion; ONC, optical nerve crush; Ac, acetylated; OGD, oxygen–glucose deprivation.

and histone methylation. In fact, this phenomenon of functional and structural cooperation between HDACs and lysine-specific demethylase (LSD) enzymes is well established, as in the multifaceted corepressor CoREST/REST/HDAC/LSD complex (71). However, complex crosstalk between lysine "readers" (enzymes that recruit PTM enzymes to acetyl-lysine residues) and "writers" (enzymes that catalyze acetylation of lysine residues) results in combinations of histone modifications that form a hierarchal landscape, which dictates the transition between silencing and activation of a certain transcription domain (72). Clearly, HDAC enzymatic crosstalk with other PTM enzymes occurs on both histones and non-histone proteins.

HDAC Inhibitors Mitigate Cardiac Infarction Following IRI

Histone deacetylase inhibitors have also shown potential in mitigating cardiac IRI (73). Importantly, HDAC activity is also upregulated in hearts after IR. Mice treated with TSA following *in vivo* IRI exhibited marked reduction of infarct area which correlated with stabilization of HIF-1a. This effect was abrogated in HDAC4 knockout cardiomycytes, in another example of the putative protective nature of a class IIa HDAC (74).

Multiple kinase pathways have been implicated in promoting myocyte survival in response to ischemic injury, including p38 MAPK (75–77), the RISK PI3K/AKT/eNOS (78–83) and RAF/MEK/ERK1/2 (84), and the survivor activating factor enhancement pathway (SAFE) (85, 86). Evidence for enzymatic crosstalk between HDACs and these pathways is growing. The cardioprotective action of HDAC inhibitors in IRI is also apparently dependent on the gp-91 subunit of NADPH oxidase (87), p38 (88), Akt1, and Mkk3 (89, 90). The transcription factor NF-kB has been suggested as a common target of multiple types of preconditioning stimuli (91). Interestingly, NF-kB is a common target of the p38 MAPK (92), Akt (93), and Erk1/2 (94) signaling pathways, and is required for p38-mediated adaptation to mechanical preconditioning in isolated hearts (92). TSA-induced pharmacological preconditioning was recently correlated with nuclear translocation, activation, and hyperacetylation of NF-kB, while specific deletion of NF-kB p50 abrogated the TSA protective effect (95). The effects of HDAC inhibition on ERK1/2 signaling have not been directly assessed in a model of IR. Intriguingly, however, HDAC inhibition prevents cell death in multiple cell lines by activating ERK1/2, while this effect is not present in several cancer cell lines (96). The absence of HDAC-mediated ERK1/2 activation in cancer cells is likely the result of suppressed HDAC1 activity (97).

Recent work in mouse tissues demonstrated that the endogenous HDAC1 inhibitor D-β-hydroxybutarate (β-OHB) caused increased acetylation at the promoter of the FOXO3a transcription factor. This led to upregulation of FOXO3a and its targets SOD2 and catalase, free radical scavengers, which further prevented paraquat-induced renal oxidative stress in mouse kidneys (98–100). Additionally, we recently reported preserved cardiac contractile function and reduced infarction following IR by pre-treating rats with Entinostat (101). This was associated with dramatic nuclear FOXO3a enrichment, along with increased transcript and protein levels of SOD2 and catalase. Nuclear enrichment of FOXO3a was likely due to decreased Akt-mediated phosphorylation at the key nuclear exclusion site S318/321 (102), suggesting that class I HDAC inhibition also influences the nuclear trafficking of transcription factors which upregulate these enzymes. HDAC4/5 are excluded from the nucleus by phosphorylation by AMPK, but localize to the nucleus in situations of low glucose, where they recruit HDAC3. Intriguingly, in this case, HDAC3 deacetylates and activates FOXO-mediated transcription of anti-apoptotic genes (103).

Conclusion

Histone deacetylases evidently form signaling hubs for cellular communication in cerebral and cardiac IRI. Class I HDACs appear to play mainly pathologic roles in IRI, by repressing transcription of genes required for cell survival, while Class IIa HDACs appear necessary for cell survival. The roles of the class IIb HDAC6 in preconditioning are not full understood, though HDAC6 is a major regulator of autophagic flux in neurodegenerative diseases (104) and a contributor to pathological responses in the heart (105). Recent studies demonstrate that the modulation of endogenous antioxidant transcription is a significant mechanism by which the inhibition of HDACs confers preconditioning protection against IRI. Neurons and cardiomyocytes may share epigenetic signaling mechanisms for activation of endogenous protection from ischemia by HDAC inhibitors.

Lines of evidence have begun to accumulate that support a role for communication between HDAC and kinase signaling networks in ischemia of the heart–brain axis. While the mechanisms behind the protective preconditioning effect of HDAC inhibition remain to be fully elucidated, it is evident that the RISK pathway and related kinases are integral components. More research into the details of the specific interactions between HDACs and other PTMs will advance our understanding of the role of HDAC inhibition in ischemic preconditioning. Dissecting the dual roles of HDACs as transcriptional repressors and as effectors of enzymatic crosstalk is needed to dissect the chronic and acute phases of preconditioning protection. Given that several small molecule inhibitors of HDAC activity are currently used in patients or in clinical trials, HDAC inhibitors represent promising treatment modalities for patients undergoing elective brain or heart surgery, or patients at high risk of stroke or cardiac arrest.

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Transcriptional response of polycomb group genes to status epilepticus in mice is modified by prior exposure to epileptic preconditioning

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Exposure of the brain to brief, non-harmful seizures can activate protective mechanisms that temporarily generate a damage-refractory state. This process, termed epileptic tolerance, is associated with large-scale down-regulation of gene expression. Polycomb group (PcG) proteins are master controllers of gene silencing during development that are re-activated by injury to the brain. Here, we explored the transcriptional response of genes associated with polycomb repressive complex (PRC) 1 (*Ring1A*, *Ring1B*, and *Bmi1*) and PRC2 (*Ezh1*, *Ezh2*, and *Suz12*), as well as additional transcriptional regulators *Sirt1*, *Yy1*, and *Yy2*, in a mouse model of status epilepticus (SE). Findings were contrasted to changes after SE in mice previously given brief seizures to evoke tolerance. Real-time quantitative PCR showed SE prompted an early (1 h) increase in expression of several genes in PRC1 and PRC2 in the hippocampus, followed by down-regulation of many of the same genes at later times points (4, 8, and 24 h). Spatio-temporal differences were found among PRC2 genes in epileptic tolerance, including increased expression of *Ezh2*, *Suz12*, and *Yy2* relative to the normal injury response to SE. In contrast, PRC1 complex genes including *Ring 1B* and *Bmi1* displayed differential down-regulation in epileptic tolerance. The present study characterizes PcG gene expression following SE and shows prior seizure exposure produces select changes to PRC1 and PRC2 composition that may influence differential gene expression in epileptic tolerance.

Keywords: epileptic tolerance, hippocampal sclerosis, neuroprotection, temporal lobe epilepsy, polycomb

INTRODUCTION

Epigenetic processes are structural modifications to chromatin which are indicative of and contribute to the transcriptional state (1). These processes include DNA methylation and histone modification and they play an important role in gene expression control. Such modifications are dynamic in the adult brain and may act as important transcriptional determinants in plasticity and memory (2, 3). Aberrant DNA methylation has been implicated in certain neurological disorders (4, 5), and other studies have emphasized the role of epigenetic mechanisms in seizures and epilepsy (6). For instance, increased levels of enzymes regulating DNA methylation has been reported in human temporal lobe epilepsy (TLE) (7), which may have important effects on gene expression (8). Further, changes in histone acetylation (at promoter elements of *Gria2*, *Bdnf*, *c-fos*, and *Creb*) and altered histone deacetylase (HDAC) activity have been noted after experimental status epilepticus (SE) (9–11) and possibly human TLE (12). Changes in the activity of transcriptional repressor RE1-silencing transcription factor (REST) also play a role in events associated with seizures (13), likely through altered histone modification (14).

Polycomb group (PcG) proteins are a large conserved family of transcriptional repressors (15). Originally described in *Drosophila melanogaster* as key silencers of Hox genes, they assemble as polycomb repressive complexes (PRCs) at the chromatin, regulating its structure and altering transcriptional activity through histone modification and effector recruitment (16–18). Deregulation of developmentally silenced genes through alteration of PcG signaling has been observed in various malignancies (19), while their contribution to lineage specification during neurogenesis is well established (20–22). Despite observations of dynamic PcG activity in postmitotic neurons (23, 24), few studies have addressed the role of PcG-mediated repression in neurological disease, and none in epilepsy. Derepression of PcG targets may be involved in L-DOPA-induced dyskinesia (25) and ischemic excitotoxicity (26). PcG-mediated repression has also been implicated in ischemic tolerance (27). This phenomenon shares many characteristics with epileptic tolerance (28, 29), where brief seizures activate a coordinated response of gene expression changes that render brain tissue refractory to subsequent insults that would otherwise be damaging (28, 30). Protection can be independent of changes to seizure severity during SE (29), likely represents the recruitment of active neuroprotective mechanisms and long-lasting changes in gene expression (28, 30) and is accompanied by a reduction in the number of spontaneous seizures evolving after SE (29).

The molecular mechanisms regulating altered gene expression in epileptic tolerance are not fully understood. Previous work suggested transcription factors such as NFκB (31) and AP1 (32) may drive gene synthesis-dependent tolerance, consistent with observations of wide-ranging divergences in gene transcription (29) and gene methylation (33) between epileptic injury (non-preconditioned animals) and epileptic tolerance (preconditioned animals). Given that epileptic tolerance is associated with a coordinated suppression of excitability- and excitotoxicity-related genes (29) and CpG island, hypermethylation is more common in tolerance that in injury (33), it seems likely that transcriptional repression is also a key modality of epileptic tolerance. Here, we performed an extensive spatio-temporal characterization of PcG transcript expression following SE, comparing responses between non-preconditioned and preconditioned mice.

MATERIALS AND METHODS

ANIMAL PROCEDURES

All animal experiments were carried out in accordance with guidelines outlined in the European Communities Council Directive (86/609/EEC) and the European Union Directive (2010/63/EU). All experimentation was approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland (REC #205) and performed under license from the relevant authority [Department of Health, Dublin, Ireland (license number B100/4423)]. Adult male C57BL/6 mice, aged 6–10 weeks (20–30 g), were obtained from Harlan (UK) and housed in a climate-controlled biomedical facility on a 12 h light/dark cycle with food and water provided *ad libitum*.

Focal-onset SE was induced by intra-amygdala (i.a.) stereotaxic microinjection of kainic acid (KA) as described previously (34). Briefly, mice were anesthetized using isoflurane (5% induction, 1.5–2% maintenance) under normothermic conditions and placed in a stereotaxic frame (Stoelting Co.). A midline scalp incision was made, Bregma located and a craniectomy performed (stereotaxic coordinates: AP = −0.95 mm; L = −2.85 mm). Next, a guide cannula was placed over the dura and the assembly fixed by dental cement. Animals were then removed from the frame and placed in an open-top container that allowed free movement for recordings. Microinjection of KA [3.75 mm subdural depth; 1 μg in 0.2 μL phosphate buffered saline (PBS)] (Sigma-Aldrich, Dublin, Ireland) was carried out in awake mice. Non-seizure control mice received injection of vehicle alone. Mice received lorazepam (6 mg/kg, intraperitoneal, i.p.) 40 min following i.a. injections to curtail seizures, reduce mortality, and restrict cerebral damage. Seizure preconditioning was accomplished by i.p. injection of KA (15 mg/kg) 24 h prior to SE induced by i.a. KA (29). Control and injury (i.e., non-preconditioned) mice were sham-preconditioned with i.p. saline.

Mice were euthanized between 1 and 24 h following i.a. injections. Saline-perfused whole brains were fresh-frozen in 2-methylbutane at −30°C and sectioned on a cryostat (12 μm) prior to immunostaining. Hippocampal subfield microdissection (of CA3-, CA1-, and DG-enriched portions) was carried out as previously described (35). In brief, following bisection of isolated whole brain and removal of the cerebellum and midbrain, the hippocampal structures were separated and removed after placing a curved forceps between the hippocampal fimbria and the lateral ventricle. The isolated tissue was rolled out over the cortex and extraneous tissue was removed, yielding intact whole hippocampus. The hippocampus was microsurgically partitioned along a longitudinal axis using fine curved forceps, with the CA1 located in the superior/posterior partition. Microdissections were immediately stored at −80°C.

GENE EXPRESSION ANALYSES

All tissue was homogenized in Trizol (Qiagen, West Sussex, UK) and RNA was isolated using a standard extraction method. Briefly, following homogenization, chloroform-mediated phase separation and isopropanol-mediated precipitation were carried out. RNA was washed and reconstituted in RNase-free H_2O. All nucleic acid extract concentrations were determined using a NanoDrop 2000 (Thermo Scientific, Reading, UK) prior to reverse transcription (RT). RNA extracts were treated with DNAse1 (Invitrogen, Dublin, Ireland) to eliminate contaminating genomic DNA and normalized to between 500 ng and 1 μg prior to RT. RT was performed using random hexamer primers (Fermentas, York, UK) and Superscript II reverse transcriptase (Invitrogen). Following RT, qPCR was carried out on a Lightcycler 2.0 (Roche, Sussex, UK) using Quantitect SYBR Green PCR kits (Qiagen) and custom designed primers (Primer 3.0, Sigma-Aldrich) for target genes. The following primer sequences were used: *Bmi1*, forward TGTCCAGGTTCACAAAACCA and reverse TGCAACTTCTCCTCGGTCTT; *Ring1a*, forward CCTGGACATGCTGAAGAACA and reverse TCCCGGCTAGGGTAGATTTT; *Ring1b*, forward ACGGACCAAAACCTCTGATG and reverse AGTGGCATTGCCTGAAGTCT; *Ezh2*, forward GGCTAATTGGGACCAAAACA and reverse GAGCCGTCCTTTTTCAGTTG; *Ezh1*, forward CTCAGTGGCAACATGCCTAA and reverse CCCACAAACACAACCAACAG; *Suz12*, forward AGAAAACGAAATCGCGAAGA and reverse CGTTGGTTTCTCCTGTCCAT; *Yy1*, forward TGAGAAAGCATCTGCACACC and reverse CGCAAATTGAAGTCCAGTGA; *Yy2*, forward GCCTCTTTTACGGGCTTTCT and reverse ACCATCGATCTGCTTCTGCT; *Sirt1*, forward GCCTGTTGAGGATTTGGTGT and reverse TAAATTTGGGGGCAATGTTC; β-actin, forward GGGTGTGATGGTGGGAATGG and reverse GGTTGGCCTTAGGGTTCAGG. β-actin was used for the normalization of mRNA expression levels. Of those PcG genes for which transcript variants exist (*Ezh2*, *Suz12*, and *Sirt1* code for two isoforms each), primers targeted regions common to both isoforms. PCR products for *Bmi1*, *Ring1a*, *Ring1b*, *Ezh2*, *Suz12*, and *Yy1* spanned exon–exon junctions, while those for *Ezh1* and *Sirt1* targeted the 3′UTR. The PCR product for *Yy2* (having just one exon) was derived from within the coding sequence. Non-reverse transcribed extracts and non-template reactions were used as negative controls. For assessment of basal transcription levels of PcG subunits, both the ΔCT value (versus the CT value of β-actin) and the $2^{-\Delta\Delta CT}$ (or RQ) value (versus a reference ΔCT value derived from the mean ΔCT value for all PcG transcripts) were plotted. Significant differences between subfields for each PcG subunit were computed using the comparative cycle threshold method ($2^{-\Delta\Delta CT}$, normalized against the average ΔCT value of the CA3). For investigation of SE-induced changes in PcG transcription,

the comparative cycle threshold method was again employed to assess the relative fold change in target transcript levels for each PcG subunit (versus the average ΔCT value of time-matched control samples). In parallel, primer specificity was investigated using Taq Polymerase PCR in a Veriti Thermocycler (Applied BioSystems, Warrington, UK). Amplification products were run in a 2% agarose gel (100 V, 15 min, with 1:10000 ethidium bromide) and imaged in a FujiFilm LAS-3000 (Fuji, Sheffield, UK) under UV (312 nm, 0.125 s exposure). For RT-qPCR analyses comparing control, injury and tolerance, three groups of mice (control, injury, and tolerance, $n = 4$) were used. For those analyses looking at differences in basal PcG expression between transcripts, control mice from across all four sampled timepoints were grouped ($n = 16$), as sham surgery did not alter basal PcG transcription in the hippocampus (Figure S1 in Supplementary Material). These samples (ipsilateral CA3, CA1, and DG) were independent of those used in immunohistochemistry and western blotting analyses.

SUBCELLULAR FRACTIONATION

Subcellular fractionation was undertaken to examine protein localization in nuclear and cytosolic compartments. Ipsilateral hippocampal tissue was pooled (two hippocampal dissections per lysate) and homogenized in M-SHE buffer [210 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH pH 7.4, 1 mM EDTA, 1 mM EGTA, and a protease inhibitor cocktail (P8340, Sigma-Aldrich)]. A nuclear-enriched pellet was isolated by repeated centrifugation and trituration (two spin cycles at 1200 × *g*, 10 min at 4°C); the supernatant, containing a crude mix of mitochondrial, microsomal, and cytoplasmic constituents, was processed as detailed below. Following further trituration and centrifugation (1000 × *g*, 10 min at 4°C), the nuclear fraction was resuspended in TSE buffer (10 mM Tris pH 7.5, 300 mM sucrose, and 1 mM EDTA) with 0.1% NP-40. A pellet bilayer emerged upon further centrifugation (8600 × *g*, 10 min at 4°C), whereby the upper opaque layer was retained and purified through repeated centrifugation. The resulting pellet, representing a nuclear-enriched fraction, was resuspended in lysis buffer and analyzed through SDS-PAGE. A crude cytoplasmic fraction was isolated and purified using repeated high-speed centrifugation steps (two spins at 1200 × *g*, 10 min; two spins 10,000 × *g*, 15 min; one spin 16,000 × *g*, 5 min; at each stage the pellet was discarded and the upper 4/5 of supernatant retained). Fraction quality was determined using immunoblotting against subcellular fraction-specific markers (Lamin A/C, nucleus; GAPDH, cytoplasm).

WESTERN BLOTTING

Western blotting was performed on hippocampal subfield microdissections, whole hippocampus, cortex, and cerebellum. Tissue was homogenized in SDS-lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1% NP-40, pH 8.0) or M-SHE lysis buffer. All lysis buffers included protease and phosphatase inhibitor cocktail (1:100, Cat P8340, Sigma-Aldrich) or individually added volumes of PMSF (1:500), aprotinin (1:1000), leupeptin (1:1000), and vanadate (1:1000). Homogenates were spun (14,000 × *g*, 10 min at 4°C) and the supernatant was retained. Nucleus-containing pellet was also retained and later resuspended in SDS-lysis buffer for certain applications. Protein concentration was determined using the micro BCA protein assay (Pierce, Rockford, IL, USA). Lysates were boiled at 95°C in gel-loading buffer and separated by SDS-PAGE (4/6–15%), before being electroporated onto nitrocellulose or PVDF membranes. Membranes were incubated overnight at 4°C with primary antibodies against: trimethyl-H3K27 (1:500), EZH2 (1:500), RING1A (1:1000), RING1B (1:1000), BMI1 (1:400), SUZ12 (1:1000), and Lamin A/C (1:1000), all rabbit polyclonal (Cell Signaling Technology, Danvers, MA, USA). Band visualization was obtained through incubation with secondary horseradish peroxidase-conjugated antibodies (20°C, Jackson Immuno-Research, Suffolk, UK), followed by Super Signal West Pico chemiluminescent substrate (Pierce). Images were captured using a FujiFilm LAS-4000 (Fuji). Densitometry was performed using ImageJ. Briefly, chemiluminescent density plots for each sample were generated and the area of the region of interest (corresponding to the expected molecular weight) was normalized to the matching loading control (Lamin A/C). For western blotting analyses, ipsilateral hippocampus from three groups of mice (control, injury, and tolerance) were used. For whole cell lysates, $n = 5$ mice per group were assessed. For analysis of nuclear-enriched fractions, $n = 3$ samples were used following pooling of two hippocampal isolates per sample. Animals were from an independent cohort to those used in RT-qPCR and immunohistochemistry analyses.

IMMUNOHISTOCHEMISTRY

Immunostaining was performed on fresh-frozen coronal sections (12 μm) prepared at the level of medial (1.9–2.1 mm posterior of Bregma) hippocampus. Sections were air-dried, fixed in 4% PFA solution, and processed for immunostaining using antibodies [anti-EZH2, anti-Trimethyl-H3K27, anti-NeuN (mouse monoclonal, Millipore, Cork, Ireland)], in 5% goat serum/0.1% Triton-X in PBS. Immunoreactivity was visualized using Alexa Fluor 488 and 568 secondary antibodies (Molecular Probes, OR, USA). Sections were labeled with Hoechst nuclear stain to visualize nuclei. Tissue was mounted in FluorSave (Sigma-Aldrich). All images were captured using a Nikon 2000s epifluorescence microscope. Control mice only ($n = 3$) were used for these analyses. Animals were from an independent cohort to those used in RT-qPCR and western blotting analyses.

STATISTICS

All data and statistical analyses were carried out using Microsoft Excel, Graphpad Prism, and Stata. Comparisons were made using one-way analysis of variance, followed by Tukey *post hoc* testing. Significance was accepted at $P < 0.05$. All data are presented as mean ± SEM.

RESULTS

POLYCOMB REPRESSIVE COMPLEX COMPONENT EXPRESSION IN THE ADULT MOUSE HIPPOCAMPUS

Previous studies have reported expression of particular PcG members within the adult brain (21, 36, 37), but there has not been a comprehensive characterization of the expression of PcG transcripts and proteins in the hippocampus of the adult C57BL/6 mouse. **Figure 1** shows the key genes forming the PRCs. The two major polycomb complexes described, defined by the major catalytic constituent, are PRC1 (containing RING1A and 1B) and

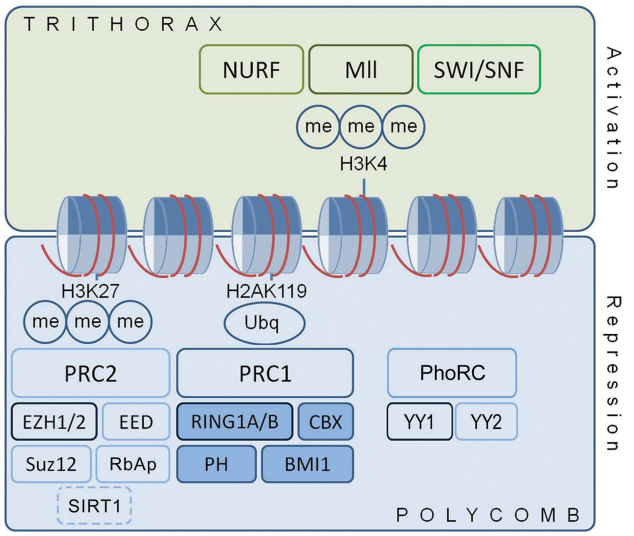

FIGURE 1 | Polycomb and trithorax proteins regulate chromatin structure and gene expression. Cartoon shows polycomb protein complex organization in relation to transcription. PcG proteins regulate chromatin structure through histone tail methylation and other post-translational modifications. PcG proteins often localize about discrete genetic elements with the functionally antagonist trithorax proteins, a diverse group of transcriptional activators, to yield a bivalent, "primed" state that is amenable to rapid alteration in response to signaling events. Ancillary DNA-binding proteins, such as YY1 of the Pho Repressive Complex, aid recruitment of PRC1 and PRC2 to target genes. They also interact with other DNA and chromatin modifiers, including DNMTs, histone deacetylases, histone acetyltransferases, and the Jarid pathway proteins. H2A and H3, histone 2A and histone 3; Me, methyl group; PRC, polycomb repressive complex; Ubq, ubiquitin.

PRC2 (containing EZH2). A third complex, known as the PhoRC (containing Yin Yang 1, YY1), may also be involved (15, 19). Transcriptional repression depends on the coordinated action of these complexes, which may display significant redundancies (38).

In order to generate a comparative profile of basal PcG expression in the hippocampus, we performed RT-qPCR on microdissected hippocampal subfields (CA3-, CA1-, and DG-enriched fractions) and calculated the difference in cycle threshold (ΔCT) value against β-actin in vehicle-injected control mice (used for subsequent investigations of polycomb regulation following SE). The transcripts analyzed were; *Bmi1*, *Ring1a*, and *Ring1b* of PRC1; *Ezh2*, *Ezh1*, *Suz12*, and *Sirtuin 1* (*Sirt1*) of PRC2; and *Yy1* and *Yy2* of the PhoRC; chosen on the basis of their roles in polycomb function, previous implications in neuronal function taken from the literature, and the availability of other resources for further study, including antibodies. It was found that all nine PcG transcripts analyzed were expressed and detectable in the hippocampus. ΔCT values for the expression of these transcripts in control mice were plotted for CA3, CA1, and DG ($n = 16$, **Figure 2A**). Qualitative assessment suggested that *Bmi1* is the highest expressed PcG transcript across all subfields, while *Sirt1* is the lowest. *Yy1* expression was higher than *Yy2* expression in both the CA3 and the DG, and in CA3 and CA1, *Ezh1* expression exceeded *Ezh2* expression. *Ring1a* and *Ring1b* were expressed at similar levels.

We then quantitatively assessed differences in expression of PcG transcripts between subfields. Again, using pooled data across all timepoints in control mice ($n = 16$), we calculated the RQ for ΔΔCT values normalized to the mean ΔCT value for all transcripts and subfields (**Figure 2B**). Normalizing to single ΔCT value ensured that all summary data in this figure were relatable. These analyses suggest that *Bmi1* is expressed significantly higher in the CA3 than in the CA1, which in turn was higher than the DG. Of the other constituents of PRC1, *Ring1a* appears to be higher in CA1 than CA3, while *Ring1b* is lowest in CA1, suggesting subfield-specific regulation of PcG components. Concerning PRC2, only *Suz12* appears differentially expressed between subfields, with significantly lower expression in CA1 when compared to CA3 and DG. Finally, *Yy2* of the PhoRC is higher in DG than in CA3. In order to account for potential effects arising from surgery- and cannula-associated manipulation prior to PBS injection, we plotted ΔCT values for each timepoint ($n = 4$) and confirmed that time elapsed after PBS injection was not likely to contribute to the observed ΔCT values (Figure S1 in Supplementary Material).

Protein levels of six PcGs were investigated by western blotting using subcellular fractions (**Figure 3A**). For three of the six, protein was only observed in the nucleus. Cytosolic expression was apparent for the remaining three proteins, though it appeared significantly lower in comparison to nuclear levels (**Figure 3A**), in keeping with functional roles in regulating transcription. We suspect that the reason some proteins are not detected in whole lysate preparations is that recovery of the nuclear fraction is poor using a general homogenization procedure.

Immunofluorescence microscopy was also performed to assess the spatial expression pattern of Ezh2 and trimethyl-histone 3 lysine 27 (trimethyl-H3K27), the characteristic histone modification catalyzed by PcG proteins. In each case, nuclear-specific immunoreactivity in neuronal populations was observed throughout the hippocampal subregions (CA3, CA1, DG, and hilus), as well as in certain non-specified glial cells (**Figure 3B**), as supported by Hoechst stain and NeuN immunoreactivity.

STATUS EPILEPTICUS INDUCES RAPID, DISTINCT CHANGES IN POLYCOMB TRANSCRIPT LEVELS

Next, a spatio-temporal expression profile of several major PcG transcripts within the hippocampus was established for mice given either i.a. vehicle (control), i.a. KA with sham-preconditioning (injury) or i.a. KA with preconditioning (tolerance). The main site of injury in this model is the CA3 subfield, with minor cell death observed in the CA1 and DG regions. As before (29), animals preconditioned by low-grade seizure activity 24 h earlier displayed approximately 50% less hippocampal damage compared to injury mice after an equally severe episode of SE (data not shown).

Using RT-qPCR, we quantified levels of nine principal PcG members within hippocampal subfields at time points up to 24 h. This included transcripts associated with PRC1 (*Ring1a*, *Ring1b*, and *Bmi1*), PRC2 (*Ezh2*, *Ezh1*, *Suz12*, and *Sirt1*) and the PhoRC (*Yy1* and *Yy2*). RT-qPCR data are summarized in **Figure 4**, with significant up-regulation (green) or down-regulation (red) indicated, as compared to control. Individual subfield data are presented for CA3 (**Figures 5A–I**), CA1 (**Figures 6A–G**), and DG (**Figures 7A–I**). Analyses show that SE induced rapid bidirectional

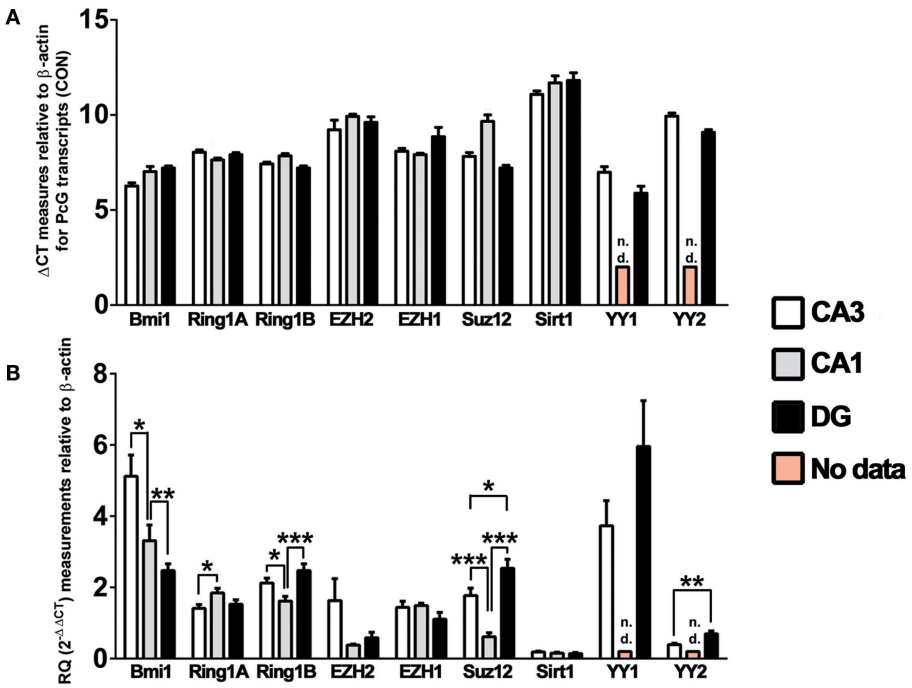

FIGURE 2 | Polycomb group transcripts expression in the mouse hippocampus. Graphs show qRT-PCR data and both the ΔCT and RQ for various core PcG transcripts, relative to β-actin. Measurements of basal PcG transcription, expressed as **(A)** the ΔCT value and **(B)** a derived RQ value ($2^{-\Delta\Delta CT}$ versus the mean ΔCT value for all transcripts and subfields) were made using hippocampus from mice that received intra-amygdala PBS injection ($n = 16$), with analysis performed on microdissected hippocampal fractions enriched for CA3, CA1, and DG. Derived RQ values in **(B)** were statistically appraised by one-way analysis of variance to determine differences between subfields in basal transcription of each PcG subunit. Data expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for $n = 16$. CA, *cornu ammonis*; CON, control; DG, dentate gyrus; n.d., no data.

changes in mRNA levels of various PcG transcripts in CA3, CA1, and DG, with down-regulation the broad response for both injury and tolerance.

Considering changes after SE, there was pronounced down-regulation of *Ring1a* in all subfields, between 4 and 24 h (**Figures 5A**, **6A**, and **7A**). *Ring1b* levels were decreased at 4 h in CA1 and 8 h in all subfields before recovering to baseline at 24 h (**Figures 5B**, **6B**, and **7B**). *Bmi1* was down-regulated in CA3 and CA1 between 4 and 24 h (**Figures 5C** and **6C**).

For PRC2 complex genes, we observed significant down-regulation of *Ezh1* at 4, 8, and 24 h across all subfields (**Figures 5E**, **6E**, and **7E**). In contrast, no instances of *Ezh2* down-regulation were observed (**Figures 5D**, **6D**, and **7D**). Similarly to *Ezh1*, *Suz12* was down-regulated in CA3 at 4, 8, and 24 h, as well as at 4 h in CA1 and 24 h in DG (**Figures 5F**, **6F**, and **7F**). *Sirt1* down-regulation was confined to CA3 at 4 h (**Figure 5G**). Finally, *Yy2* of the PhoRC was decreased at 4 and 8 h post-SE in the CA3 only (**Figure 5I**). In general, down-regulation seemed more prevalent in the CA3, but was seen in DG and CA1 at later timepoints between 4 and 24 h (**Figure 4**). However, down-regulation of *Sirt1* and *Yy2* was specific to the CA3 at all timepoints (**Figure 4**). The number of down-regulation events in the CA3/CA1/DG was 1/0/0 (1 h), 11/9/0 (4 h), 7/6/3 (8 h), and 8/4/6 (24 h), respectively, suggesting that the extent of down-regulation peaked at 4 h in the CA3 and CA1, but was greatest at 24 h in the DG. In contrast, up-regulation was seen to occur dominantly at 1 h (12 of 14 analyzed), and was generally restricted to the CA3 (9 of 12 such events). Levels of *Ring1b* (**Figure 5A**) and *Bmi1* (**Figure 5C**) of PRC1 were increased at 1 h, while up-regulation of *Ezh2* (**Figures 5D**, **6D**, and **7D**), *Suz12* (**Figures 5F** and **6F**), and *Sirt1* (**Figure 7G**) of PRC2 was observed. *Yy1* and *Yy2*, of the PhoRC, were also seen to be significantly higher at 1 h (**Figures 5H,I**). With the exception of *Ezh2*, no PcG transcript was increased at 4 h or later following SE.

DIFFERENTIAL EXPRESSION OF PcG GENES IN EPILEPTIC TOLERANCE

Seizure preconditioning had relatively modest effects on SE-induced changes in PcG transcript expression with notable exceptions. For transcripts of PRC1 components, decreases were more associated with tolerance than injury. *Ring1b* down-regulation at 8 h was hippocampus-wide in tolerance but restricted to the CA3 in injury (**Figures 5B**, **6B**, and **7B**). *Ring1b* up-regulation at 1 h, meanwhile, was injury-specific (**Figure 5B**), and further, there was tolerance-specific down-regulation of *Bmi1* at 4 h (CA3, **Figure 5C**) and 8 h (CA1, **Figure 6C**). Notably, there was no instance of injury-specific down-regulation or tolerance-specific up-regulation for transcripts of the PRC1. Conversely, down-regulation of *Suz12* (of PRC2) was more associated with injury.

Suz12 up-regulation was seen in the CA3 and CA1 of tolerance mice at 1 h but was restricted to CA3 in injury and at lower levels than seen in tolerance (**Figures 5F** and **6F**). Down-regulation of *Suz12* at 4 h (CA1, **Figure 6F**) and 24 h (CA3, **Figure 5F**)

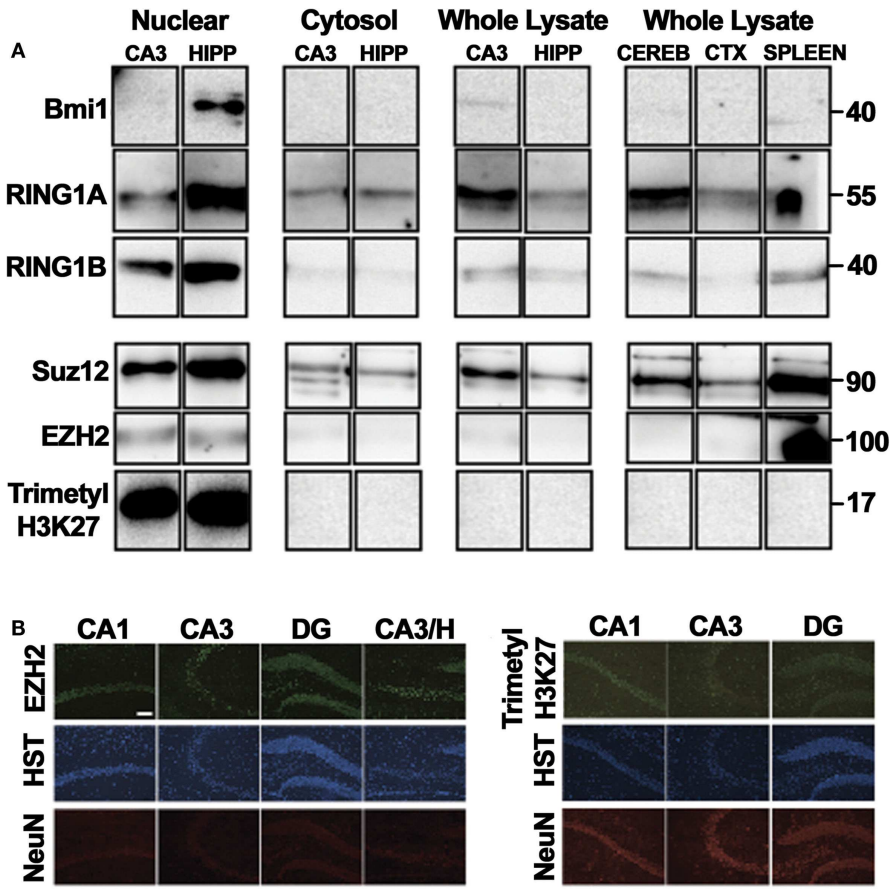

FIGURE 3 | Principal polycomb group proteins in the mouse hippocampus. Western blot depictions here were obtained following multiple exposures. For nuclear and cytosolic fractionation, either three CA3 tissue samples or two hippocampal tissue samples were pooled and run numerous times for each immunoblot. For analysis of whole lysates, lysates were from a single animal. Each column represents one lysate, with each row representing an independent immunoblot and exposure. **(A)** Western blotting was performed on whole cell lysates of the CA3, whole hippocampus, cerebral cortex, cerebellum, and spleen as well as cytosolic and nuclear fractions of the CA3 and the whole hippocampus to confirm the expression of PcG proteins in the adult brain. Observed molecular weights based on the migration of a protein ladder are given (right). **(B)** The neuronal expression of the polycomb repressive complex 2 (PRC2) catalytic subunit EZH2 was validated by immunofluorescence, with a similar pattern of expression observed for the PRC2-associated epigenetic histone modification, trimethylation of lysine27 in histone 3. CA, *cornu ammonis*; CEREB, cerebellum; CTX, cortex; DG, dentate gyrus; H, hilus; trimethyl-H3K27, trimethylated lysine 27 of histone 3; HIPP, hippocampus; HST, Hoechst. Scale, 50 μm.

was injury-specific. There were also minor differences seen with *Sirt1* and *Yy2* (see **Figure 4**). Finally, *Ezh2* was seen to increase in both seizure groups in a time- and subfield-specific manner (**Figures 5D**, **6D**, and **7D**); at 1 h, up-regulation was specific to the DG in injury, but was restricted to the CA3 in tolerance. *Ezh2* was also increased at 8 and 24 h, in the CA1 of injury mice (8 h only, **Figure 6D**) and in the DG of seizure-tolerant mice (24 h only, **Figure 7D**). Interestingly, *Ezh2* up-regulation in the CA3 at 1 h in tolerance was coupled to a tolerance-specific down-regulation of *Ezh1* in CA3 at 1 h (**Figure 5E**).

EXPRESSION OF INDIVIDUAL PRC COMPONENTS AFTER STATUS EPILEPTICUS REFLECTS COMPLEX-WIDE EFFECTS IN THE HIPPOCAMPUS

To determine if down-regulation of PcG transcripts aligned with those associated with the same PRC, the mean RQ per complex was calculated using RQ scores for each of the PRC constituents analyzed, across the CA3, CA1, and DG hippocampal subfields. These scores revealed that expression of individual PRC constituents predicts complex-wide effects in the hippocampus. Following transient up-regulation at 1 h following SE, scores for PRC1, PRC2, and PhoRC demonstrate coordinated down-regulation of polycomb transcription from 4 h onward, with some divergence between injury and tolerance (summarized in **Figure 8A**; see also Figure S2 in Supplementary Material). As with individual components, down-regulation of PRC1 was more extensive in tolerance than with injury. For instance, in the CA3, there was a significant increase in PRC1 scores in injury at 1 h ($P < 0.01$, compared to control, **Figure 8A**; also Figures S2A,B in Supplementary Material) and a tolerance-specific decrease in PRC1 score at 4 h in tolerance ($P < 0.05$, compared to control, injury). PRC1 down-regulation also appeared to be more widespread

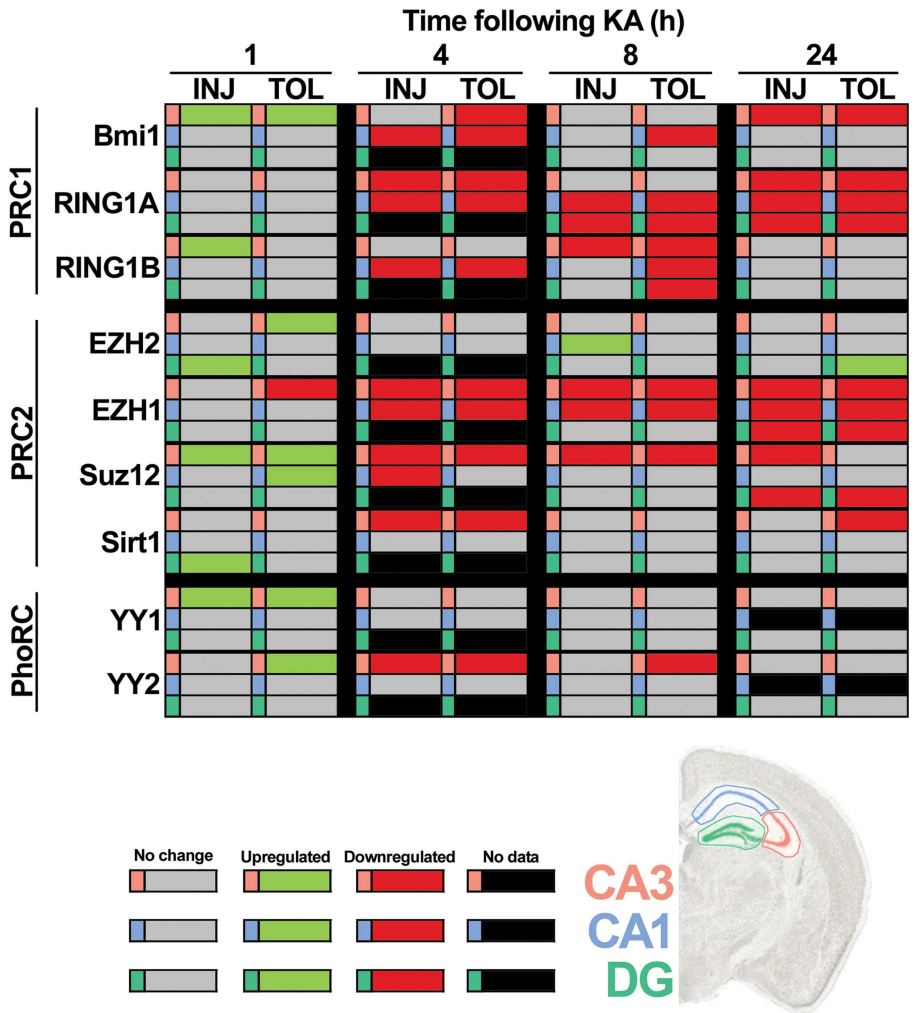

FIGURE 4 | Focal-onset SE causes rapid changes in polycomb transcript levels in the hippocampus of sham-preconditioned and seizure-tolerant mice. Relative polycomb transcript expression, as compared to control and corrected to β-actin, was determined using qRT-PCR. All significant differences are indicated in green (up-regulation) or red (down-regulation), as determined versus control following one-way analysis of variance with Tukey *post hoc* test. Gray cells indicate no significant difference versus control samples and black cells indicate that no data are available for that particular cell. Polycomb members/complexes are indicated and tabulated by timepoint (1, 4, 8, and 24 h), experimental group (epileptic injury and tolerance), and hippocampal subfield (CA3, CA1, and DG). CA, *cornu ammonis*; DG, dentate gyrus; INJ, injury; KA, kainic acid; PRC, polycomb repressive complex; TOL, tolerance.

throughout the hippocampus when considering tolerance, with numerous decreases at 4 and 8 h following SE (**Figure 8A**). Conversely, PRC2 down-regulation was more prevalent at 4 and 8 h after SE in injury groups, with injury-specific decreases in PRC2 score in CA1 (4 h, $P < 0.05$, compared to control, **Figure 8A**; also Figures S2C,D in Supplementary Material) and CA3 (8 h, $P < 0.05$, compared to control, **Figure 8A**; also Figures S2A,B in Supplementary Material). As with individual constituents, changes in PcG transcription in the DG were minimal before 24 h (**Figure 8A**; also Figures S2E,F in Supplementary Material). In sum, it was apparent that divergences between injury and tolerance concerned either up-regulation (at 1 h) or diminished down-regulation (between 4 and 24 h) of specific PRCs, where increased PRC1 score was associated with injury and increased PRC2 score was associated with tolerance. There were no observed divergences between injury and tolerance in the DG at 8 or 24 h (**Figure 8A**; also Figures S2E,F in Supplementary Material). A full breakdown of significance is shown in Figures S2B,D,F in Supplementary Material.

Given this association of changes in PRC component transcription with either injury or tolerance, we analyzed protein levels at 24 h following SE, when preceding changes in mRNA expression might be expected to manifest. Through western blotting and subcellular fractionation, SE-mediated effects on PcG protein expression were assessed in injury and tolerance mice. Assessment included the use of whole cell protein lysates as well as nuclear fractions, isolated from whole hippocampus. Elution of nuclear-specific Lamin A/C is shown (**Figure 8E**). For nuclear fractionation, hippocampal samples were pooled (two hippocampal samples per lysate). We assessed protein levels of RING1A and RING1B of PRC1, EZH2, and SUZ12 of PRC2 and

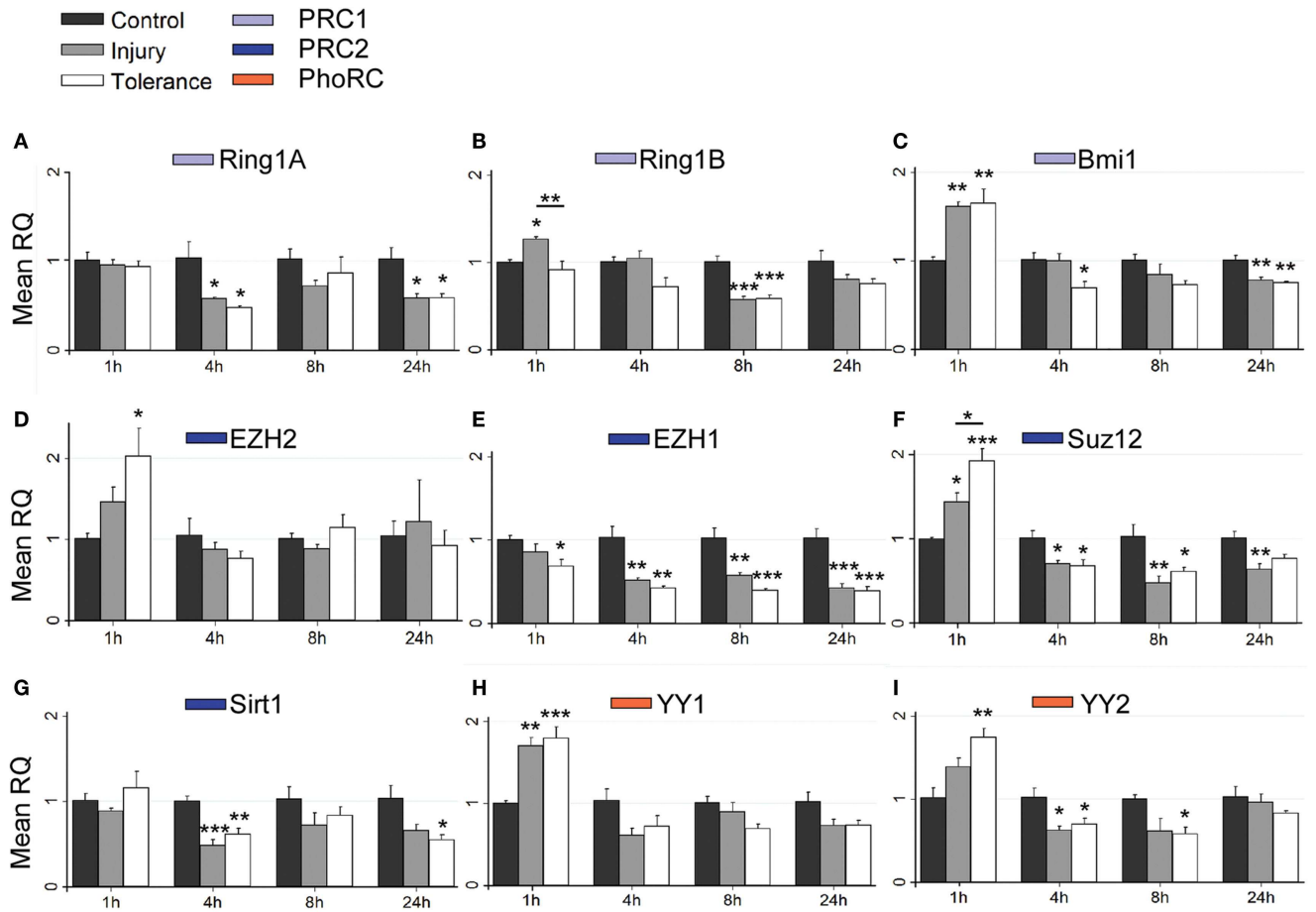

FIGURE 5 | Focal-onset SE causes rapid changes in polycomb transcript levels in the CA3 of sham-preconditioned and seizure-tolerant mice. **(A–I)** Relative fold expression of various PcG mRNA transcripts was measured in the CA3 using qRT-PCR in vehicle-injected (control), sham-preconditioned (injury), and seizure-tolerant (tolerance) mice at 1, 4, 8, and 24 h following SE. Expression was corrected to β-actin and normalized to control ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (versus control unless indicated), one-way analysis of variance with Tukey *post hoc* test. PRC, polycomb repressive complex.

trimethyl (Lys27)-Histone 3, the polycomb-associated chromatin mark (**Figures 8B–F**). In whole cell lysates, RING1A (PRC1) levels were higher in injury than tolerance at 24 h in the hippocampus ($P < 0.05$, **Figures 8B,F**). In nuclear-enriched fractions, there were no significant differences in RING1B (PRC1), EZH2 (PRC2), or SUZ12 (PRC2) protein levels (**Figures 8B,C,F**). There was an apparent threefold increase in RING1B levels in injury compared to tolerance, as well as a twofold increase in both EZH2 and SUZ12 levels in tolerance compared in injury, a PRC-specific alignment that was also seen in transcript analysis. Notably, we did not observe an associated loss of trimethylation of lysine 27 in histone 3 in nuclear-enriched hippocampal lysates at 24 h after SE, suggesting that acute global loss of this modification is not a feature of SE (**Figures 8D,F**).

DISCUSSION

Polycomb group proteins are a conserved family of transcriptional silencers which were recently linked to the neuroprotection observed in ischemic tolerance (27). Here, we report the effect of prolonged seizures on PcG expression in the adult mouse hippocampus and how this is altered in the setting of epileptic tolerance. The present study also comprises the first detailed comparison of the relative expression of the various transcripts of the PRCs between hippocampal subfields. We found that in injury-group mice, SE induced rapid, bidirectional changes in levels of PcG family gene expression. We found an almost uniform pattern of rapid up-regulation (1 h) followed by later down-regulation (4 h and thereafter) of various transcripts. Broad down-regulation was seen to continue to 24 h post-SE which may be relevant to gene expression patterns beyond the initial period of cell death in the model (34). The amount of time elapsed after SE is the major determinant of the changes, though there are region-specific changes evident. Alterations in PcG transcript levels were more extensive and more rapid in the CA3 and CA1 than the DG, suggesting that SE-elicited gene expression responses are more pronounced in those populations most vulnerable to damage in this model (29). This is predictable, since transcription would be expected to be reduced in damaged cells. Nevertheless, the finding that

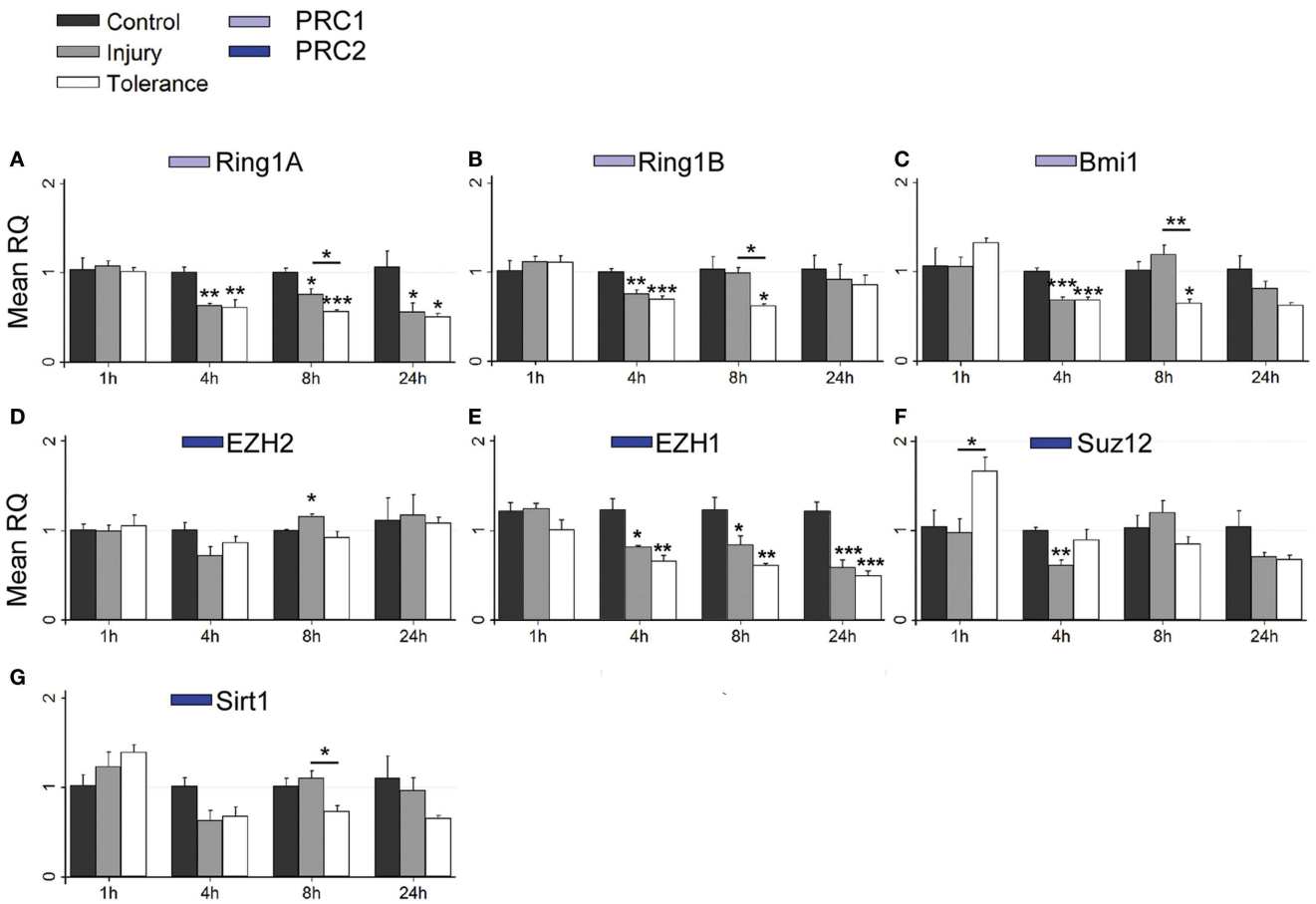

FIGURE 6 | Focal-onset SE causes rapid changes in polycomb transcript levels in the CA1 of sham-preconditioned and seizure-tolerant mice. **(A–G)** Relative fold expression of various PcG mRNA transcripts was measured in the CA1 using qRT-PCR in vehicle-injected (control), sham-preconditioned (injury), and seizure-tolerant (tolerance) mice at 1, 4, 8, and 24 h following SE. Expression was corrected to β-actin and normalized to control ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (versus control unless indicated), one-way analysis of variance with Tukey *post hoc* test. PRC, polycomb repressive complex.

all populations displayed reductions in transcription suggests the prolonged seizures, rather than cell death *per se*, is responsible for many changes.

Spatio-temporal-specific changes in gene expression may be a common and important feature of SE. In a comprehensive microarray study conducted across hippocampal subfields and several timepoints, subfield represented a more discriminatory parameter than seizure frequency when comparing changes in gene expression following SE (39). Distinct functional clusters were also associated with each of the sampled timepoints, representing acute, latent, and chronic stages of evoked epilepsy (39). Other microarray studies on individual subfields of the hippocampus including the CA3 (40), the CA1 (41), and the DG (42) recapitulate this general trend, with each study noting clustered expression changes in largely non-overlapping functional groups. In terms of the well-characterized PcG proteins YY1 and SIRT1, such temporal and regional dynamics in expression are also evident. Region-specific expression of YY1 has been reported to underlie differential expression of adenosine A2A receptors in the brain (43). YY1 and its binding partners have also demonstrated stimulus-specific patterns of expression and activity following electroconvulsive shock, KA, and PTZ (44–46). Evoked changes in SIRT1 expression, meanwhile, were noted *in vitro* and *in vivo*, following induced epileptiform activity, SE, and electroconvulsive shock (47–49). Changes to YY1 and SIRT1 were bidirectional and evolved over time, depending on the model and stimulus.

The present study did not explore whether altered PcG expression influences the levels of genes under the control of PRCs. This will require further studies targeting specific PcG genes or entire PRC complexes in the model. We can, however, postulate mechanisms by which altered PcG expression could influence cellular outcomes after SE. In addition to direct control of genes regulating apoptosis, various PcG proteins have been observed to directly bind and modify p53, a protein implicated in seizures and damage after SE. BMI1, for instance, can bind p53 with PRC1 subunits RING1A and RING1B, leading to ubiquitination and degradation of p53 (50). This activity was directly attributed to RING1B, an E3 ubiquitin ligase, where RING1B knockdown

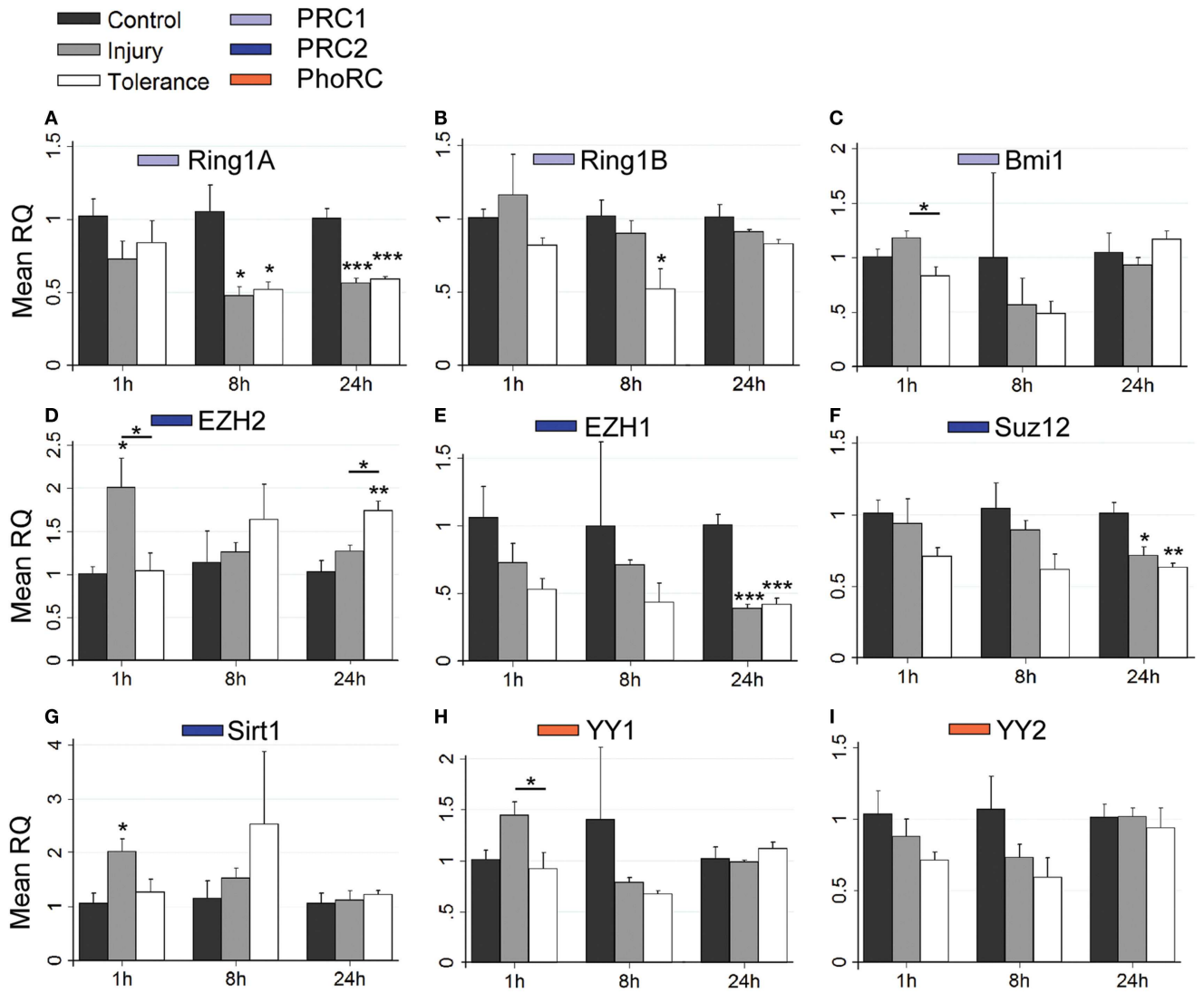

FIGURE 7 | Focal-onset SE causes rapid changes in polycomb transcript levels in the DG of sham-preconditioned and seizure-tolerant mice. **(A–I)** Relative fold expression of various PcG mRNA transcripts was measured in the DG using qRT-PCR in vehicle-injected (control), sham-preconditioned (injury), and seizure-tolerant (tolerance) mice at 1, 8, and 24 h following SE. Expression was corrected to β-actin and normalized to control ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (versus control unless indicated), one-way analysis of variance with Tukey *post hoc* test. PRC, polycomb repressive complex.

sensitized cells to apoptosis (51). BMI1 deficiency is associated with p53 accumulation, Bcl-2 down-regulation, and increased hippocampal apoptosis (52). Sirt1 inhibition of p53 function is well documented (53), while EZH2 has been noted to indirectly modulate p53 signaling and depletion of EZH2 leads to increased apoptosis in response to DNA damage (54). PcG repression has also been linked to REST function. Transcriptional regulation by REST has been implicated in epileptogenesis after SE (13) and REST has been observed to bind and recruit both PRC1 and PRC2 to certain REST target genes (55). Indeed, REST depletion causes loss of trimethyl-H3K27 in murine stem cells, and transgenic insertion of promoter fragments containing REST-binding elements can recruit trimethyl-H3K27 (56).

Changes to PcG expression may influence axonal and dendritic structures, which could also be important after SE and for later development of epilepsy. EZH2 recruitment to the *Bdnf* locus has been implicated in nominal restriction of dendritic arborization and activity-induced BDNF expression was linked to derepression of the *Bdnf* locus following reduced EZH2 binding (24). EZH2 is crucial to the regulation of hippocampal neurogenesis and axonal guidance, with downstream deficits in memory function following conditional knockout (22, 23). Neurogenesis is enhanced following seizures and may contribute to seizure-related pathologies (57) and polycomb as well as trithorax group proteins have been implicated in neurogenic processes (20). Together, the wide-ranging reductions in PcG expression that we observed

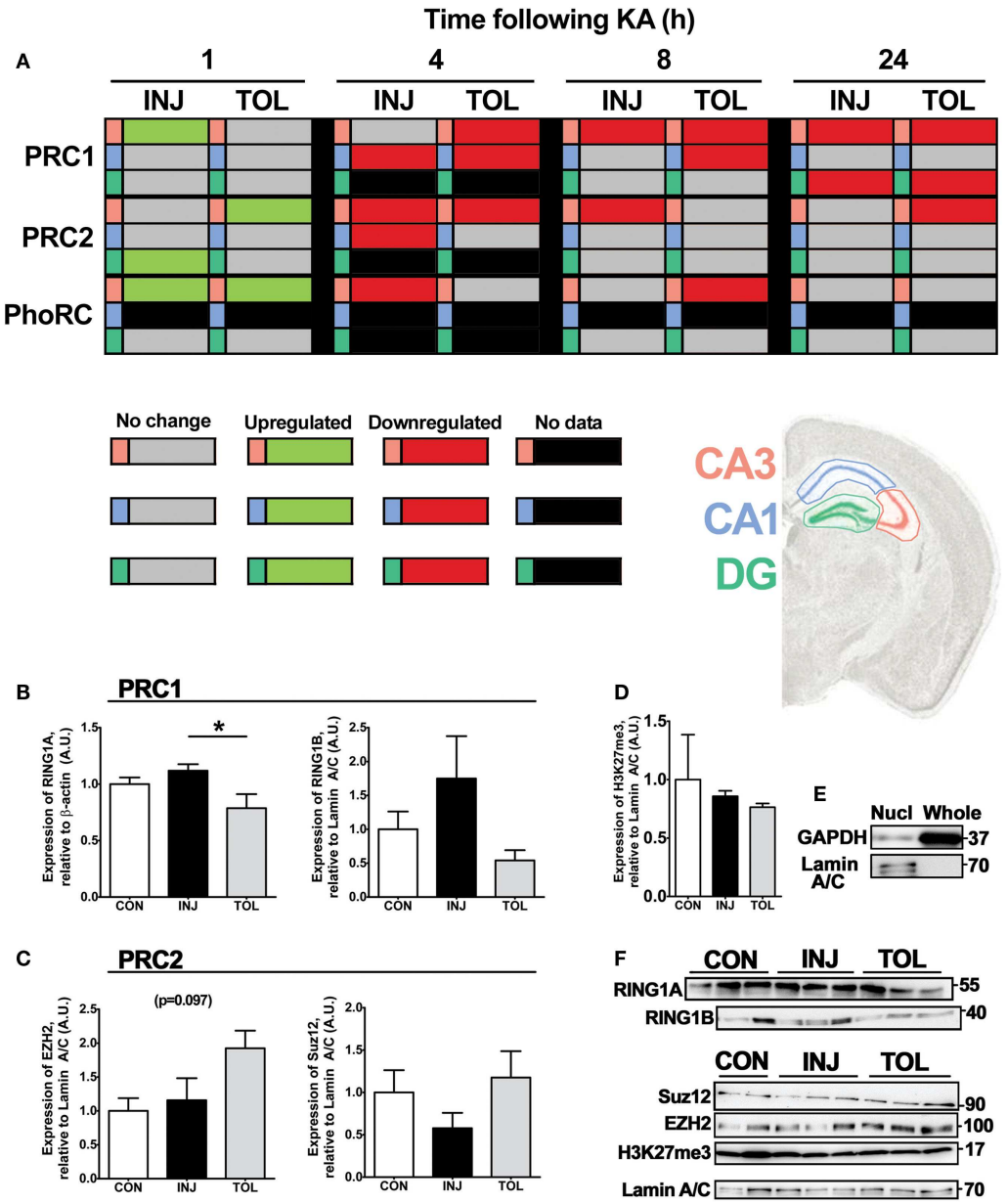

FIGURE 8 | Expression of individual PRC constituents following SE predicts complex-wide effects in the hippocampus. **(A)** Relative polycomb repressive complex score was calculated using the mean RQ value for member constituents determined using qRT-PCR, as compared to control and corrected to β-actin. All significant differences are indicated in green (up-regulation) or red (down-regulation) as determined versus control following one-way analysis of variance with Tukey *post hoc* test. Gray cells indicate no significant difference versus control samples and black cells indicate that no data are available for that particular cell. Polycomb complexes are indicated and tabulated by timepoint (1, 4, 8, and 24 h), experimental group (epileptic injury and tolerance), and hippocampal subfield (CA3, CA1, and DG). **(B–F)** Semi-quantification of **(B)** RING1A and RING1B, **(C)** EZH2 and SUZ12, and **(D)** trimethyl-H3K27 was performed using western blotting, relative to the endogenous controls β-actin (for RING1A) or Lamin A/C (all others), in whole or nuclear-fractionated lysates of the hippocampus ($n = 3$–5). **(E)** Markers of cellular compartments Lamin A/C and GAPDH were eluted for nuclear-enriched and whole cell lysates. Representative blots used in semi-quantification analysis are depicted in **(F)**, $n = 1$ per lane. Observed molecular weights based on the migration of a protein ladder are shown (right). Data expressed as mean ± SEM in **(B–D)**. $^*P < 0.05$, one-way analysis of variance. CA, *cornu ammonis*; CON, control; DG, dentate gyrus; H3K27me3, trimethylated lysine 27 of histone 3; INJ, injury; KA, kainic acid; Nucl, nuclear-enriched lysate; PRC, polycomb repressive complex; TOL, tolerance.

following SE suggest functional studies could focus on the potential relationship between PcGs and p53 accumulation, alterations in REST, and changes in neuronal morphology or neurogenesis.

A major focus of the present study was the differential expression of PcG family genes in epileptic tolerance. A damage-tolerant state was generated by exposing mice to seizure preconditioning

a day before SE. Tolerant mice display only ~50% of the normal damage to the CA3 subfield after SE and previous work has shown the neuroprotection is associated with widespread transcriptional silencing (29). More recently, genome-wide methylation analysis revealed a small number of genes were differentially hypermethylated in tolerance compared to injury (33), suggesting epigenetic processes are important in the protection. PcG proteins, as transcriptional repressors, may contribute to this tolerant phenotype. Indeed, BMI1 and Scmh1 have been implicated as contributing factors in ischemic tolerance through their repressive action on potassium channel proteins (27). Accordingly, a reasonable prediction would have been to see up-regulation of PcG genes in epileptic tolerance. In fact, we generally found down-regulation of PcG transcripts in both injury and tolerance. However, the extent of down-regulation between PRC1 and PRC2 diverged for injury and tolerance. The number of down-regulation events across hippocampal subfields and timepoints was greater for injury than tolerance for PRC2. Changes in protein expression of RING1A and RING1B (of PRC1) and EZH2 and SUZ12 (of PRC2) were concordant with this observation. The possibility of divergences in activity of PRC1 and PRC2 between injury and tolerance is interesting. Although polycomb complexes are generally recruited together in a stepwise fashion, it has been shown that PRC2 can occupy certain genomic sites independently of PRC1 (58), while PRC1 has been shown to be capable of chromatin binding in the absence of PRC2 (59). Given that bivalent domains with recruitment of both complexes may mediate stricter transcriptional silencing (58) and that REST can differentially recruit PRC1 in a context-specific manner (60), SE-induced changes in the balance of PRC1 and PRC2 are likely to have significant effects on transcriptional regulation. This is borne out by observations that differential expression of PRC1 subunit BMI1 and PRC2 subunit EZH2 results in repression of different targets (61).

Given its putative role in the regulation of neuronal morphology and cell death pathways, the apparent increase of EZH2 in tolerance at 24 h is particularly intriguing. We observed that, in control mice, *Ezh1* mRNA expression exceeds that of *Ezh2*, particularly in the CA3 and CA1, in keeping with previous profiles of PcG expression in non-proliferative tissues (62). *Ezh1* expression is profoundly decreased throughout the hippocampus following SE, regardless of preconditioning. Does tolerance-specific up-regulation of *Ezh2* at 24 h following SE offset this *Ezh1* deficiency? EZH1 and EZH2 are homologous members of the PRC2 that may have partially overlapping roles and redundancy. For instance, global K3K27me3 can be preserved by EZH1-containing PRC2 in $EZH2^{-/-}$ cells, where subsequent depletion of EZH1 leads to translational derepression in these cells (63). Redundancy in EZH1 and EZH2 is supported by other double knockout studies (64), but there is also conflicting data suggesting that EZH2 deficiency is sufficient to destabilize global trimethyl-H3K27 (62). Notably, none of these studies have reported extensive demethylation of H3K27 following EZH1 depletion alone, in keeping with our observations that trimethyl-H3K27 levels are unchanged after SE. However, some sub-functionalization of these proteins is apparent. EZH1 may only target a subset of EZH2 genes and has inferior methyltransferase activity (62). It has been suggested that PRC2-EZH2 is more active in *de novo* H3K27 di- and trimethylation, while PRC2-EZH1 is involved in maintenance of the mark and transcriptional repression (65). Further, EZH1 and EZH2 have different chromatin blinding and compaction properties. Our transcript and protein analysis suggests that tolerance may be associated with an increased retention of EZH2 following widespread loss of *Ezh1* expression. As such, changes to PRC2–EZH2 activity in both injury and tolerance represents an ideal next step in delineating possible transcriptional regulatory changes arising after SE. The lack of destabilization in trimethyl-H3K27 at 24 h after SE suggests that such changes, if any, may be restricted to a small number of sites.

The mechanisms of PcG recruitment to distinct genomic elements are also unclear (15) and it is not yet known whether PRE are widespread in mammalian genomes (15). However, a recent study confirmed that rapid derepression of PcG target genes can occur following a pathological insult in the brain and confirmed several neuronal PcG targets (25). Subsequent investigations involving chromatin immunoprecipitation and proteomic analyses represent a viable means of further delineating the structure, interactions, and targets of PcG complexes in the adult brain.

In summary, we have characterized regional and temporal changes in PcG expression in the adult mouse hippocampus. Our data show that SE produces immediate changes in the transcription of polycomb genes, with divergence noted for preconditioned animals. Functional data are now required to link the observed gene changes for PcG proteins to cell injury and other outcomes after SE in injury and tolerance.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fneur.2015.00046/abstract

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The relationship between truncation and phosphorylation at the C-terminus of tau protein in the paired helical filaments of Alzheimer's disease

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† This article is dedicated to our colleague and friend, Raúl Mena, who sadly passed away on June 11th, 2014.

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We previously demonstrated that, in the early stages of tau processing in Alzheimer's disease, the N-terminal part of the molecule undergoes a characteristic cascade of phosphorylation and progressive misfolding of the proteins resulting in a structural conformation detected by Alz-50. In this immunohistochemical study of AD brain tissue, we have found that C-terminal truncation of tau at Asp-421 was an early event in tau aggregation and analyzed the relationship between phospho-dependent tau epitopes located at the C-terminus with truncation at Glu-391. The aim of this study was to determine whether C-terminal truncation may trigger events leading to the assembly of insoluble PHFs from soluble tau aggregates present in pre-tangle cells. Our findings suggest that there is a complex interaction between phosphorylated and truncated tau species. A model is presented here in which truncated tau protein represents an early neurotoxic species while phosphorylated tau species may provide a neuroprotective role in Alzheimer's disease.

Keywords: tau protein, truncation, neurotoxicity, neurofibrillary tangles, PHFs, tau oligomers, Alzheimer's disease

INTRODUCTION

Tau pathology, a principal hallmark of Alzheimer's disease, is characterized by abnormal hyperphosphorylation and truncation of tau proteins (Wischik et al., 1988a; Goedert et al., 1992; Novak et al., 1993; Mena et al., 1996). Both molecular events are associated with the formation of paired helical filaments (PHFs) and the appearance of the neurofibrillary tangles (NFTs) within the cytoplasm of vulnerable cells (Wischik et al., 1988b; Novak et al., 1993; Mena et al., 1995, 1996; Guillozet-Bongaarts et al., 2005; Luna-Munoz et al., 2007). Although it has been suggested that phosphorylation of tau is associated with PHF assembly, evidence to confirm this has not been substantiated, since these studies have been based upon *in vitro* experiments and animal models rather than Alzheimer's disease tissue (Alonso et al., 1996, 2001). Wischik and colleagues demonstrated that the insoluble PHF consists of a protease-resistant core that retains the characteristic structural features of the intact PHF after Pronase digestion, but lacks a fuzzy outer coat (Wischik et al., 1988a,b; Novak et al., 1993). The tau fragment within the PHF core, corresponds to a sequence of 93–95 amino acids in length that are C-terminally truncated at Glu-391 (Wischik et al., 1988a; Novak et al., 1993). PHFs can be distinguished by their differential solubility. While sarkosyl-soluble PHFs are mainly constituted of tau protein in a hyperphosphorylated state, insoluble PHF fractions from Alzheimer's disease brains are Pronase resistant and phosphorylated tau accounts for no more than 15% of the content of such PHFs (Wischik et al., 1995). Furthermore, rather than promoting the aggregation of tau, phosphorylation of tau is inhibitory for aggregation *in vitro* (Schneider et al., 1999).

The monoclonal antibody (mAb) 423 specifically identifies the C-terminal truncation of tau at Glu-391 in Alzheimer's disease brains (Novak, 1994). We have demonstrated that mAb 423-immunoreactive deposits are present in both intra- and extra-cellular NFTs (Mena et al., 1991, 1995; Garcia-Sierra et al., 2003; Luna-Munoz et al., 2007). Furthermore, C-terminally truncated tau protein has a greater affinity for binding full-length tau molecules (Abraha et al., 2000; Berry et al., 2003) and core PHF-tau over-expressed in transfected COS cells induces apoptosis (Fasulo et al., 1998).

We have combined the techniques of double- and triple-labeled immunohistochemistry with confocal microscopy to examine the molecular changes that arise in pre-tangle cells (Luna-Munoz et al., 2005, 2007). Such cells display the majority of epitopes present in PHFs but in the absence of cytoplasmic fibrillary structures. Examining pre-tangle cells has enabled us to study stages of phosphorylation along the N-portion of tau protein in Alzheimer's disease (Luna-Munoz et al., 2007). In particular, we used four phospho-dependent tau antibodies, namely p-231, TG-3, AT8, and AT100. In addition to mAb 423, TauC3 was used to identify C-terminal truncation of the protein at Asp-421 (Gamblin et al., 2003c; Guillozet-Bongaarts et al., 2005; Luna-Munoz et al., 2007; Basurto-Islas et al., 2008). Early stages of tau processing in pre-tangle cells are characterized by a specific cascade of events in which phosphorylation of the N-terminal domain of tau plays a major role. In addition, we found that Asp-421 truncation at the C-terminus of tau molecule is also present at early stages of tau aggregation (Luna-Munoz et al., 2007).

In this study, we have analyzed the relationship between phosphorylation and truncation in pre-tangle cells. Under normal conditions, truncation at Glu-391 is not detected in aggregates of tau within the cytoplasm of pre-tangle cells. We discuss the consequences of the presence of a highly toxic PHF core in the early stages of tau processing in Alzheimer's disease and discuss how phosphorylated tau may act in a protective manner against the toxicity of the PHF core.

MATERIALS AND METHODS

BRAIN TISSUE

Brain tissue from six Alzheimer's disease patients (**Table 1**) was examined in this study (ages, 47–93 years; mean 78.33 years; 2–6 h post-mortem delays) All tissues were obtained from the Brain Bank-LaNSE CINVESTAV-IPN, Mexico in accordance with the institutional bioethics guidelines. The diagnosis of Alzheimer's disease was obtained using the NINCDS-ADRDA group criteria (McKhann et al., 1984), and all samples were Braak stages 5–6. Blocks of hippocampus and adjacent entorhinal cortex were fixed by immersion in a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4°C for 7 days.

ANTIBODIES

The characteristics of the antibodies used in this study are listed in **Table 2** and the location of their epitopes shown in the schematic representation of tau protein in **Figure 1**.

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Free-floating sections (50 μm-thick) were exposed to Pronase (0.05% diluted in PBS; Type XIV, Sigma, St Louis, Mo) at 37°C for 30 min, washed with PBS then formic acid (98–100%; Merck, Germany) at room temperature for 3 min. Sections were blocked with a solution of 0.2% IgG-free albumin (Sigma Chemical Co.) in PBS for 20 min at room temperature. Sections were then incubated with the primary antibodies pS396 and mAb 423 (both are IgG subtypes) overnight at 4°C, and then with FITC-tagged goat-anti-rabbit IgG secondary antibody and TRITC-tagged goat-anti-Mouse IgGγ secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). PBS containing 0.2% Triton X-100 (Sigma Chemical Co.) solution was used in all of the immunolabeling steps for 1 h. Primary antibodies were used at the dilutions indicated in **Table 2**).

Triple immunolabeling was performed using different combinations. (a) Alz-50 with AD2 and TG3. The corresponding secondary antibodies used were FITC-tagged goat-anti-rabbit IgG, TRITC-tagged goat-anti-mouse IgGγ, and Cy5-tagged goat-anti-mouse IgMμ, respectively. (b) For the combination of TauC3, pS396, and TG-3, FITC-tagged goat-anti-mouse IgGγ, TRITC-tagged goat-anti-rabbit IgG, and Cy5-tagged goat-anti-mouse IgMμ where used. (c) Some sections were immunolabeled with TauC3, AD2, and mAb 423 and counterstained with thiazin red (for the staining of β-pleated structures). These three antibodies are considered to detect late stages of tau processing in NFTs (Galván et al., 2001; Garcia-Sierra et al., 2003, 2008). Control sections were included in which incubations with Pronase and formic acid were omitted. Selected sections were counterstained with thiazin red (TR) to differentiate non-fibrillar from fibrillar states of tau aggregates (Mena et al., 1995, 1996).

CONFOCAL MICROSCOPY

Double and triple immunolabeled sections were mounted with anti-quenching media (Vectashield; Vector Laboratories, Burlingame) and viewed through a confocal laser scanning microscope (TCS-SP2, Leica, Heidelberg) using a 100× oil-immersion plan Apochromat objective (NA 1.4). Ten to fifteen consecutive single sections were sequentially scanned at 0.8–1.0 μm intervals for two or three channels throughout the z-axis of the sample. The resultant stack of images was projected and analyzed onto the two-dimensional plane using a pseudocolor display of green (FITC), red

Table 1 | Characteristics of the 6 Brain Samples used in this study.

Case #	Brain area	Sex	Age (years)
1.	Hippocampus CA1	M	80
2.	Hippocampus CA1	F	90
3.	Hippocampus CA1	M	81
4.	Hippocampus CA1	F	47
5.	Hippocampus CA1	F	93
6.	Hippocampus CA1	M	79

All samples were Braak stages 5–6. Tissues were from the LANSE Brain Bank, CINVESTAV, Mexico City.

Table 2 | Tau-specific antibodies.

Antibody	Tau epitope	Species/sub-class	Dilution	References
(A) NON-PHOSPHORYLATED EPITOPES				
Tau-12	N-terminal (residues 9-18)	Mo/IgG	1:1000	(Horowitz et al., 2006)
T46	C-terminal (residues 404-441)	Mo/IgG	1:100	(Preuss et al., 1995)
(B) CONFORMATIONAL EPITOPE				
Alz-50	Conformational epitope (residues 5-15 and 312-322)	Mo/IgM	1:500	(Carmel et al., 1996)
(C) PHOSPHO-DEPENDENT N-TERMINAL EPITOPE				
TG-3	p-Thr-231	Mo/IgM	1:60	(Jicha et al., 1997b)
(D) PHOSPHO-DEPENDENT C-TERMINAL EPITOPES				
pS396	p-Ser-396	Rb/IgG	1:500	(Bramblett et al., 1993)
pS400	p-Ser-400	Rb/IgG	1:100	(Liu et al., 2005)
pS404	p-Ser-404	Rb/IgG	1:100	(Augustinack et al., 2002)
pS409	p-Ser-409	Rb/IgG	1:100	(Jicha et al., 1999b)
AD2	p- Ser-396/p-Ser-404	Mo/IgG	1:500	(Buee-Scherrer et al., 1996)
pS422	p-Ser-422	Rb/IgG	1:1000	(Bussiere et al., 1999)
(E) C-TERMINAL TRUNCATION EPITOPES				
TauC3	C-terminal truncation at Asp-421	Mo/IgG	1:1000	(Gamblin et al., 2003c)
mAb 423	C-terminal truncation at Glu-391	Mo/IgG	1:20	(Novak, 1994)

Abbreviations: Mo, mouse; Rb, rabbit; IgG, immunoglobulin G; IgM, immunoglobulin M.

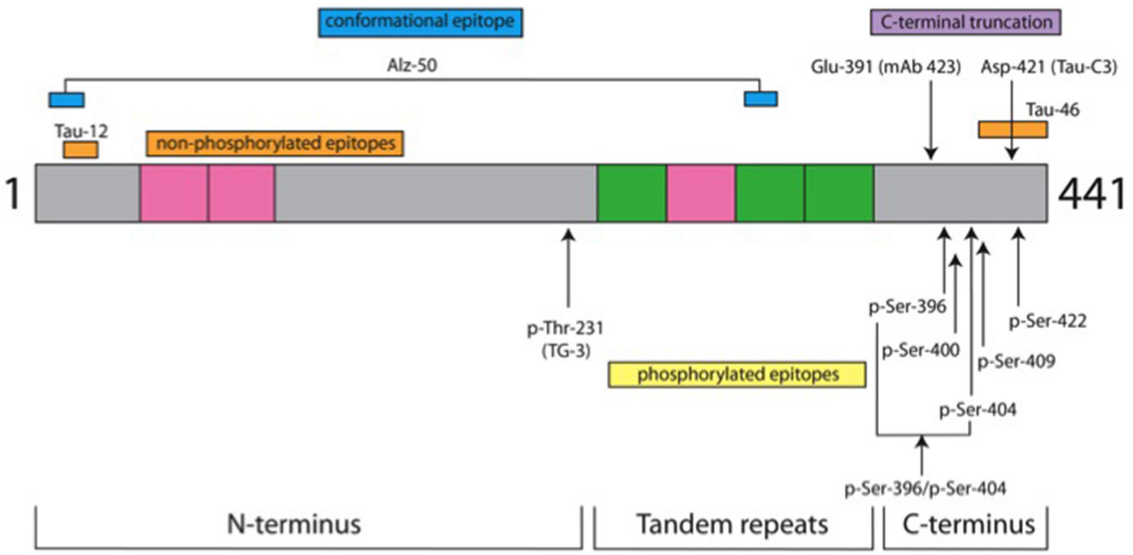

FIGURE 1 | Tau protein domains and location of antibody epitopes. The schematic representation shows three domains of tau protein and the location of epitopes for the antibodies used in this study. It is depicted as the longest tau isoform in the central nervous system of 441 amino acids (grey). Inserts from alternatively spliced exons are denoted in pink. There are two in the N-terminus and one in the tandem repeat domain; the other 3 repeat domains are shown in green. Non-phospho-dependent epitopes are shown above the drawing, whereas phospho-dependent epitopes are indicated below. Details for antibodies are listed in **Table 2**. In green are depicted the repeat domains of tau protein and gray is the tau protein amino acid sequence.

(TRITC) and blue (CY5). Fluorochromes were excited at 488 nm (for FITC), 540 nm (for TRITC) and 650 nm (for CY5).

One hundred images in the areas studied for each combination of antibodies were analyzed (**Table 3**). From each field in 100× oil objective, we quantified the number of NFTs in both channels and the percentage of colocalization of the signal for all channels. The fields were randomly chosen within the sections. Colocalization analysis was carried out by quantifying the total number of neurons positive for tau protein phosphorylated at S396 and the percentage value of neurons positive to other tau markers, including: Tau-12, T46, TauC3, AD2, and mAb 423, were determined to make 100 visual fields at 100×. The data was normalized to a percentage measure of the number of tau pS396-positive cells that were simultaneously recognized by other tau epitope analyzed.

Table 3 | Proportion of NFTs co-labeled with pS396 and other tau antibodies.

Antibody combination	Co-localization of antibodies in NFTs (%)*
pS396/Tau-12	7.0
pS396/T46	6.6
pS396/TauC3	47.0
pS396/AD2	83.8
pS396/mAb 423	85.7

**Co-localization measured for 100 fields at 100× magnification.*

RESULTS

DOUBLE IMMUNOLABELING OF NFTs WITH pS396 AND AD2, COUNTERSTAINED WITH THIAZIN RED

It has been shown that the antibodies AD2 and pS396, having similar epitopes, display a similar pattern of immunoreactivity in tangles (Buee-Scherrer et al., 1996; Galván et al., 2001; Augustinack et al., 2002; Mondragon-Rodriguez et al., 2008). Through double-labeling experiments with AD2 and pS396, we defined two sub-populations of NFTs: one was characterized by the co-localization of pS396 and TR (**Figures 2A,B**) but lacking AD2 immunolabeling (**Figure 2B**); the other sub-population of NFTs was distinguished by the co-localization of all three markers (AD2, pS396, and TR). The co-localization of the latter combination of antibodies was strongest in the proximal processes of neurons (**Figure 2A**; arrowhead). Analysis of pS396 and AD2 double-immunolabeled sections showed that there was co-localization in 84% of tangles (**Table 3**).

DOUBLE IMMUNOLABELING WITH TauC3 AND pS396 IN NFTs

Truncation at Asp-421, detected using mAb TauC3, is an early event in tau processing in pre-tangle cells (Rissman et al., 2004; Luna-Munoz et al., 2007; Mena and Luna-muñoz, 2009). In this study, we analyzed the spatial association between pS396 and TauC3 antibodies in NFTs and pre-tangle cells. While the NFT observed in **Figure 2C** was detected by both antibodies, the tangle observed in **Figure 2D** was detected only by pS396. Taking into account that the tangle in **Figure 2C** is intracellular, whereas that in **Figure 2D** is extracellular, we conclude that the epitope identified by pS396 was more resistant to the extracellular proteolysis than the epitope detected by TauC3. There was 47% of co-localization observed in the NFTs labeled with pS396 and TauC3 (**Table 3**).

TRIPLE IMMUNOLABELING OF NFTs

To determine whether phosphorylation at pS396 was associated with full-length tau, we performed triple immunolabeling with the antibodies for pS396 together with Alz-50 and either Tau-12 or T46 (to non-phosphorylated N- and C- termini, respectively). Alz-50 was included as a marker of conformational changes in the tau molecule. We have previously demonstrated the loss of both C- and N- termini in the protein conformation identified by Alz-50 and that these changes are associated with early events of tau processing present in pre-tangle neuronal cells (Jicha et al., 1997a,b, 1999a; Weaver et al., 2000; Guillozet-Bongaarts et al., 2005; Luna-Munoz et al., 2007). pS396 immunoreactivity (green

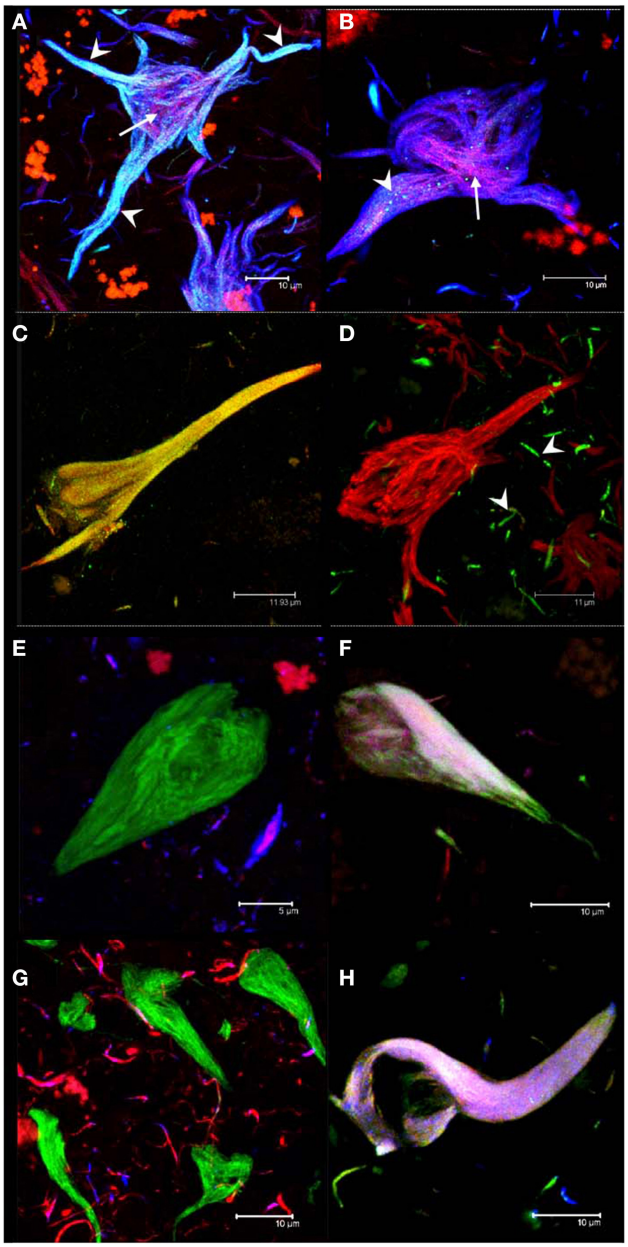

FIGURE 2 | Double and triple immunolabeling of tangles with antibodies. Intracellular NFTs were detected by both pS396 and AD2 **(A)**. Extracellular NFTs, however, displayed intense immunoreactivity with pS396 but only sparse and granular staining with AD2 (**B**, arrowheads). Thiazin red detected some PHF bundles located at the center of both intra- and extra-cellular NFTs (**A,B**, arrows). Double immunolabeling with TauC3 and pS396 identified two tangle subtypes: one in which both antibodies were co-localized **(C)** and another that was reactive only with pS396 **(D)**. TauC3 immunoreactivity was restricted to short, thin neurites located in the vicinity of NFTs (arrowheads). Triple immunolabeling with pS396 and Alz-50 with either Tau-12 **(E,F)** or T46 **(G,H)**; and double immunolabeling with pS396 and AD2, counterstained with thiazin red **(A,B)**; and pS396 with TauC3 **(C,D)**. Two subtypes of tangles were observed. (i) The first was recognized by pS396 (green), but not by Alz-50 (blue) either with Tau-12 (red) **(E)** or with T46 (red) **(G)**. (ii) A second subtype was defined as the one which displayed immunoreactivity with all three markers **(F,H)**.

channel) was present in tangles which were not labeled by Tau-12 (**Figure 2E**, red channel), T46 (**Figure 2G**, red channel) or Alz-50 (**Figures 2E,G**, blue channel). A sub-population of tangles, however, displayed immunoreactivity with all three antibodies (**Figures 2F,H**). These findings suggest that the epitopes of antibodies Tau-12, T46 and Alz-50 but not that of pS396, are lost during the early stages of tangle formation. For pS396 with AD2 and TauC3, a high level of co-localization in tangles was observed. This was in contrast to the low level of co-localization (6–7%) of pS396 with N- and C-termini (Tau-12 and T46) (**Table 3**).

DOUBLE IMMUNOLABELING WITH pS396 AND mAb 423 IN EARLY INTRACELLULAR TANGLES

The labeling of intracellular tangles showed co-localization for pS396 and mAb 423 immunoreactivity, as previously described (Garcia-Sierra et al., 2003, 2008). In these early tangles, pS396 immunoreactivity was observed either in an isolated form in the perinuclear area or organized in net-like structures (**Figure 3A**, arrow). The mAb 423 failed to identify these structures (**Figure 3A**, arrow).

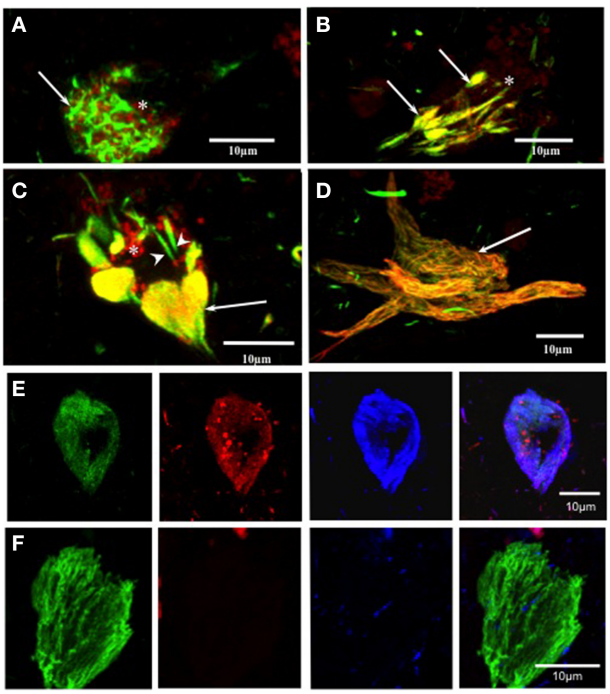

FIGURE 3 | Double and triple immunolabeling with pS396 and mAb 423, and Alz-50 in neuronal cells with tau degeneration in Alzheimer's disease. (A) pS396 immunoreactivity was observed in the intracellular early tangles, being present in the perinuclear area (arrows) and closely associated with lipofuscin (arrow, red channel). The mAb 423 failed to identify these structures (red channel, **B, C, D**) In tissue pre-treated with Pronase/formic acid, pS396 and mAb 423 antibodies co-localized in early tangles (**B**, arrows), confluent tangle bundles (**C**, arrows), and NFT (**D**, arrow). **(E, F)** Triple immunolabeling with mAb 423, pS396, and Alz-50 in Pronase/formic acid treated brain tissue, show all three antibodies co-localized in the intracellular tangle **(E)**. An extracellular tangle **(F)** displayed immunoreactivity with only mAb 423. * lipofuscin granules.

DOUBLE IMMUNOLABELING WITH pS396 AND mAb 423 IN ALZHEIMER'S DISEASE TISSUE PRE-TREATED WITH PRONASE/FORMIC ACID IN EARLY TANGLES

When brain tissue was treated with Pronase and formic acid prior to immunohistochemistry, mAb 423 immunoreactivity was observed in early tangles and was co-localized with pS396 (**Figure 3B**, arrows). Clustered, doubly labeled, early tangles were regularly observed. Double immunolabeling was also found in elongated tangle bundles that were associated with autofluorescent lipofuscin granules located in the vicinity (**Figure 3C**, arrow). Some isolated early tangles were detected only by pS396 (**Figure 3C**, arrowheads). Extracellular tangles were frequently labeled by both pS396 and mAb 423 (**Figure 2H**, arrow). Eighty-six percent of tangles showed co-localization of these two antibodies (**Table 3**). The remaining tangles were labeled only by mAb 423, indicating that these were extracellular NFTs.

TRIPLE IMMUNOLABELING WITH ANTIBODIES pS396, mAb 423 AND Alz-50 IN PRONASE/FORMIC ACID PRE-TREATED BRAIN TISSUE IN NFTs

An intracellular tangle detected by the mAb 423, pS396, and Alz-50 is shown in **Figure 3E**. There was a trend, however, for the Alz-50 immunoreactivity to be slightly stronger when compared with that of mAb 423 and pS396. Alz-50 and TG-3 are specific markers of intracellular NFTs (Jicha et al., 1997a), and the presence of these two epitopes has been implicated in the early stages of tangle formation (Luna-Munoz et al., 2005). A sub-population of extracellular NFTs was identified by mAb 423 but not by pS396, as illustrated in **Figure 3F**.

TRIPLE IMMUNOLABELING WITH ANTIBODIES mAb 423, pS396, AND Alz-50 IN PRONASE/FORMIC ACID-TREATED BRAIN TISSUE IN PRE-TANGLE CELLS

When we performed triple immunolabeling with mAb 423, pS396, and Alz-50 in Pronase/formic acid-treated brain tissue, pre-tangle cells, such as the one illustrated in **Figure 4**, displayed a diffuse granular cytoplasmic immunolabeling with mAb 423 (green channel) and Alz-50 (blue channel), but were not reactive with pS396 (red channel). Although many immunolabeled granular deposits were detected by mAb 423 alone, the majority of such structures that were immunolabeled by Alz-50 were also detected by mAb 423. Some neurons show cytoplasmic clusters of granular appearance.

In early investigations, combining confocal microscopy and electronmicroscopy, we were able to demonstrate that mAb 423 immunoreactivity was present in a diffuse granular pattern in the cytoplasm of putative pre-tangle cells (Mena et al., 1991, 1995), although this immunolabeled pattern has been difficult to find. After the Pronase/formic acid procedure, however, mAb 423 immunoreactivity was revealed in diffuse granular structures in the perinuclear area of the neuronal cell (**Figure 4A**; arrows). Some of these structures also co-localize with Alz-50 immunoreactivity (**Figure 4C**; arrows).

DISCUSSION

The present investigation was focused on the analysis of tau protein processing and aggregation in neuronal soma from the

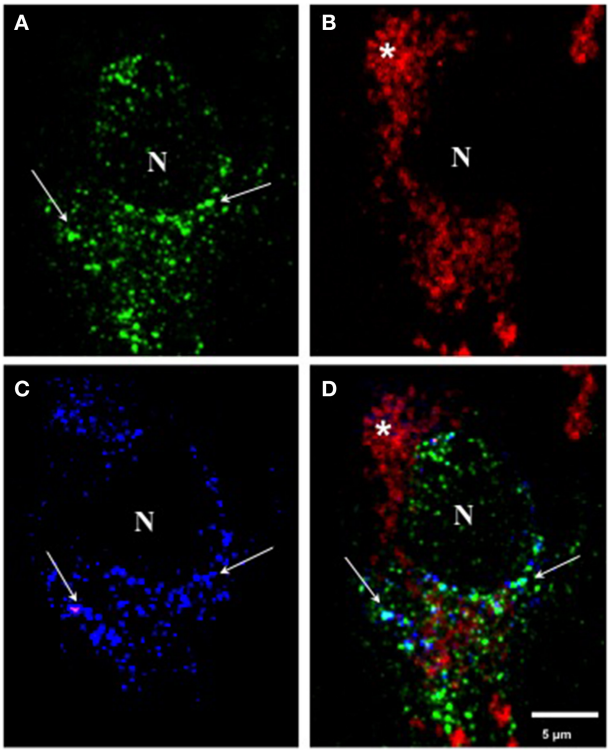

FIGURE 4 | Triple immunolabeling with antibodies mAb 423, pS396, and Alz-50 of pre-tangle cells after Pronase/formic acid treatment. **(A–D)** Triple labeling with mAb 423 (green), Alz-50 (blue) and pS396 (red). A pre-tangle cell displayed diffuse granular cytoplasmic immunolabeling with mAb 423 in the green channel (arrows) the majority of which was also detected by Alz-50 in the blue channel (**B**; arrows). However, pS396 failed to detect such granular structures in the red channel **(B)**. Autofluorescent lipofuscin granules were also observed (*). N, nucleus.

earliest stages (pre-tangle) of granular aggregation prior to the formation of PHFs, which are the components of intracellular and extracellular NFTs. Although NFTs are the structures that best correlate with the evolution of dementia in Alzheimer's disease, we were interested in addressing the early steps involved in their formation.

Our aim was to study the processing and aggregation of tau protein in the neuronal soma at early stages (pre-tangle) to determine the functional role of phosphorylated and truncated tau in the aggregation process at the cellular level.

We previously described that tau protein undergoes a cascade of events in Alzheimer's disease characterized by phosphorylation at specific sites and conformational changes along its N-terminus in pre-tangle neurons (Luna-Munoz et al., 2007). In this report, we have examined early events in tau protein processing along the C-terminal domain of tau and describe that, with the exception of phosphorylation at p-Ser-422, phospho-dependent tau epitopes were not associated with C-terminal truncation at Glu-391. Reactivity with mAb 423 was also absent from these structures. Interestingly, mAb 423 immunoreactivity was evident as granular diffuse deposits in pre-tangle cells after pre-treatment with Pronase/formic acid (**Figure 4A**), whereas immunoreactivity with pS396, pS400, pS404, and pS409 was absent in these conditions. Immunoreactivity with these C-terminal, phospho-dependent antibodies was observed in structures that represent tau protein aggregation in the pre-tangle stage (Luna-Munoz et al., 2007; Mena and Luna-muñoz, 2009).

The findings described here and in our earlier studies are summarized in a scheme that links the histological observations with the molecular changes in tau protein (**Figure 5**). Tau protein is predominantly found associated with axonal microtubules in unaffected neurons (Stage 0) but in Alzheimer's disease it accumulates in the somatodendritic compartment of neurons (Binder et al., 1985). The scheme in **Figure 5** depicts this process through six stages that are characterized by differential immunoreactivity with antibodies to distinct epitopes along the tau molecule.

The first abnormal event, occurring in the cytoplasm of neuronal cells prone to degeneration in Alzheimer's disease, is characterized by the appearance of the minimal PHF-tau core unit (truncated at Glu-391) within the cytoplasm (Stage 1). This pathological event appears to determine subsequent stages that are characterized by the binding of intact tau molecules to the PHF core. An early series of phosphorylation events, first observed along the N-terminus (Stage 2) (Luna-Munoz et al., 2007; Mena and Luna-muñoz, 2009), would favor the action of caspase-3 that cleaves at Asp-421 (Gamblin et al., 2003c) (Stage 3). These initial steps would go undetected by TR (Uchihara et al., 2001; Luna-Munoz et al., 2007) because the early clusters of both aggregated and less polymerized tau molecules leading to the formation of proto-PHF (tau oligomers of different lengths) (Mena and Luna-muñoz, 2009) are still randomly distributed and do not have fully formed β-pleated sheet conformational structures (Jicha et al., 1997a,b, 1999a; Uchihara et al., 2001). Thus, they have insufficient affinity for the binding of TR (Jicha et al., 1997b; Uchihara et al., 2001; Luna-Munoz et al., 2005). The resultant tau oligomers would act as a template that grows bi-directionally as further tau molecules become sequestered and structurally integrated into the proto-PHF.

According to previous studies of pre-tangles (Mena et al., 1996; Galván et al., 2001; Luna-Munoz et al., 2005), membranous organelles, including mitochondria, nucleus and rough endoplasmic reticulum could serve as a substrate on which the emerging proto-PHF could grow, providing proteolytic stability during elongation of the assembled filaments (Galván et al., 2001). The ability of C-terminally truncated PHF-core tau to capture full-length tau *in vitro* enables sequential cycles of binding, truncation and binding. The origin of this process is not known, but it does not necessarily need to be restricted to a single specific membrane protein alteration; several different proteins or macromolecular complexes could serve as substrates for the initial binding of tau protein. After the initial capture of tau and its proteolysis, the truncated PHF-core tau can bind further tau molecules with increased avidity (Wischik et al., 1996), generating oligomeric aggregates that eventually develop into fibrillary aggregates. Truncation at Glu-391, would confer a special conformation to enable the formation of PHFs, their anti-parallel structural alignment and the restriction of access for the proteolytic enzymes to these oligomers/aggregates/filaments

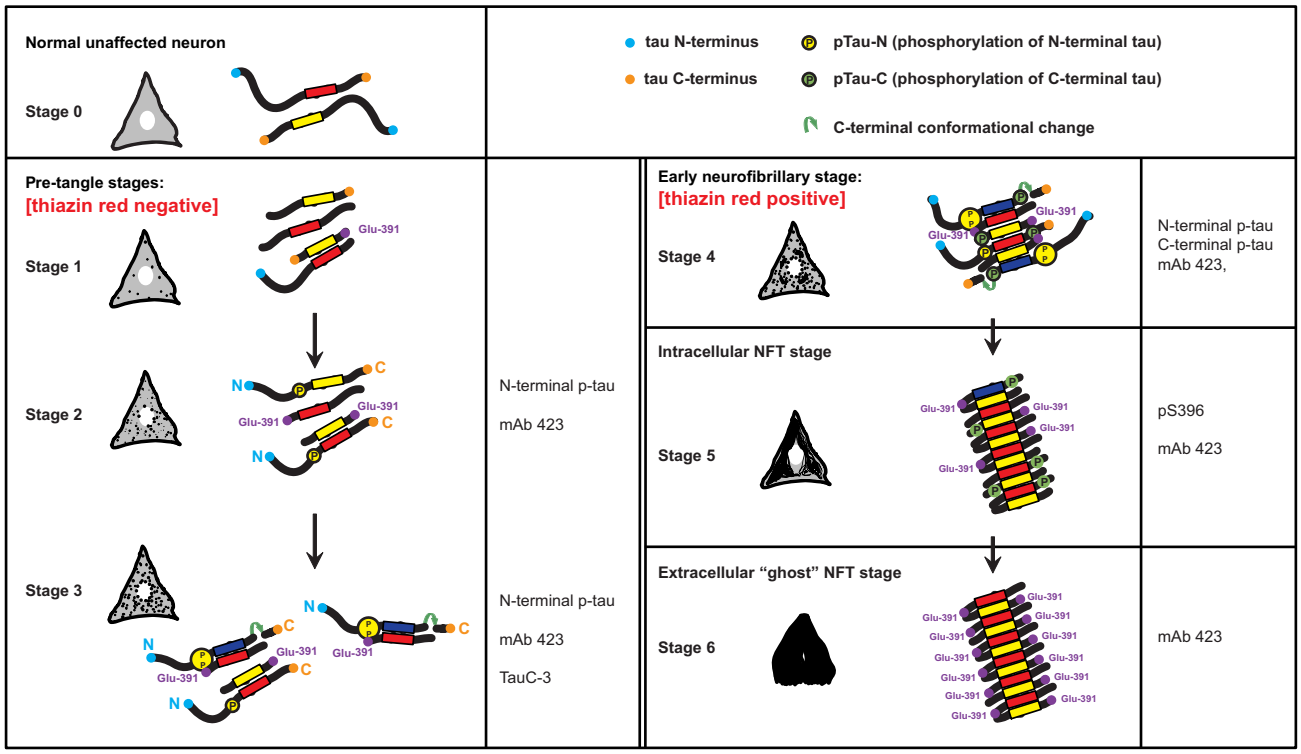

FIGURE 5 | A model for the stages of tau assembly into PHFs in pre-tangle neurons in Alzheimer's disease. Unaffected neurons (Stage 0) will have normal tau (neither truncated or hyperphosphorylated) that is associated predominantly with axonal microtubules. Stages 1–3 represent early stages that precede the appearance of fibrillary inclusions. Stage 1 is characterized by the appearance of the PHF core, namely a fragment of 93–95 amino acids in length corresponding to the repeat domain of tau. Stage 2 is defined by the cytoplasmic aggregation of tau molecules resulting from the binding of PHF-core fragments with full-length tau and that is reactive with the N-terminal phospho-dependent antibody. This stage corresponds to the diffuse granular structures seen by confocal microscopy. The PHF-core tau is not evident at this stage, but can be exposed after Pronase/formic acid treatment. Stage 3 corresponds to a further stage in which C-terminal truncation at Asp-421 appears. By Stage 4, intracellular PHFs and fibrils are present, which are phosphorylated at both N- and C-termini and showed variable immunoreactivity to mAb 423. The fibrillar nature of the tangles was confirmed by labeling with TR. As the tangle develops intracellularly in Stage 5, the N-terminal portions are removed and proteolysis reveals the epitope recognized by mAb 423. By Stage 6, the plasma membrane has been disrupted and the extracellular "ghost" tangles are evident, which are comprised of highly insoluble tau, reactive with mAb 423 but with only occasional pS396 epitopes remaining. See text for more detailed description.

in affected cells (Wischik et al., 1996). Fragments of tau truncated at Glu-391 favor tau polymerization in Alzheimer's disease (Wischik et al., 1996; Berry et al., 2003; Gamblin et al., 2003b). In early tangles, the C-terminal portion of tau would be phosphorylated at Ser-396, Ser-400, Ser-404 and Ser-409 (Stage 4). Interestingly, phosphorylation at Ser-396 seems to be very stable and co-localizes with the PHF core (mAb 423) in the later stages of NFT formation, even after neuronal death (Stages 5 and 6).

The proposed model explaining early mechanisms of tau aggregation in pre-tangle cells, prior to its polymerization into PHFs, deals with two different issues: the toxic and neuroprotective capacities of tau protein (Gamblin et al., 2003a; Castellani et al., 2008; Congdon and Duff, 2008; Garcia-Sierra et al., 2008; Wang and Liu, 2008; Nunomura et al., 2009). Based upon our findings and those from other studies, we propose a model that integrates these processes together with the events of truncation and phosphorylation. We propose that the polymerization of tau protein into PHFs, in Alzheimer's disease, involves complex interactions of truncated and phosphorylated tau species within the cytoplasm of vulnerable neurons (Zilkova et al., 2011). The toxic PHF-core tau fragment would be expected to trigger a cellular protective response, including phosphorylation of normal tau protein and activation of caspases, including caspase-3 (Fasulo et al., 2000, 2005; Gamblin et al., 2003c). Once sequestered in a highly stable and resistant PHF core, full-length tau protein would then become phosphorylated, thereby hiding the toxic species (Bretteville and Planel, 2008; Castellani et al., 2008; Su et al., 2008; Wang and Liu, 2008; Zhang et al., 2009).

This implies that phosphorylation in the early stages of PHF formation would play a protective role, based upon *in vitro* and animal models (Arendt et al., 2003; Stieler et al., 2009, 2011). Moreover tau hyperphosphorylation had been proposed as a protective mechanism, because it is known that this phenomenon could occur in normal (no pathological) conditions, such as during development or hibernation, and in these cases tau hyperphosphorylation does not lead to aggregation or neurodegeneration (Goedert et al., 1993; Arendt et al., 2003; Bretteville and Planel, 2008).

It is important to emphasize that the phosphorylation of the N-terminal portion of the protein tau is a very early event in tau

aggregation and abnormal accumulation in Alzheimer's disease (Luna-Munoz et al., 2005, 2007). However, our observations in all Alzheimer's disease cases analyzed here showed that tau phosphorylation at the C-terminus appears to be associated with early stages of tau aggregation when the formation of small TR-reactive tangles occurs. Tau phosphorylation at Ser-422 is observed from the earliest stages of tau aggregation. Previous studies suggested that tau phosphorylation at the Ser-422 can prevent the action of caspase-3, which is very active in Alzheimer's disease and truncates tau at Asp-421 (Guillozet-Bongaarts et al., 2006). Truncated tau is present from early to late stages of tau aggregation. This is why we considered that tau processing and phosphorylation occurs throughout the entire formation and evolution of the NFTs. Two events are relevant for this process: 1) tau processing and 2) sequestration of intact tau and its phosphorylation. This implies that the more internal epitopes of the NFT are the more stable and resistant to proteolysis and not necessarily the last to have been generated. These epitopes could be present throughout the evolution of the NFT, and they become more evident when the NFTs have been treated with Pronase/formic acid, thus exposing the PHF core. In a single tangle, it is possible to find many different tau epitopes corresponding to various stages of processing and phosphorylation of tau protein. Thus, NFTs and pre-tangle structures can be considered as dynamic structures in which multiple pathological processes occur that affect tau protein.

In addition, as proposed by Bondareff and colleagues, a progressive sequence of binding and proteolysis, would eventually provide a template upon which further intact tau molecules can be incorporated into the PHF core and become phosphorylated (Bondareff et al., 1990). Because of the proteolytically resistant conformation adopted by emerging oligomers, all those characterized by Glu-391 truncation would form PHFs. A similar cascade of events will characterize the formation of the NFTs. The predicted effect of both phosphorylation and NFT formation would be the protection of the neuron (Morsch et al., 1999). Eventually, however, the tangled-neuron would die because of abnormal metabolism and/or the release of NFTs into the extracellular space (Mena et al., 1996; Garcia-Sierra et al., 2003; Mena and Luna-muñoz, 2009). The subsequent action of proteolytic enzymes would expose the PHF core as the ghost or extracellular NFT appears (Garcia-Sierra et al., 2003, 2008; Guillozet-Bongaarts et al., 2005). It remains to be determined whether NFTs, purified from Alzheimer's disease brains, are toxic. These studies, however, provide important insight into the characterization of tau aggregates as they exist *in vivo*.

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Non-coding RNAs in stroke and neuroprotection

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This review will focus on the current state of knowledge regarding non-coding RNAs (ncRNA) in stroke and neuroprotection. There will be a brief introduction to microRNAs (miRNA), long ncRNAs (lncRNA), and piwi-interacting RNAs (piRNA), followed by evidence for the regulation of ncRNAs in ischemia. This review will also discuss the effect of neuroprotection induced by a sublethal duration of ischemia or other stimuli given before a stroke (preconditioning) on miRNA expression and the role of miRNAs in preconditioning-induced neuroprotection. Experimental manipulation of miRNAs and/or their targets to induce pre- or post-stroke protection will also be presented, as well as discussion on miRNA responses to current post-stroke therapies. This review will conclude with a brief discussion of future directions for ncRNAs studies in stroke, such as new approaches to model complex ncRNA datasets, challenges in ncRNA studies, and the impact of extracellular RNAs on human diseases such as stroke.

Keywords: cerebral ischemia, neuroprotection, preconditioning, non-coding RNA, microRNA

INTRODUCTION

Despite advances in preclinical studies that have established mechanisms of cell death in brain ischemia and identified potential strategies to prevent or treat brain injury following stroke, few of these advances have successfully translated into clinical practice. Endogenous neuroprotective responses have been studied in experimental stroke largely using a model wherein a sublethal duration of ischemia is given before a stroke (preconditioning). However, additional methods of preconditioning are also effective in reducing stroke injury, i.e., hypoxia, anesthetic agents, and activation of Toll-like receptors, and natural states of neuroprotection in animals such as hibernation that lend important insight to the topic. More recent efforts have focused on strategies for neuroprotection after a stroke (post-conditioning), which is more relevant to the clinical presentation in human patients. This review will present the current state of knowledge regarding evidence for specific families of non-coding RNAs (ncRNA) in experimental stroke and neuroprotection: microRNAs (miRNA), long non-coding RNAs (lncRNA), and piwi-interacting RNAs (piRNA). The literature search was performed using PUBMED queried with a combination of terms listed in **Table 1**, and the number of references returned for each query.

In recent years, there has been increasing focus on the role of ncRNAs as regulators of post-transcriptional gene expression, and of responses to ischemia and neuroprotection (1). Spinal cord injury and traumatic brain injury also lead to changes in miRNA expression that precedes injury (2). Further, dysregulation of ncRNA activities has been closely linked to the pathophysiology of cerebral vascular endothelium and neurologic functional disorders in the brain's response to ischemia (3). Experimental stroke models have been used for global profiling of brain or blood miRNAs in animals, or to manipulate specific miRNAs or their targets to examine their potential as neuroprotective strategies for stroke. Given the current published reviews for the role of miRNAs in the occurrence and development of post-stroke depression (4), and in the regulation of miRNAs that lead to angiogenesis and remyelination (5), neurogenesis (6), and spontaneous recovery (7), they will not be discussed here. Also, outside the scope of this review are many miRNAs studies on cardiac ischemia and protection that may provide important insights on miRNAs in cerebral ischemia and neuroprotection (8).

NON-CODING RNAs

Early biochemistry studies identified three families of RNA that function cooperatively in the process of protein synthesis: messenger, transfer, and ribosomal RNA. Messenger RNAs (mRNA) carry genetic information copied from DNA in a series of three-base codes that specify a particular amino acid, specifying the polypeptide sequence. Transfer RNAs (tRNA) carry and bind a complementary amino acid to the growing end of a polypeptide chain. Ribosomal RNAs (rRNA) bind to protein complexes, which physically move along mRNAs and catalyze the assembly of amino acids into polypeptide chains (9). Additional families of ncRNAs that play essential roles in the processes of protein synthesis were subsequently discovered (10). For example, small nuclear RNAs (snRNA) bind to proteins to form spliceosomes that process pre-mRNA into mature mRNA in the nucleus (11), while small nucleolar RNAs (snoRNA) guide chemical modifications of other RNAs (12). This review will focus on three newer families of ncRNAs; (1) miRNAs that regulate post-transcriptional gene expression, largely by direct effects on mRNAs (13), (2) lncRNAs that can regulate transcription and translation, and act as epigenetic modifiers, and (3) piRNAs that largely exert effects on transcription and genomic maintenance. The reader is directed to a 2014 Frontiers in Cellular Neuroscience Research Topic entitled "*Regulatory RNAs in the Nervous System*" for comprehensive discussions regarding the biosynthesis and function of these ncRNAs, as well as a recent review entitled "*Non-Coding RNAs as Potential Neuroprotectants against Ischemic Brain Injury*," which beautifully

Table 1 | Literature search strategy.

Search terms			Number of references
Ischemic preconditioning	Cerebral	Review	104
Neuroprotection	Cerebral ischemia	MiRNA	10
Preconditioning	Cerebral ischemia	MiRNA	5
	Stroke	MiRNA	4
Post-conditioning	Cerebral ischemia	MiRNA	0
	Stroke	MiRNA	1
MiRNA	Stroke		78
	Ischemia		147
	Preconditioning		23
	Neuroprotection		28
	Neuroprotection	Ischemia	12
	Neuroprotection	Cerebral ischemia	46
	Neuroprotection	Cerebral ischemia	10
		Ischemic stroke	9
	Biomarker	Cerebral ischemia	2
	Biomarker	Stroke	9
PiRNA	Brain		9
	Stroke		1
	Ischemia		1
	Preconditioning		0
	Neuroprotection		0
	Neuroprotection	Ischemia	0
		Cerebral ischemia	1
	Neuroprotection	Cerebral ischemia	0
	Neuroprotection	Ischemic stroke	0
LncRNA	Brain		56
	Stroke		2
	Ischemia		3
	Preconditioning		0
	Neuroprotection		0
	Neuroprotection	Ischemia	0
	Cerebral ischemia		2
Exosome	MiRNA	Cerebral ischemia	0
		Cerebral ischemia	0
	Stroke		4
Post-stroke	MiRNA		1

PUBMED was queried with the following combination of search terms, and the resultant numbers of references returned for each combination are listed.

depicts the current understanding of the biogenesis pathways for each of these three ncRNAs discussed herein (14).

MicroRNAs

The miRNAs are the most well-characterized family of ncRNA to date. Mature, functional miRNA sequences are ~21–23 nucleotides (nt) in length, and are most recognized for their roles in the regulation of post-transcriptional gene expression via direct effects on the 3′-untranslated region (3′UTR) of mRNAs that lead to translational repression or mRNA degradation. MiRNA biogenesis has been previously described and will not be presented here, but the reader is guided to a recent comprehensive review of this topic (15). The miRNAs are particularly abundant in the nervous system where they serve as effectors of brain development (16–18) and maintenance of the neuronal phenotype (19, 20). MiRNAs also drive the maturation of dendrites and spines (21–23) and serve as effectors of synaptic plasticity and function (24–30). Emerging studies highlight roles for miRNAs in extracellular vesicles (EVs) where they contribute to cell–cell communication in the brain (31–33) and throughout the nervous system (34). As such, dysregulation of EVs may underlie diverse neurological disorders including brain tumors (35), neurodegeneration, and neuroinflammation (34), and EVs in biofluids are currently being evaluated for their potential as biomarkers or therapeutic strategies for the treatment or prevention of human diseases, including many brain disorders (36).

Long non-coding RNAs

The lncRNAs are >200 nt in size, and they differ greatly from each other with respect to their size, interacting partners, and modes of action (37). The lncRNAs are processed through pathways similar to those of protein-coding genes, and have similar histone-modification profiles, splicing signals, and exon/intron lengths. The complex intergenic, overlapping, and antisense patterns of lncRNAs relative to adjacent protein-coding genes suggests that many lncRNAs regulate the expression of adjacent protein-coding genes. LncRNAs are also implicated in the regulation of transcription and post-transcriptional gene expression, as comprehensively reviewed (38). LncRNAs can stabilize or promote the translation of target mRNAs through extended base pairing, or they can inhibit target mRNA translation or facilitate mRNA decay via partial base pairing. Even in the absence of any base pair complementarity, lncRNAs can suppress precursor mRNA splicing and translation by acting as decoys for RNA-binding proteins or miRNAs, or compete for miRNA-mediated inhibition of mRNAs resulting in increased expression of corresponding proteins. Over one-half of all lncRNAs are expressed in the brain (39) and they regulate many CNS processes (40). Hence, dysregulation of lncRNAs can also contribute to CNS pathologies such as neurodegeneration, neuroimmunological disorders, primary brain tumors, and psychiatric diseases (41). As such, lncRNAs present new targets for the understanding, diagnosis, and treatment of CNS disorders (42).

Piwi-interacting RNAs

The piRNAs are ~25–33 nt in length, depending on the PIWI protein group they bind to, and they lack sequence conservation between organisms. Recent reviews highlight the complexity of piRNA biogenesis pathways, which have just begun to be elucidated (43, 44). PiRNA biogenesis pathways are complicated as they are distinct in individual organisms, and they are distinct from miRNA pathways in that there is no evidence for a double-stranded RNA precursor and biogenesis is independent of Dicer (45–47). There are two proposed pathways for generating piRNAs: a primary processing pathway and a "ping-pong" amplification loop, as recently reviewed (14). PiRNA clusters are transcribed in the sense or antisense direction, and the long single-stranded RNA serves as the basis for piRNA production.

The piRNAs protect germline cells from transposons in organisms as they guide PIWI proteins to complementary RNAs derived from transposable elements, and then the PIWI proteins cleave transposon RNA. Transposons are genetic elements that copy themselves to RNA, then back to DNA, and then integrate back into the genome where they may induce mutations that cause disease by inserting near or within other genes to disrupt their function. The piRNAs have been dubbed as the guardians of the genome because of their ability to silence deleterious retrotransposons (48). Very recent studies also show piRNAs as effectors in the epigenetic control of long-term memory storage in the brain (49).

Together, these three newer families of ncRNAs serve to regulate many aspects of cellular biology that ultimately control phenotype, function, and genomic integrity. As such, dysregulation of any or all of these ncRNAs will likely have profound impact on CNS disorders.

ISCHEMIA

OVERALL EFFECT OF ISCHEMIA ON miRNA EXPRESSION

The overall effect of stroke on miRNA expression in brain and blood has been examined in experimental rodent models and in human subjects. Most rodent studies have used transient middle cerebral artery occlusion (MCAO) to induce focal cerebral ischemia. The first such study used microarray analysis to examine overall expression of miRNAs in rat brain and blood at 24 and 48 h after ischemia and showed that miRNAs are regulated by focal ischemia and may potentially serve as blood biomarkers for ischemia (50). Focal ischemia studies in spontaneously hypertensive rats also showed altered miRNA profiles at all reperfusion times examined (3 h to 3 days), with several miRNAs altered at multiple time points (51). Bioinformatic analysis predicted mRNA targets of the ischemia-regulated miRNAs as proteins known to mediate inflammation, transcription, neuroprotection, receptor function, and ionic homeostasis (51). Further, *in silico* analysis of 8 ischemia-induced miRNAs revealed sequence complementarity to 877 gene promoters, suggesting that ischemia-regulated miRNAs directly modulate gene expression. Importantly, these studies also showed that focal ischemia did not alter mRNA levels for Drosha, Dicer, Pasha, and Exportin-5, proteins involved in the miRNA biogenesis pathway (51). Studies focused on humans revealed that circulating blood miRNA profiles were altered in young ischemic stroke patients (18–49 years) and that specific miRNAs that could classify subtypes of stroke (large-vessel atherosclerosis vs. small-vessel disease vs. cardioembolism) were still detectable in blood several months after injury (52). The first RNAseq studies identified significant changes in 78 known and 24 novel miRNAs in rat brain following ischemia, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis predicted that protein targets of these miRNAs function in signaling transduction, MAPK signaling, NF-kappaB signaling, and neurotrophin signaling (53).

Together, these studies show that miRNAs are regulated in brain and blood where they likely regulate translation, and transcription, in post-ischemic brain. These studies also established the potential for blood miRNAs to be developed as clinical biomarkers for the diagnosis and prognosis of ischemic stroke in humans.

SEX-SPECIFIC miRNA RESPONSES TO ISCHEMIC INJURY

Females are more resistant to stroke injury than males until menopause (54), and responses to experimental stroke and cell death are sexually dimorphic (55). In males, stroke initiates mitochondrial release of apoptosis-inducing factors that result in caspase-independent cell death, while in females stroke primarily triggers mitochondrial cytochrome c release and subsequent caspase activation. At baseline, females have higher levels than males of brain mRNA for X-linked inhibitor of apoptosis (XIAP), the primary endogenous inhibitor of caspases, and stroke significantly decreases XIAP mRNA in females but not in males. However, XIAP protein levels are decreased in both sexes after stroke, likely due to different levels of miR-23a, which binds to the XIAP 3′UTR, in male and female ischemic brain (55). Subsequent studies also revealed distinct differences in miRNA responses to ischemia in male and female brain, as well as a signature miRNA response to ischemia common to males and females (56). The finding that there are sex differences in miRNA expression during development in rat cortex may bear on differential responses to ischemia in adults (57). Accordingly, a recent study investigated the impact of age and sex on miRNA expression in adult (6 months) and middle-aged (11–12 months) female and male rats subjected to the endothelin-1 model of MCAO (58). Consistent with MCAO injury, infarct volume and sensory-motor deficits were significantly reduced in adult females compared with middle-aged females, adult males, or middle-aged males. Analysis of blood at 2 days post-stroke revealed 21 differentially regulated circulating miRNAs, and principal component analysis demonstrated that most of the variance was due to age. At 5 days post-stroke, analysis of blood revealed 78 differentially regulated circulating miRNAs, and principal component analysis confirmed that most of the variance was associated with sex. Brain miRNAs analyzed at 5 days post-stroke revealed a small cohort of miRNAs (miR-15a, miR-19b, miR-32, miR-136, and miR-199a-3p) highly expressed exclusively in adult females. These patterns of circulating miRNA expression suggest that age is the primary influence on the initial severity of stroke pathology, but sex is the primary influence on recovery from stroke. Together, these studies support that miRNA expression can be different between males and females, which may contribute to sexually dimorphic responses to ischemia.

EFFECT OF ISCHEMIA ON SPECIFIC miRNAs AND TARGETS

Subsequent studies began to investigate the functional significance of individual ischemia-regulated miRNAs on the molecular mechanisms that lead to brain cell death. In addition to those described above, several of these specific miRNAs are discussed below.

miR-497

The expression of miR-497 was significantly induced in mouse brain 24 h after MCAO, and in mouse N2A neuroblastoma (N2A) cells after *in vitro* ischemia induced by oxygen-glucose deprivation (OGD) (59). Yin and colleagues showed a direct correlation between miR-497 and cell death: gain or loss of miR-497 promoted or decreased OGD-induced neuronal cell loss, respectively. Luciferase assays showed direct binding of miR-497 to the 3′UTR of B-cell lymphoma 2 (Bcl-2) and Bcl-w mRNAs, whose proteins function as key regulators in attenuating stroke-induced

apoptotic cell death (60–63). Inhibition of miR-497 in mouse brain enhanced Bcl-2/-w protein expression in the ischemic region, attenuated ischemic brain infarction, and improved neurological outcomes in response to focal ischemia.

miR-29b

Subsequent studies showed that miR-29b promoted ischemic cell death by targeting Bcl-2. MiR-29b expression was significantly increased in rat brain by focal ischemia, and in primary cultured neurons by OGD (64). Cell death was also directly correlated with miR-29b expression: gain or loss of miR-29b promoted or decreased OGD-induced neuronal cell loss, respectively. Additionally, miR-29b directly bound to the 3′UTR of Bcl-2 mRNA, and overexpression of the *bcl-2* gene rescued neuronal cell death induced by miR-29b.

miR-134

Overexpression of miR-134 promoted hippocampal cell death in cultured neurons subjected to OGD, while inhibition of miR-134 was protective. Expression of cyclic AMP (cAMP) response element-binding protein (CREB), a putative target of miR-134, was inversely regulated by miR-134 (65).

miR-21

In situ hybridization showed increased expression of miR-21 in rat neurons in the ischemic boundary zone at 2 and 7 days after embolic MCAO, and RT-qPCR studies verified a quantitative increase in mature miR-21 in neurons isolated from the ischemic boundary zone (66). Overexpression of miR-21 protected cortical neurons from cell death induced by OGD, while inhibition of miR-21 promoted OGD-induced cell death. Overexpression of miR-21 in neurons significantly reduced levels of Fas Ligand (FASLG), a cell death-inducing ligand, and luciferase assays showed that miR-21 binds to the 3′UTR of FASLG mRNA.

miR-347

Permanent focal cerebral ischemia in rat brain also leads to regulation of miRNAs between 3 h and 14 days following MCAO (67). Of particular interest was miR-347 that was upregulated at both acute and late phases following ischemia. Overexpression of miR-347 increased neuronal apoptosis and induced the expression of novel mRNA targets including acyl-CoA synthetase long-chain family member 4 (Acsl4), Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3-like (Bnip3l), and phytanoyl-CoA 2-hydroxylase interacting protein (Phyhip) (67).

These independent studies demonstrate that although there are diverse miRNAs regulated by ischemia, multiple miRNAs act directly and indirectly on cell-death proteins and can influence both pro- and anti-apoptotic pathways.

TEMPORAL AND REGIONAL EXPRESSION OF SPECIFIC miRNAs IN ISCHEMIA

MiRNAs can induce distinct outcomes in a temporal- and cell-specific manner. Cultured rat cortical neurons and astrocytes subjected to OGD exhibit unique miRNA expression patterns. OGD increased miR-29b expression in neurons by twofold after 6 h and by fourfold after 24 h compared to miR-21 that remained unchanged until after 24 h of OGD (68). However, OGD increased miR-29b and miR-21 in astrocytes only after 12 h, and further, altered expression of miR-30b, miR-107, and miR-137 was specific to astrocytes. Responses to ischemia in mouse brain also showed increased expression of miR-181 in the core, where cells die, and decreased expression of miR-181 in the penumbra, where cells survive (69). Reduction in miR-181a was correlated with increased levels of glucose-regulated protein, 78 kDa (GRP78), a heat shock protein that functions within the chaperone network to increase resistance to and improve recovery from ischemic brain injury (69, 70). Further studies compared miRNA expression induced by OGD in rat cortical neurons to *in vivo* miRNA expression in mice exposed to the three-vessel occlusion model of ischemia (71). These studies showed that while multiple miRNAs were differentially regulated by ischemia in each model, miR-19b, miR-29b-2*, and miR-339-5p were all significantly upregulated in common to both models (71). These studies suggest that there are signature miRNA responses to ischemia in salvageable brain regions, information that may lend new insights into cell- and temporal-specific protective strategies for the treatment of ischemia.

lncRNAs IN ISCHEMIA

The effect of ischemia on lncRNA expression was first examined in adult male spontaneously hypertensive rats at 3, 6, and 12 h after MCAO (72). The results showed that of 8314 lncRNAs analyzed, 443 were significantly changed at all time points (359 upregulated and 84 downregulated). Of these, 61 stroke-responsive lncRNAs showed >90% sequence homology with exons of protein-coding genes, but *in vitro* translation of these lncRNAs did not form any protein products (72). Based on the roles of lncRNAs in cellular homeostasis during development and disease via interactions with chromatin-modifying proteins (CMPs), the effect of ischemia on the association of lncRNAs with CMPs, Sin3A, and corepressors of the RE1 (Repressor Element 1)-silencing transcription factor (coREST) was examined (73). These studies revealed that 177 of the 2497 lncRNAs in rat cerebral cortex showed increased binding to either Sin3A or coREST following ischemia, and that of these, 26 lncRNAs enriched with Sin3A and 11 lncRNAs enriched with coREST were upregulated by ischemia. Thus, these studies suggest that ischemia-induced lncRNAs might associate with CMPs to modulate the post-ischemic epigenetic landscape.

piRNAs IN ISCHEMIA

The first evidence for expression of piRNAs in the nervous system was revealed by RNAseq analysis of mouse hippocampal RNA (74). PiRNA expression in adult male spontaneously hypertensive rats examined 24 h after focal ischemia revealed that of ~40,000 piRNAs analyzed, 105 piRNAs were significantly altered in cerebral cortex with 54 upregulated and 51 downregulated (75). Bioinformatic analysis of four of the top stroke-responsive piRNAs (two up, two down) revealed that their transposon targets belong exclusively to the class of retrotransposons. Further, analysis of the promoter region of 10 stroke-responsive piRNAs revealed binding sites for 159 transcription factors, including the zinc finger, Kruppel, and E2F transcription factor-like families, supporting that transcription factors control the expression of stroke-responsive piRNAs.

NEUROPROTECTION

PRECONDITIONING

The studies discussed here will focus only on miRNA responses to preconditioning models that lead to neuroprotection against stroke as there are no published studies regarding the impact of post-conditioning on miRNA, lncRNA, or piRNA in cerebral ischemia to date. Preconditioning is a phenomenon whereby a brief, non-lethal duration of a stimulus induces a transient state of resistance (tolerance) to a subsequent lethal duration of a stimulus. Ischemic preconditioning (IPC) is an experimental technique induced by a short duration of ischemia that protects the brain from a subsequent injurious duration of ischemia (76, 77). New protein synthesis is required for IPC-induced tolerance (78), and the signature of ischemic tolerance is global repression of gene expression (79–81). However, additional preconditioning stimuli such as hypoxia, anesthesia, and activation of the Toll-like receptors can induce tolerance and protection against ischemia, as discussed below.

Ischemic preconditioning

Ischemic tolerance was first examined in a gerbil model of global cerebral ischemia (82, 83). The first studies to examine miRNA responses to ischemia in mice evaluated their expression in response to IPC (15 min MCAO), ischemia (60 min MCAO), and tolerance (15 min MCAO, 72 h reperfusion, 60 min MCAO). Microarray analysis of cortical tissue isolated from adult male mice 24 h after each treatment revealed that miRNAs were regulated by each treatment with 192 upregulated and 95 downregulated by IPC, and that there was very little overlap in the miRNAs regulated between all three treatment groups (84). Bioinformatic analysis focused on those miRNAs downregulated by IPC that could result in increased protein synthesis revealed that predicted targets included transcriptional regulators such as methyl cytosine-phosphate-guanine (CpG) binding protein 2 (MeCP2) (84). MecCP2 is a global transcriptional regulator that largely serves to repress gene expression but can also activate gene expression (85). MeCP2 protein, but not mRNA expression, increased in mouse cortex by 8 h after IPC, and MeCP2 knockout mice showed increased sensitivity to the preconditioning stimulus and were not tolerant to a subsequent ischemic event (84). Recent studies in a cardiac ischemia model showed that mesenchymal stem cells, which protect ischemic cardiomyocytes by secretion of paracrine factors, secreted exosomes enriched with miR-22 following IPC, and uptake of these exosomes by cardiomyocytes reduced ischemia-induced apoptosis via direct targeting of MeCP2 (86). These studies support a protective role for MeCP2 in both cerebral and cardiac ischemia, although the specific gene targets in each model need to be identified.

Analogous studies to examine miRNA expression in a rat model of IPC induced in adult male spontaneously hypertensive rats by 10 min MCAO revealed significant changes in 51 miRNAs (26 upregulated and 25 downregulated) at 6 h of reperfusion, and 20 of these miRNAs remained altered 3 days after IPC (87). Bioinformatic analysis of the miRNAs and KEGG Pathway analysis of their predicted targets showed MAP-kinase and mammalian target of rapamycin (mTOR) signaling as the top two pathways targeted by the upregulated miRNAs, and Wnt and gonadotropin releasing hormone (GnRH) signaling as the top two pathways targeted by the downregulated miRNAs. Interestingly, these studies also showed that multiple miRNAs downregulated by IPC were predicted to target MeCP2 in rat, consistent with previous studies on IPC in mouse (84).

Additional studies in adult male mice subjected to IPC or ischemia revealed 8 miRNAs that together comprise the miR-200 and miR-182 families of miRNAs that were selectively upregulated 3 h after IPC, and 2 miRNAs (miR-681 and miR-197) that were selectively upregulated 24 h after IPC (88). No miRNAs were downregulated at 3 h after IPC, and only 1 miRNA (miR-468) was downregulated at 24 h after IPC. Transfection of the 8 IPC upregulated miRNAs into N2A cells revealed 5 miRNAs that increased cell survival in response to OGD, and decreased expression of prolyl hydroxylase 2 (PHD2), a predicted target of the miR-200 family involved in the ubiquitin/proteosomal pathway. Reduced levels of PHD2 protein were also detected 24 h after IPC, but not after ischemia.

Hypoxic preconditioning

Hypoxic preconditioning (HPC) induced by autohypoxia (acute and repetitive exposure to progressive hypoxia) in adult male mice significantly decreased ischemic injury by mechanisms involving multiple conventional protein kinase C βII (cPKCβII)-interacting proteins (89). Bioinformatic analysis of 17 miRNAs (4 upregulated and 13 downregulated) in mouse cortex between HPC and ischemia predicted that cPKCβII, γ, and novel protein kinase Cε (nPKCε)-interacting proteins as targets of the HPC-specific miRNAs (90). Together, these studies support that HPC-induced protection against ischemia involves regulation of cPKC proteins and modulation of calcium signaling in mouse brain.

Anesthetic preconditioning

Several experimental studies show that inhalational anesthetics serve as preconditioning agents that lead to neuroprotection against stroke. Administration of isoflurane, halothane, sevoflurane, desflurane, nitrous oxide, and other agents is protective in the setting of stroke, and potential mechanisms of anesthetic preconditioning include antagonism of AMPA and NMDA receptors and reduced excitotoxicity, modulation of cerebral catecholamine release, direct anti-oxidant effects of anesthesia, and reduction of the cerebral metabolic rate (91). Recent studies to examine the contribution of miRNAs to anesthetic preconditioning revealed that isoflurane significantly increased expression of miR-203 in rat B35 neuron-like cells, and there was a trend toward increased miR-203 expression in rat cerebral cortex after isoflurane exposure (92). Overexpression of miR-203 in B35 cells increased both tolerance to ischemia and expression of Phospho-Akt, a protein kinase that promotes cell survival. The effect of sevoflurane preconditioning on rat brain miRNA expression following transient cerebral ischemia revealed 3 upregulated and 9 downregulated miRNAs (93). Among these, miR-15b expression was increased after ischemia, while sevoflurane preconditioning downregulated miR-15b expression and increased expression of Bcl-2, a target of miR-15b. These studies suggest that sevoflurane may exert anti-apoptotic effects by inhibiting miR-15b expression leading to increased expression of Bcl-2.

Toll-like receptor preconditioning

Preconditioning can be induced by activation of the Toll-like receptors (94, 95). CpG oligodeoxynucleotide is a Toll-like receptor 9 agonist that when administered 72 h prior to ischemia can reduce injury in rodent and non-human primate models of experimental stroke (96, 97). MiRNA expression studies in adult male mice revealed that miRNAs are not regulated at 3, 24, or 72 h after CpG preconditioning (98), suggesting that alterations in miRNA expression in the brain are not necessary for the neuroprotection induced by systemic CpG preconditioning. Interestingly, CpG-preconditioned mice had fewer total miRNAs regulated in response to MCAO compared with saline-treated mice (57 vs. 73 miRNAs), suggesting that CpG preconditioning alters the miRNA response to stroke (98). Subsequent analysis of the miRNAs differentially expressed in CpG-preconditioned and saline-treated mice at 24 h after stroke revealed that miRNA suppression correlated with the upregulation of genes involved in neuroprotection, suggesting that suppression of differential miRNAs in CpG preconditioning may reprogram the genomic response to stroke to favor a protective outcome.

Hibernation torpor

Torpor is a form of sleep that helps animals conserve valuable resources in times of stress, such as in cold or very hot, dry weather. Hibernation torpor (HT) is used to model endogenous mechanisms of tolerance, as metabolic rates decrease in order to conserve energy in both conditions. HT induces massive global SUMOylation in ground squirrels, and overexpression of Ubc9, SUMO-1, or SUMO-2/3 is protective against ischemic injury in cortical neurons subjected to OGD, and in mouse brain (99, 100). SUMO conjugation and global protein conjugation by ubiquitin-like modifiers (ULMs) including NEDD8, ISG15, UFM1, and FUB1 significantly increased in ground squirrel brain during HT. MiRNA analysis of ground squirrel brain during HT revealed that the miR-200 and miR-182 families were among the most consistently depressed miRNAs relative to active animals (101). Inhibition of these miRNA families in SHSY5Y cells resulted in increased global protein conjugation and tolerance to OGD-induced cell death (101). These studies link endogenous tolerance to brain ischemia in hibernating animals to the regulation of a broad range of ULMs via repression of miRNAs.

NEUROPROTECTION VIA MANIPULATION OF miRNAs AND/OR THEIR TARGETS

The previous studies support that manipulation of specific miRNAs or their targets altered by IPC or ischemia may be translated into viable neuroprotective strategies for stroke. Identified targets thus far include epigenetic modifiers, heat shock proteins, ubiquitin modifiers, DNA repair and cell cycle proteins, autophagosome assembly proteins, and mediators of inflammatory responses to injury in astrocytes.

miR-29

Previous studies showed that miR-29 is highly expressed in adult rat brain, and is significantly downregulated by focal ischemia (51). Bioinformatic analysis revealed DNA (cytosine-5)-methyltransferase 3a (DNMT3a) as a target of miR-29c, suggesting that increased DNMT3a expression in ischemic brain may contribute to stroke injury (102). Indeed, treatment with premiR-29c was protective against ischemia both *in vitro* (PC12 cells) and *in vivo,* suggesting that strategies to replace miR-29c may decrease cell death following ischemia. In addition, the miR-29c gene promoter region contains binding sites for REST, and treatment of PC12 cells with REST siRNA prevented ischemia-induced downregulation of miR-29c, which maintained repression of DNMT3a resulting in significantly reduced ischemic cell death (102).

miR-132

Studies show that miR-132 is a neuron-specific miRNA that contains a RE1 site within its promoter regions in mouse and is a validated target of REST in mammalian cells (19). Transient global ischemia markedly decreased miR-132 expression in rat hippocampal CA1 cells at 24 and 48 h after ischemia, due to ischemia-induced enrichment of REST at the miR-132 promoter and repressed expression of miR-132 (103). REST-dependent silencing was specific to miR-132, as REST did not alter expression of miR-9 or miR-124a that also has RE1 sites in their promoter regions. In addition, the repression of miR-132 was selectively induced in vulnerable CA1 neurons, but not in CA3 neurons, supporting that REST-dependent repression of miR-132 is critical to ischemia-induced neuronal death (103). Depletion of REST *in vivo* blocked ischemia-induced loss of miR-132 in hippocampal neurons, while overexpression of miR-132 *in vitro* and *in vivo* afforded robust protection against ischemia-induced neuronal death. These studies support that REST-dependent repression of miR-132 induces neuronal death in global ischemia.

miR-181b and miR-134

The expression of miR-181b and miR-134 is decreased by HPC and ischemia in adult mouse cortex, but increased in tolerant brain (90). Targets for miR-181b include heat shock protein A5 (HSPA5) and ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1), and expression of miR-181b in N2A cells repressed HSPA5 and UCHL1 and protected cells against OGD-induced cell death. Further, miR-181b antagomirs reduced caspase-3 cleavage and neuronal cell loss in ischemic cortex and improved neurological deficits in mice after ischemia (104). A subsequent study showed that downregulation of miR-134 in mouse N2A cells is protective against OGD by upregulating heat shock protein A12B (HSPA12B) (105). HSPA12B was validated as a target of miR-134, and increased expression of HSPA12B correlated with attenuation of neural cell damage in OGD-treated N2A cells, and reduced infarct size and improved neurological outcomes in mice with ischemic stroke (4).

miR-30a

Downregulation of miR-30a following ischemia in N2A cells and in mouse brain correlated with an increased conversion ratio of LC3 (microtubule-associated protein 1 light chain 3)-II/LC3-I and increased expression of Beclin 1, which are required for formation of the autophagosome (106).

miR-124

Ischemia leads to reduced expression of miR-124 in rat brain (107, 108) and downregulation of miR-124 in rats subjected to focal ischemia correlated with increased expression of Ku70, a DNA

repair protein. *In vivo* administration of a miR-124 antagomir into rat brain increased expression of Ku70 mRNA and protein in the ischemic region, resulting in reduced cell death and infarct size, and improved neurological outcomes in rat (109).

miR-424

Expression of miR-424 is decreased in the plasma of patients with acute ischemic stroke, and in mouse plasma and brain after ischemia (110). Overexpression of miR-424 decreased infarct size and brain edema due to MCAO when administered either pre- or post-treatment. MiR-424 mimics reduced mRNA and protein levels of cell division cycle 25 homolog A (CDC25A), cyclin D1, and cell division protein kinase 6 (CDK6) in BV2 microglial cells, all of which are upregulated in ischemic brain (110).

miR-103-1

Recent studies examined miR-103-1 as a treatment strategy for stroke, based on its potential to regulate the expression of the Na^+/Ca^{2+} exchanger (NCX1), which mediates bidirectional flux of calcium and sodium across the synaptic membrane and increases ischemic damage in rat brain (111). NCX1 was validated as a target of miR-103-1 in cortical neurons, and intracerebroventricular infusion of anti-miR-103-1 not only prevented NCX1 reduction induced by stroke but also significantly reduced the extent of brain ischemia when infused 24 h before stroke onset (112).

Astrocyte miRNAs

Ischemic brain extract contains factors that elicit increased expression of stromal cell-derived factor-1 (SDF-1), a chemokine that is induced by proinflammatory stimuli and that activates leukocytes, in C6 cells and primary astrocytes (113). SDF-1 expression increased in preconditioned astrocytes contributes to brain tolerance via the regulation of the miR-223/miR-27b pathways (113). Neuroprotection by manipulation of miRNA-related pathways are also found in astrocyte-rich miRNAs, including miR-181 and miR-29 families, and miR-146a, and their validated targets, GRP78, and Bcl-2 family members (114).

EFFECTS OF POST-STROKE NEUROPROTECTIVE THERAPIES ON miRNAs

Recent studies show that post-stroke therapies can effectively regulate the expression of miRNAs and their targets, and these miRNAs may serve as direct targets for clinical intervention in stroke. For example, post-stroke treatment of rats with valproic acid, a histone deacetylase inhibitor and a mood stabilizer, upregulated the expression of several miRNAs including miR-331 and miR-885-3p, and improved neurological deficits and motor performance following ischemia (115). In addition, studies show that using Velcade (a proteasome inhibitor that may also suppress TLR signaling) administered in combination with tissue plasminogen activator (tPA) is neuroprotective against ischemia in aged rats (116). The combination of Velcade plus tPA resulted in increased levels of miR-l46a on cerebral endothelial cells, which express TLRs, and outcomes were associated with reduced expression of vascular interleukin-1 receptor-activated kinases 1 (116). Further, it is well known that ischemic injury is more severe in older animals as compared to younger animals, and this difference is associated with reduced availability of insulin-like growth factor-1 (IGF-1) whose levels decrease with age. IGF-1 infusion following stroke, which prevents estrogen neurotoxicity in middle-aged female rats, altered the expression of two conserved IGF pathway regulatory miRNAs, Let-7f, and miR-1. Adult female rats treated with anti-miR-1 as late as 4 h after ischemia had significantly reduced cortical infarct volume, and anti-Let-7 robustly reduced both cortical and striatal infarcts and preserved sensorimotor function and inter-hemispheric neural integration (117). However, anti-Let-7 was only effective in intact females and had no effect on males or ovariectomized females, indicating that the gonadal steroid environment critically modifies miRNA action (117). Further studies examined the effect of post-stroke IGF-1 treatment on miRNA expression in middle-aged female rats (118). IGF-1 administered immediately after ischemia significantly reduced infarct volume in 9- to 11-month female rats 24 h after stroke. Post-stroke IGF-1 treatment significantly downregulated 8 out of 168 disease-related miRNAs in tissue isolated 4 h after ischemia. KEGG pathway analysis predicted that targets of these miRNAs include proteins involved in PI3K–Akt signaling, cell adhesion/ECM receptor pathways, and T-and B-cell signaling. Further, Phospho-Akt was reduced by 4 h after ischemia, but was elevated by IGF-1 treatment at 24 h after ischemia. IGF-1 also induced Akt activation, an effect that was preceded by a reduction of BBB permeability and global suppression of cytokines at 4 h post-stroke (118).

CONCLUSION

The goal of this review was to present the current state of knowledge regarding ncRNAs and their role(s) in stroke and neuroprotection. However, of these three relatively new families of ncRNAs, miRNAs are the most well studied to date. Yet the lncRNAs and piRNAs are certain to contribute to the setting of ischemia and neuroprotection, and there are several important future lines of investigation for all ncRNA studies. The foregoing studies highlight the difficulty of analyzing miRNA expression data, due to the diversity of ischemia and neuroprotection models, the time points examined, and the fact that since miRNAs can act in concert to exert effects on target mRNAs, focus on a single miRNA is unlikely to realistically capture endogenous responses. Thus, the first such studies must focus on ways to meaningfully translate the outcome of multiple expression profiling studies from diverse labs in order to identify global signaling pathways or targets that have been underappreciated to date. To address this issue, Xiao and colleagues sought to model complex interactions in order to identify synergistic effects within miRNA responses to brain ischemia (119). miRNA array expression data submitted to the Gene Expression Omnibus database from multiple studies in different labs were examined to identify mouse brain miRNAs differentially expressed between ischemia and control. Common predicted targets of the miRNAs were then used as a candidate subset to identify functional modules. An miRNA functional synergistic network, constructed by assembling all miRNA synergistic pairs, revealed 51 differentially expressed miRNAs identified between ischemia and control (32 upregulated and 19 downregulated). Among them, 24 miRNAs commonly regulating at least one target gene were used to construct a network. Subsequent analyses revealed 16 miRNAs forming 20 miRNA

interaction pairs that participated in inflammatory responses, suggesting these 16 miRNAs as underlying targets for prevention and/or treatment of stroke (119). These analyses highlight that the power of global miRNA and ncRNA studies lies in their potential to identify proteins or pathways involved in responses to ischemia. The second such studies must address the need to identify the effects of ncRNAs acting in concert to regulate cellular phenotypes or responses to stress. To date, each family of ncRNA has largely been examined individually, due to technical barriers to combinatorial analysis of all ncRNAs. RNAseq studies can be used to identify all RNA species in a given sample set, but even this method is limited in the ability to analyze distinct sizes of RNA species. Thus, lncRNAs (>200 nt) are not yet typically sequenced together with small ncRNAs such as the miRNAs and piRNAs (~20–36 nt). A third line of exciting studies will come from the not-so-recent finding that extracellular RNAs exist in biofluids, mostly contained within EVs such as microvesicles, and can serve as biomarkers of neurological disorders (120). EVs and their molecular components are increasingly shown to be effectors of cell–cell communication in the brain and their uptake can alter the phenotype of a recipient cell (32). Thus, future lines of investigation should include examining the consequences of disrupted blood flow and metabolism, such as during a stroke, on communication mediated by EVs. Further, given that EVs mediate communication between distinct cell types in the brain, future therapeutic strategies for the treatment or prevention of stroke and other brain disorders might include focused delivery of molecular or pharmaceutical agents by EVs, potentially using vesicles isolated from ones own biofluids. The importance and excitement of such studies is highlighted by the recent formation of the Extracellular RNA Communication program (http://commonfund.nih.gov/Exrna/index) supported by the National Institutes of Health Common Fund that aims to discover fundamental biological principles about the mechanisms of exRNA generation, secretion, and transport; to identify and develop a catalog of exRNA in normal human body fluids; and to investigate the potential for using exRNAs as therapeutic molecules or biomarkers of disease. Thus, there are many new and exciting future opportunities for research and development in the field of ncRNAs, and in particular for their role in, contribution to, and regulation of the treatment of human diseases, including stroke.

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REVIEW
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Role of MicroRNAs in innate neuroprotection mechanisms due to preconditioning of the brain

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Insults to the brain that are sub-threshold for damage activate endogenous protective pathways, which can temporarily protect the brain against a subsequent harmful episode. This mechanism has been named as tolerance and its protective effects have been shown in experimental models of ischemia and epilepsy. The preconditioning-stimulus can be a short period of ischemia or mild seizures induced by low doses of convulsant drugs. Gene-array profiling has shown that both ischemic and epileptic tolerance feature large-scale gene down-regulation but the mechanism are unknown. MicroRNAs are a class of small non-coding RNAs of ∼20–22 nucleotides length which regulate gene expression at a post-transcriptional level via mRNA degradation or inhibition of protein translation. MicroRNAs have been shown to be regulated after non-harmful and harmful stimuli in the brain and to contribute to neuroprotective mechanisms. This review focuses on the role of microRNAs in the development of tolerance following ischemic or epileptic preconditioning.

Keywords: preconditioning, microRNAs, ischemia, epilepsy, neuroprotection

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Preconditioning

The concept of preconditioning was first described in a model of heart ischemia. Here, brief ischemic episodes protected against a subsequent ischemic insult (Murry et al., 1986), opening a new way for the treatment of heart diseases. Several preconditioned treatment paradigms have been used in the clinic (including remote preconditioning stimulus) to protect patients against an ischemic insult in heart pathologies (McCafferty et al., 2014).

Preconditioning is an adaptive response where a small dose of a harmful substance protects the brain from a subsequent damaging insult (Murry et al., 1986; Stenzel-Poore et al., 2007; Lin et al., 2008; Dirnagl et al., 2009; Assaf et al., 2011). The fact that any injury to the brain applied below the threshold of cell damage, including seizures, will induce preconditioning and neuroprotection to the brain shows the importance of understanding the mechanism underlying preconditioning and its potential as novel treatment option in brain disorders.

The preconditioning stimulus induces a transient state of protection which is called tolerance. Early work demonstrated that de novo protein synthesis was required for tolerance; this implied regulation of gene expression in a time-specific manner and having an effect only several days after the precondition stimulus (Barone et al., 1998).

Preconditioning can induce neuroprotection over two time-frames: (1) Rapid tolerance, which happens in a short period of time and is independent of protein production and is associated with synapse remodeling (Meller et al., 2008). (2) Delayed (classical) tolerance, which evolves over 1–3 days post-preconditioning and requires de novo protein production which peaks after 3 days

and diminishes over the course of the following week (Stenzel-Poore et al., 2007).

Preconditioning Stimuli

Preconditioning can be induced by several stimuli in the brain, including non-injurious ischemia, cortical spreading depression, brief episodes of seizures and low doses of endotoxins (LPS, lipopolysaccharide) (Kitagawa et al., 1990; Simon et al., 1993; Kobayashi et al., 1995; Chen and Simon, 1997; Towfighi et al., 1999). A cross-tolerance phenomenon has been recognized where stimuli and challenge are from different nature. For example, a brief-seizure stimulation or LPS injection can protect against a subsequent ischemic injury (Plamondon et al., 1999; Towfighi et al., 1999).

Data has shown that the reprogramming of genes involved in cellular response to excitotoxic insults plays an important role in the preconditioning process of different tissues (Mirrione et al., 2010). Evidence from genomic studies has demonstrated that diverse stimuli which trigger neuroprotection achieved by preconditioning may share a common process which depends on a fundamental reprogramming of the response to injury, e.g., in ischemia or epileptic tolerance has been shown that 80% of genes are down-regulated. This reprogramming process can induce novel neuroprotective pathways which in turn lead to the synthesis of new proteins changing the molecular and genetic response to subsequent injury (Stenzel-Poore et al., 2004, 2007).

In experimental models of *status epilepticus*, several preconditioning stimuli including bicuculline, electroshock, kindling, and low dose injection of the convulsant kainic acid, has shown to reduce brain damage after *status epilepticus* (Amini et al., 2014). Gene profiling has shown that down-regulation of gene expression is a general mechanism underlying the tolerance process (Borges et al., 2007; Jimenez-Mateos et al., 2008; Meller et al., 2008; Della-Morte et al., 2012), including downregulation of genes involved in ion channels, calcium signaling and excitability (Jimenez-Mateos et al., 2008).

microRNAs

The term MicroRNA, previously called small temporal RNAs (stRNAs) due to their role in the regulation of developmental timing of *Caernohabditis elegans,* was first introduced in 2001 (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Originally microRNAs were defined as small non-coding RNAs (∼20–22 nucleotides) that regulate post-transcriptional gene expression in a sequence-specific manner. Since their discovery in *C. elegans*, microRNAs were found to be expressed in invertebrates and vertebrates, including humans, and many show highly-conservation during evolution (Lagos-Quintana et al., 2001).

MiRNAs regulate gene expression via translational inhibition, mRNA degradation or a combination of both mechanisms (O'Carroll and Schaefer, 2013). In the brain, however, miRNA targeting is frequently not associated with reduced mRNA levels of targets (Klein et al., 2007). Almost 50% of all identified miRNAs are expressed in the mammalian brain and there is significant cell- and region-specific distribution reflecting roles in gene expression that direct the functional specialization of neurons and the morphological responses that are required to adapt to their continuously changing activity state (Siegel et al., 2011; O'Carroll and Schaefer, 2013). MiRNAs and their biogenesis components display select localization within neurons, with significant enrichment in dendrites, enabling local, activity-dependent miRNA regulation of protein levels (Lugli et al., 2005, 2008; O'Carroll and Schaefer, 2013). Recent work demonstrated that certain pre-miRs have localization signals which translocate them to the synaptic sites where they are processed into mature miRNA (Bicker et al., 2013).

Most miRNA genes are expressed as a cluster within a single poly-cistronic transcript (Baskerville and Bartel, 2005) and transcribed by RNA polymerase II, resulting in the primary sequence (Cai et al., 2004; Lee et al., 2004). In the nucleus the primary sequence is recognized by the nuclear microprocessor complex containing Drosha and DGCR8 (DiGeorge syndrome critical region 8) which generate the precursor-miRNA (pre-miR) of ∼60–70 nt length (Lee et al., 2003). Next, the precursor is exported into the cytoplasm by Exportin-5 in a GTP-dependent manner (Bohnsack et al., 2004). Once in the cytoplasm, microRNAs are further processed by Dicer to a single-strand RNA mature form (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Finally, a single strand of the mature miRNA is selected and loaded into the RISC complex and bound to the members of the Argonaute (Ago) protein family. This miRNA-bound-RISC complex is the functional and active form of the miRNA which then targets the mRNA. Binding of a 7–8 nt seed region, usually within the 3′UTR of the mRNA, results in either inhibition of the translation or mRNA degradation. This process requires the activation of de-cap enzymes and removal of the poly-A tail (Kwak and Tomari, 2012).

Several studies have shown the role of miRNAs in the brain by using mainly genetic tools in mice, including conditional or full deletion of biogenesis enzymes in the microRNA pathway. Deletion of DGCR8, which affects the production of the precursor microRNA, results in a reduction in brain size and loss of inhibitory synaptic neurotransmission (Babiarz et al., 2011; Hsu et al., 2012). Conditional deletion of Drosha in neural progenitors did not affect neurogenesis in the developing brain, but did affect differentiation and migration of neurons (Knuckles et al., 2012). Deletion of Dicer from neurons produces severe brain abnormalities, including microencephaly and defects in dendritic arborization in cortex and hippocampus (Davis et al., 2008; De Pietri Tonelli et al., 2008; Babiarz et al., 2011; Dorval et al., 2012). Mice lacking Dicer in astrocytes develop spontaneous seizures and suffer from increased premature mortality (Tao et al., 2011). These data imply that the miRNA biogenesis system is essential for brain development and function. Surprisingly, one study reported that specific deletion of Dicer in the adult mouse forebrain transiently enhanced learning and memory, although these animals later displayed degeneration of neurons in the cortex and hippocampus (Konopka et al., 2010).

Analysis of Argonaute (Ago1–4) proteins, main components of the RISC complex, has given more diverse results (Burroughs et al., 2011). Ago-2 is the most abundant expressed member in

various tissues including the brain (Liu et al., 2004) and critical for miRNA-mediated repression of mRNAs (Czech and Hannon, 2011). Deficiency in Ago-2 results in death of mice during early embryogenesis or mid-gestation (Morita et al., 2007). This reflects not only the essential role of Ago2 in embryonic development but perhaps an effect of impaired microRNA generation (Morita et al., 2007). In contrast, studies in conditional mutants showed individual deficiencies in Ago 1, 3, and 4 does not produce obvious effects in mice, suggesting a redundancy among Ago family members (O'Carroll et al., 2007).

microRNA in Ischemia Preconditioning

Three different publications show the regulation of microRNAs after an ischemic preconditioning stimulus in parallel (**Table 1**) (Dharap and Vemuganti, 2010; Lee et al., 2010; Lusardi et al., 2010).

Lusardi et al., using a brief ischemic stimulus in mice, showed that preconditioning at the same time up-regulated and down-regulated different microRNAs in the mouse cortex (Lusardi et al., 2010). A common target-gene of the down-regulated microRNAs was the transcriptional regulator MeCP2 (methyl CpG binding protein 2) (Lusardi et al., 2010). Similar results were found when hypertension was used as preconditioning stimulus in rats, where multiple down-regulated microRNAs target MeCP2 (Dharap and Vemuganti, 2010). MeCP2 was first described as a potent transcriptional repressor (Nan, 1998). Mutations in MeCP2 cause Rett syndrome, mental retardation, Angelman syndrome, and autism (Hite et al., 2009; Gonzales and LaSalle, 2010). The up-regulation of MeCP2 via microRNAs corroborated the general gene-repression in tolerance reported before (Lusardi et al., 2010), as increase levels of MeCP2 resulted in a down-regulation of the transcriptome.

In contrast to these studies, Lee et al. showed that neuroprotection achieved by brief ischemic preconditioning stimuli in mice could be due to a family member of miR-200 which targets PHD2 (prolyl hydroxylate 2), which in turn has been involved in the regulation of HIF1α after ischemia (Lee et al., 2010). Up-regulation of miR-200 shortly after the preconditioning stimulus reduced the levels of PHD2 and increased HIF1α. These results were corroborated *in vitro*, where the up-regulation of miR-200 using mimic decreased levels of PHD2 (Lee et al., 2010). Supporting these results, transgenic mice lacking HIF1α exposed to hypoxic preconditioning presented less neuroprotection than control mice after an hypoxia-ischemic insult (Sheldon et al., 2014).

TABLE 1 | MicroRNAs in preconditioning.

Preconditioning stimulus	microRNAs	Target gene	References
Ischemia	Pull ofmiRs	MeCP2	Lusardi et al., 2010
Ischemia	miR-200	PhP2	Lee et al., 2010
Hybernation	miR-200	SUMO	Lee et al., 2012
Epileptic	miR-184	Not determined	McKiernan et al., 2012

More recently, a model of hibernation, a natural model of tolerance to ischemia, was used to study the role of microRNAs during neuroprotection in the brain (Lee et al., 2012). During torpor stages, hibernating animals lower their energy consumption, blood flow, and body temperature to an otherwise lethal level which is similar to levels during an ischemic insult (Lee et al., 2012). Microarray analysis from ground squirrel brains were performed during the active and the torpor phase and 405 microRNAs were different between both stages, with the family of miR-200 being the most representative, consistent with Lee et al. (2010). In this study the authors suggest that miR-200 family could regulate members of the SUMO family which is involved in the ubiquitin regulation and protein degradation, thereby explaining the reduction in protein levels during tolerance (Lee et al., 2012).

microRNA in Epileptic Preconditioning

Similar to ischemia, several studies analyzed the role of microRNAs after a preconditioning-seizure stimuli (**Table 1** and Hatazaki et al., 2007; Kretschmann et al., 2015).

McKiernan and colleagues by using low dose of systemic KA in mice showed a de-regulation of microRNA after epileptic preconditioning. Here, a general up-regulation of microRNAs was the main response in preconditioned mice compared to control mice (McKiernan et al., 2012). Whereas 9 microRNAs were uniquely present in the preconditioning group, 39 microRNAs showed increase and 6 reduced levels in the pre-conditioning group. From the up-regulated microRNAs, 25 of the 39 were significantly regulated (McKiernan et al., 2012). Between the up-regulated microRNA group, the highest levels were found for miR-184. Inhibition of miR-184 *in vivo* resulted in neuronal death after a normally non-damaging preconditional stimulus (McKiernan et al., 2012) and an increase in neurodegeneration during the tolerant state after status epilepticus (damaging injury stimulation). Together, these results show a neuroprotective role of miR-184 during preconditioning (McKiernan et al., 2012).

Similar results have been found in acute seizure models not associated with neuronal death (Kretschmann et al., 2015). Kretschman and colleagues showed that 6 h after the induction of a single seizure via electrical stimulation up-regulation of microRNAs was the main response. 146 microRNAs were de-regulated, with almost 140 microRNAs being up-regulated and only few of them down-regulated (Kretschmann et al., 2015).

In a more detailed study, the expression levels of microRNAs were analyzed after electroshock stimulation, showing that microRNA levels follow three distinct patterns after non-damage neuronal activity (Eacker et al., 2011). MicroRNA regulation can be clustered in three distinct groups: Class 1, microRNAs which progressively increase their expression levels after electrical stimulation, including miR-134; Class 2, microRNAs which are strongly up-regulated in the first hours after stimulation with levels decreasing back to normal rapidly; Class 3, microRNAs that increase initially but decrease at a later time-point (12 or 24 h after the stimulation) (Eacker et al., 2011). This study shows the specific response of microRNAs after brain stimulation, and its possible role in the different tolerant-stages.

Targeting microRNAs on Brain

Several approaches for manipulating miRNA levels in neuronal cells *in vitro* and *in vivo* have been described, including recent applications of miRNA antisense oligonucleotides, miRNA gene knockout and miRNA sponges in neuronal cells. The efficiency of microRNAs inhibitors has been reported several times, inhibition of miR184 show a loss in the preconditioning neuroprotection without any toxicity (McKiernan et al., 2012). In contrast, no many approaches have shown over-expression of microRNAs in the brain, possible related with the toxic effect increase levels of RNA in the brain. The study of new drugs which target microRNAs in the central nervous system will be one of the main fields of future investigations.

Conclusions

Studying the molecular mechanism underlying the innate neuroprotection can help to understand how the brain protects itself against a damaging insult. Analysis of tolerant states has shown that a general down-regulation of genes is the main response in the brain, independently of the original insult, ischemic, or seizures. MicroRNAs are master regulators of gene-expression, its regulation after the preconditioning stimulus could be causative of the general suppression of gene-expression and being a good candidate for future target-therapy. Still several questions are remaining: Can we regulate microRNAs in the brain? Systemic administration of microRNA mimics or microRNA inhibitors will be necessary. Can we regulate microRNA expression in a time dependent manner? Tolerant is a time-dependent effect which only last for several days, a longer inhibition or over-expression of microRNAs could be toxic for the brain.

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REVIEW
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Ionic homeostasis in brain conditioning

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Most of the current focus on developing neuroprotective therapies is aimed at preventing neuronal death. However, these approaches have not been successful despite many years of clinical trials mainly because the numerous side effects observed in humans and absent in animals used at preclinical level. Recently, the research in this field aims to overcome this problem by developing strategies which induce, mimic, or boost endogenous protective responses and thus do not interfere with physiological neurotransmission. Preconditioning is a protective strategy in which a subliminal stimulus is applied before a subsequent harmful stimulus, thus inducing a state of tolerance in which the injury inflicted by the challenge is mitigated. Tolerance may be observed in ischemia, seizure, and infection. Since it requires protein synthesis, it confers delayed and temporary neuroprotection, taking hours to develop, with a pick at 1–3 days. A new promising approach for neuroprotection derives from post-conditioning, in which neuroprotection is achieved by a modified reperfusion subsequent to a prolonged ischemic episode. Many pathways have been proposed as plausible mechanisms to explain the neuroprotection offered by preconditioning and post-conditioning. Although the mechanisms through which these two endogenous protective strategies exert their effects are not yet fully understood, recent evidence highlights that the maintenance of ionic homeostasis plays a key role in propagating these neuroprotective phenomena. The present article will review the role of protein transporters and ionic channels involved in the control of ionic homeostasis in the neuroprotective effect of ischemic preconditioning and post-conditioning in adult brain, with particular regards to the $Na^+/Ca2^+$ exchangers (NCX), the plasma membrane $Ca2^+$-ATPase (PMCA), the Na^+/H^+ exchange (NHE), the $Na^+/K^+/2Cl^-$ cotransport (NKCC) and the acid-sensing cation channels (ASIC). Ischemic stroke is the third leading cause of death and disability. Up until now, all clinical trials testing potential stroke neuroprotectants failed. For this reason attention of researchers has been focusing on the identification of brain endogenous neuroprotective mechanisms activated after cerebral ischemia. In this context, ischemic preconditioning and ischemic post-conditioning represent two neuroprotecive strategies to investigate in order to identify new molecular target to reduce the ischemic damage.

Keywords: transporters, ionic exchangers, stroke, preconditioning/post-conditioning, brain ischemia

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Ischemic Preconditioning

The concept of ischemic preconditioning was introduced first in the heart (Murry et al., 1986) and later on in the brain (Murry et al., 1986; Schurr et al., 1986). Ischemic preconditioning is neuroprotective strategy in which a subliminal stimulus is applied before a subsequent harmful ischemia (Kirino, 2002; Dirnagl et al., 2003; Gidday, 2006). The state of tolerance mediated by this strategy is composed of two phases of neuroprotection. The first phase, named *rapid or acute preconditioning* (Perez-Pinzon et al., 1997), starts 3–5 min after the preconditioning stimulus and ends 1 h later and is due to rapid post-translational protein modifications (Barone et al., 1998; Meller et al., 2006). The second phase, named *delayed preconditioning* (Perez-Pinzon et al., 1997), starts 2–3 days after preconditioning and ends 1 week later and mainly involves *de novo* protein synthesis (Barone et al., 1998; Dirnagl et al., 2003; Meller et al., 2005).

Mechanisms Involved in Ischemic Preconditioning Neuroprotection

The molecular mechanisms underlying ischemic tolerance are not yet fully understood because of its extreme complexity, involving many signaling pathways and alterations in gene expression. Several studies have investigated the signaling cascades and key molecules both in *in vitro* and *in vivo* models of ischemic preconditioning. Most of the stressors induce both rapid and delayed tolerance. As recently reviewed (Obrenovitch, 2008), the features of ischemic tolerance partially are similar to naturally occurring adaptive mechanisms, including at cellular levels:

1. Alterations in cellular energy metabolism: preconditioning preserves mitochondrial function (Dave et al., 2001; Racay et al., 2007) and mediates an increase in expression of genes playing a role in energy metabolism.
2. Preservation of mitochondrial membrane potential: preconditioning preserves respiratory complexes, mitochondrial oxidative phosphorylation and antioxidant enzymes such as superoxide dismutases (SODs), catalase, glutathione peroxidase, and thioredoxin system (Danielisová et al., 2006; Racay et al., 2007).
3. Modulation of neuronal excitotoxicity: preconditioning mediates a shift to inhibitory neurotransmission, suppressing glutamate release, downregulating AMPA and NMDA receptors, and glial glutamate transporters, increasing GABA release and GABA-A receptors expression (Dave et al., 2005).
4. Suppression of cell death and apoptotic mechanisms: preconditioning reduces the release of cytochrome c, inhibits caspases, and proapoptotic genes, and activates survival pathways such as serine/threonine activated kinases (Akt) and extracellular signal-regulated kinases (ERK) and trophic factors (Lehotský et al., 2009).
5. Activation of the mechanisms mediating DNA repair and plasticity by increasing the activity nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Gidday, 2006; Lehotský et al., 2009).
6. Reduction of inflammatory response: preconditioning activates Toll-like receptor, suppresses induced proinflammatory cytokines (TNFs) and activates transcription nuclear factor κB (NFκB) (Gidday, 2006).
7. Cerebrovascular adaptation by vascular remodeling: preconditioning activates vascular endothelial growth factors (VEGF), erythropoietin and heme oxygenase 1 as targets of hypoxia inducing factor 1α (HIF-1α). The blood–brain barrier was preserved thanks to the reduced activity of matrix metalloproteinases and cell adhesion molecules (Gidday, 2006).
8. Improved capacity to preserve cellular ionic and pH homeostasis: preconditioning regulates expression and activity of ion transport systems ($Ca2^{+}$-ATPase, acid-sensing ion channels, Na^{+}/H^{+} exchange, $Na^{+}/K^{+}/2Cl^{-}$ cotransport and $Na^{+}/Ca2^{+}$ exchanger) and transporters for metabolites.

Ischemic Post-conditioning

Unlike ischemic preconditioning, ischemic post-conditioning is a relatively novel neuroprotective strategy (Zhao et al., 2006; Pignataro et al., 2008). Rapid revascularization of the occluded vessels represent one of the most effective approaches currently used for the treatment of acute ischemic stroke. However, during the early reperfusion phase an increase in reactive oxygen species and intracellular free Ca^{2+} may occur, thus leading to additional injury (Kuroda and Siesjö, 1997). Ischemic post-conditioning is realized by applying brief interruptions of reperfusion after ischemia. That a protocol of cycles of brief reperfusion followed by re-occlusion was able to reduce the ischemic damage was first demonstrated during cardiac ischemia (Zhao et al., 2003). More recently, it has been demonstrated that ischemic post-conditioning induces neuroprotection also in a rat hippocampal slice model of cerebral ischemia (Scartabelli et al., 2008), in rodent models of spinal cord injury (Jiang et al., 2006), and in models of focal (Zhao et al., 2006; Pignataro et al., 2008, 2013b; Xing et al., 2008), and global ischemia (Pateliya et al., 2008; Wang et al., 2008).

Mechanisms Involved in Ischemic Post-conditioning Neuroprotection

At present a few information are available about the mechanisms involved in the neuroprotection mediated by ischemic post-conditioning. Since post-conditioning is applied after the ischemic injury, the changes observed in the brain have to be ascribed to the combined effects of post-conditioning and stroke, rather than the effect of the post-conditioning only. Thus, it is difficult to sunder the protective mechanisms of post-conditioning from the consequences of its protection. However, up until now, many mechanisms have been suggested (Zhao, 2009), such as:

1. Reduction of hyperemia consequent to the reperfusion, that may be accompanied other toxic events, such as the production of free radicals, loss of blood–brain barrier integrity, and activation of inflammation cascade (Schaller and Graf, 2004).

2. Blocking of apoptosis. Ischemic post-conditioning reduced ROS production (Zhao et al., 2006), attenuates lipid peroxidate levels (Xing et al., 2008) and increased the activities of antioxidant enzymes, such as superoxide dismutase and catalase (Danielisová et al., 2006). Furthermore, rapid post-conditioning reduced the release of cytochrome c from the mitochondria to the cytosol, a critical cascade for the induction of apoptosis, thus suggesting that post-conditioning may reduce ischemic injury by blocking apoptosis (Zhao et al., 2012).
3. Inhibition of inflammation. Rapid post-conditioning inhibits inflammation after stroke. Indeed, rapid post-conditioning inhibits myeloperoxidase activity, an indicator of leukocyte accumulation (Xing et al., 2008), attenuates the expressions of IL-1β, TNF-α, and the ICAM-1 protein expression (Xing et al., 2008).
4. Activation of Akt Pathway. In agreement with previous studies demonstrating that the Akt pathway promotes neuronal survival after stroke (Zhao et al., 2006), rapid post-conditioning increases Akt phosphorylation and Akt activity (Gao et al., 2008; Pignataro et al., 2008). Furthermore, the protection mediated by rapid post-conditioning was prevented by Akt inhibition (Gao et al., 2008; Pignataro et al., 2008) and abolished the protective effect post-conditioning in hippocampal slice cultures (Scartabelli et al., 2008), overall, ischemic post-conditioning exerts its neuroprotective effectiveness also through Akt activation.
5. Cellular ionic homeostasis and energy metabolism. Post-conditioning-mediated neuroprotection is correlated to the maintainance of ionic homeostasis occurring by modulating expression and activity of several proteins involved in ionic homeostasis, some of which will be treated below (**Figure 1**, **Table 1**).

Brain Ionic Transporters during Ischemic Conditioning

Ischemic brain conditioning activates intracellular biological responses to a potential lethal insult; therefore it is predictable that organs reinforce their tolerance when exposed to a sublethal insult prior or after a harmful episode of ischemia by increasing energy metabolism or delaying anoxic depolarization after the onset of the subsequent ischemic insult (Stenzel-Poore et al., 2003; Pignataro et al., 2008, 2009). Indeed, an impairment in the activity of voltage-gated potassium channels has been observed in cortical neurons exposed to a brief non-injurious oxygen and glucose deprivation (OGD). Moreover, it has been demonstrated that ischemic conditioning prevented the inhibition of Na^+/K^+-ATPase activity after brain ischemia in hippocampal and cortical neurons of rats subjected to global forebrain ischemia. Instead, *in vivo* experiments in gerbils showed that both Ca^{2+}-ATPase activity and mitochondrial calcium internalization increased in CA1 hippocampal neurons exposed to preconditioning (Ohta et al., 1996). Accordingly, in hippocampal neurons obtained from preconditioned gerbils $[Ca^{2+}]_i$ increased after anoxic and aglycemic episodes whereas this increase was inhibited in ischemic-tolerant animals (Shimazaki et al., 1998). The molecular mechanisms of this phenomenon are still under investigation. An explanation may be the increase in the expression of the plasma membrane calcium ATPase 1 (PMCA-1) as recently demonstrated (Kato et al., 2005). Moreover, many evidence support the idea that other transporters are involved in the protection mediated by brain conditioning. In this regard, a key role is exerted by the sodium calcium exchanger (NCX), in fact its expression was reduced during cerebral ischemia (Pignataro et al., 2004; Boscia et al., 2006). More important, NCX downregulation was prevented when rats were subjected either to ischemic preconditioning or post-conditioning (Pignataro et al., 2013a). In addition Na^+/H^+ exchanger and $Na^+/K^+/2Cl^-$ cotransporter are critical ion transporters in the context of cerebral ischemia, as they contribute to the regulation of intracellular pH and cell volume (Pedersen, 2006). Finally, acid-sensing cation channels (ASIC) have also been studied during ischemic conditions since they represent key targets in many different aspects of acidosis, a condition that occurs during ischemic stroke. Brain preconditioning as an adaptive response to stroke requires in particular new protein transcription and translation; giving that HIF-1–dependent transcription is being clearly common in activating pathways of several exchangers/transporters/ionic channels involved in stroke, scientific community has assigned a key role to HIF-1α, as a master transcriptional regulator of cellular and developmental response to hypoxia. In effect stable HIF-1 can bind to its heterodimeric partner HIF-1β, and together these proteins, once stabilized during hypoxic-ischemic conditions, can act in the nucleus to transactivate genes involved in adaptation to hypoxic-ischemic stress. HIF represents the "hyphen" that definitely binds to each other all the different mechanisms converging on the same signaling cascade, responsible for the homeostatic regulation in protected brain (Ratan et al., 2004). In the light of these premises, the present article will review the main role of protein transporters and exchangers controlling ionic homeostasis in the neuroprotective effect of ischemic preconditioning and post-conditioning, with particular regards to the Na^+/Ca^{2+} exchangers (NCX), the plasma membrane Ca^{2+}-ATPase (PMCA), the Na^+/H^+ exchange (NHE), the $Na^+/K^+/2Cl^-$ cotransport (NKCC) and the acid-sensing cation channels (ASIC).

Role of Na^+/H^+ Exchange (NHE) and $Na^+/K^+/2Cl^-$ Cotransport (NKCC) In Brain Conditioning

The ubiquitous plasma membrane Na^+/H^+ exchanger is protein highly conserved across vertebrate species and characterized as a major membrane transporter involved in the regulation of cellular pH and volume in the nephron of the kidney. Specifically it is present in the intercalary cells of the collecting duct (Pedersen, 2006). The $Na^+/K^+/2Cl^-$ cotransporter (NKCC) is a protein that aids in the active transport of sodium, potassium, and chloride into and out of cells; it is widely distributed throughout the body and it has important functions in organs that secrete fluids. NKCC2 is found specifically in the kidney,

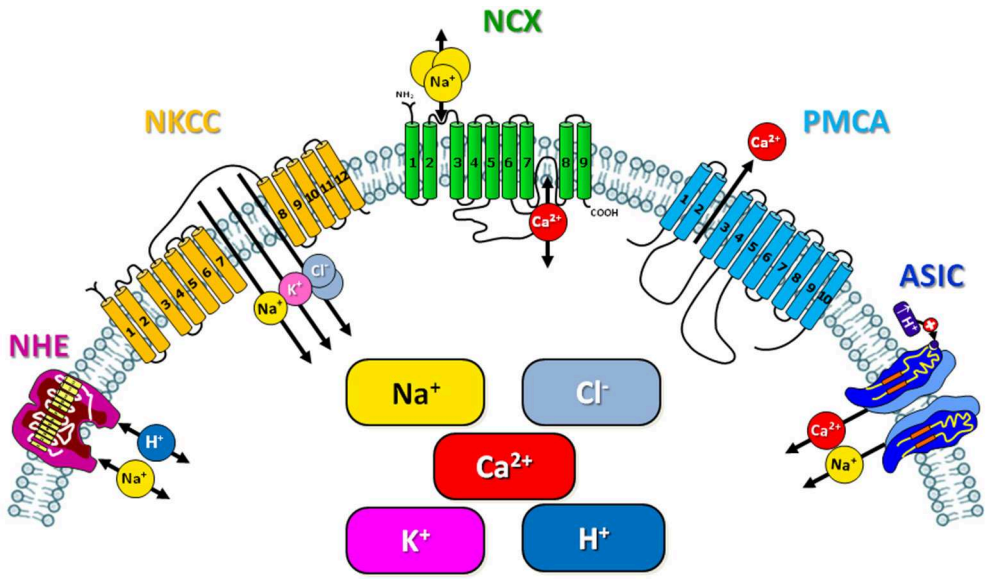

FIGURE 1 | Schematic representation of the ionic exchangers/transporters involved in the neuroprotection mediated by ischemic preconditioning and post-conditioning.

TABLE 1 | Role of ionic exchangers/transporters during pre-and post-conditioning.

Ionic exchanger/ Transporter	Role during preconditioning	Role during post-conditioning	References
$Na^+/K^+/2Cl^-$ Cotransport (NKCC)	**Neuroprotective role:** Activation of NKCC participates in the protection mediated by ischemic cardiac preconditioning	**Neuroprotective role:** Activation of NKCC1 mimiks an *in vitro* post-conditioning	Schaefer et al., 1995; Yang et al., 2013
Na^+/H^+ Exchange (NHE)	**Neurodegenerative role:** NHE inhibition delays pH recovery thus mediating protection	**Neurodegenerative role:** NHE inhibition delays pH recovery thus mediating protection	Shipolini et al., 1997; Goldberg et al., 2002
ASIC	**Neurodegenerative role:** ASIC expression decrease during conditioning	**Neurodegenerative role:** ASIC expression decrease during conditioning	Pignataro et al., 2011a
NCX	**Neuroprotective role:** NCX expression increase during conditioning and its inhibition or silencing prevents neuroprotection	**Neuroprotective role:** NCX expression increase during conditioning and its inhibition or silencing prevents neuroprotection	Pignataro et al., 2011b, 2012
PMCA	**Neuroprotective role:** PM CA 1 is increased in the hippocampus following preconditioning	Role not known	Shimazaki et al., 1998

where it serves to extract sodium, potassium, and chloride from the urine so that they can be reabsorbed into the blood (Haas, 1994).

Na^+/H^+ exchanger and $Na^+/K^+/2Cl^-$ cotransporter are critical ion transporters in the context of cerebral ischemia, as they contribute to the regulation of intracellular pH and cell volume (Pedersen, 2006). In particular, while the mammalian NHE is an integral membrane transport protein that mediates an electroneutral 1:1 exchange of intracellular H^+ for extracellular Na^+ (Xia et al., 2003), the electroneutral NKCC contransporter mediates the influx of Na^+, K^+, and Cl^- with a stoichiometry of $1Na^+:1K^+:2Cl^-$.

Due to this dual role it is difficult to predict whether a change in the expression and in the activity of these transporters may ameliorate or impair the susceptibility of cells to ischemia; for example, enhanced Na^+/H^+ exchange would help to reduce the intracellular pH but, on the other hand, it would promote cell swelling and further Ca^{2+} influx through Na^+/Ca^{2+} exchange. In particular, in cortical astrocyte cultures obtained from Na^+/H^+ exchanger isoform 1-deficient NHE1(-/-) mice the lack of NHE1

attenuated the damage induced by conditions mimicking cerebral ischemia *in vitro*, and this effect was confirmed also *in vivo* (Kintner et al., 2004). Indeed, the pharmacological blockade of NHE-1 reduces infarct volume in animal models of brain ischemia. In addition, ischemia/reperfusion causes an increase in NHE-1 expression/activity (Ramasamy et al., 1995).

Although NHE activation is essential for the restoration of physiological pH_i, hyperactivation of NHE1 in neurons, in response to the metabolic acidification associated with an ischemic–hypoxic insult (Vornov et al., 1996; Luo et al., 2005; Hwang et al., 2008; Kersh et al., 2009), disrupts the intracellular ion balance, causing intracellular Na^+ and $Ca2^+$ overload (Matsumoto et al., 2004), which eventually leads to cell death. Consequently the *in vitro* and *in vivo* approach of several studies have demonstrated neuroprotection with NHE inhibitors.

Similar data were obtained with genetic manipulations of NKCC1 in mice and by the inhibition of NKCC1 using bumetanide (Chen and Sun, 2005). NKCC1 is expressed in neurons throughout the brain where it contributes to the maintenance of $[Cl^-]_i$. Thus, it may affect neuronal excitability by regulating $[Cl^-]_i$. Expression of NKCC1 protein has also been found in astrocytes and oligodendrocytes (Lenart et al., 2004). The pharmacological inhibition or transgenic ablation of NKCC1 significantly attenuates infarction and swelling after brain ischemia induced by transient middle cerebral artery occlusion (tMCAO) (Chen and Sun, 2005). Therefore, it may be hypothesized that NKCC1 represents an attractive target for conditioning strategies against stroke.

NHE and NKCC Role during Ischemic Preconditioning

NHE and NKCC role during ischemic preconditioning is still controversial and very little is known about their role in the processes triggered by brain preconditioning, since all the studies were conducted in the heart. According to a first theory, activation of both transporters participates in the protection mediated by ischemic cardiac preconditioning. Indeed, the reduced acidification occurring during ischemia (Schaefer et al., 1995) along with the increase in intracellular sodium following preconditioning is consistent with a modulation of these proteins by preconditioning (Ramasamy et al., 1995). These mechanisms may include the activation of both NKCC and NHE, potentially resulting in reduced intracellular acidosis during ischemia. NKCC regulation is multifactorial, with evidence that the cotransporter is regulated by changes in cell volume (O'Donnell, 1993) and cAMP-dependent (Pewitt et al., 1990; Lytle et al., 1992; Klein, 1993) and non cAMP-dependent protein phosphorylation as well as ionic concentration gradients. Increased inward cotransporter flux during ischemia after preconditioning could be beneficial in protecting the heart through its functional coupling with the $Cl^-/HCO3^-$ exchanger (Anderson et al., 1990). In fact, chloride transported into the cell by the Na^+-K^+-$2Cl^-$ cotransporter may exit via Cl^-/HCO_3^- exchange, thus increasing intracellular $HCO3^-$ and limiting acidosis.

The primary factors regulating NHE include the intracellular proton concentration and the phosphorylation state. Under baseline conditions, the exchanger contributes to the extracellular/intracellular proton gradient with net outward transport of protons (Piwnica-Worms et al., 1986). Under conditions of ischemia in which protons are generated and intracellular pH decreases, the exchanger is stimulated to increase extrusion of protons, thus increasing the inward transport of sodium ions. However, this hypothesis has been more recently disproved. In fact, it has been highlighted that the above discussed study was carried out in hearts perfused with a medium containing $HCO3^-$ and the method used to induce acute intracellular acidosis resulted in greater acidosis in preconditioned than in control hearts. For these reasons, the faster initial recovery from acidosis in preconditioned hearts may be a consequence of the lower starting pH_i. Indeed, de Albuquerque et al. (1995) recently showed that, in a similar condition, the rate of pH_i recovery is similar in control and preconditioned rat hearts. More recently, it has been demonstrated that NHE activity did not contribute to the cardioprotection mediated by ischemic preconditioning. Indeed, inhibition of Na^+/H^+ exchanger isoform-1 (NHE1) is protective in adult myocardium in a model of ischemia followed by reperfusion; however, the effect is unclear in immature myocardium (Cun et al., 2007). More important, the administration of HOE, a NHE inhibitor, did not prevent the protection induced by preconditioning, thus suggesting that NHE inhibition during the prolonged ischemic period may enhance the protection afforded by preconditioning (Shipolini et al., 1997). Accordingly, another group demonstrated that both NHE inhibition and ischemic preconditioning eliminated the increase in intramyocyte Na^+ content that otherwise occurred with cardioplegic arrest and reperfusion in a porcine model. Because their mechanisms are distinct, the authors proposed that an additive beneficial effect against ischemia-reperfusion injury can be achieved by using NHE inhibition together with a preconditioning stimulus as prereperfusion therapy (Goldberg et al., 2002).

NHE and NKCC Role during Ischemic Post-conditioning

Up until now, there is few information about the role of NHE1 exchanger in brain post-conditioning since many studies have demonstrated its neurodegenerative role in brain ischemia upon activation. According to these data, the ischemic neuroprotection induced by post-conditioning may be associated with the downregulation of NHE1 expression or activity. These hypothesis are supported by few experimental evidences. In this regard, some information were obtained in the heart where it has been demonstrated the role of NHE in a delayed recovery of pH_i after heart post-conditioning. Indeed, it has been suggested that prolongation of acidosis during reperfusion is important for the post-conditioning-mediated neuroprotection. Inserte et al. demonstrated that such post-conditioning protocols that significantly prolong pH_i recovery during initial reperfusion are protective for the heart. Accordingly, there is a narrow inverse correlation between the level of the delay and the extent of cell death, demonstrating the key role of the delay of intracellular acidosis in the protection mediated by post-conditioning (Inserte

et al., 2009). The normalization of pH_i during reperfusion is mediated by the combined action of different transport systems, including Na^+/H^+-exchanger, $Na^+/HCO3^-$ symport, and H^+-coupled lactate efflux (Vandenberg et al., 1993). These systems appear to be mainly redundant, since inhibition of one of them does not result in significant delay in pH_i recovery. For instance, inhibition of Na^+/H^+-exchanger by selective agents administered at the onset of reperfusion has a very small effect on pH_i recovery (Docherty et al., 1997; Ten Hove and Van Echteld, 2004). By contrast, simultaneous inhibition of Na^+/H^+-exchanger and $Na^+/HCO3^-$ symport delay normalization of pH_i (Docherty et al., 1997; Schäfer et al., 2000; Inserte et al., 2008).

The concept that pH_i correction was correlated to the washout of lactate, H^+ and CO_2 was supported by the strong correlation between the delay in pH_i recovery and the levels of lactate measured in the coronary effluent (blood outflow through coronaries) during the first 2 min of reperfusion (Vandenberg et al., 1993; Inserte et al., 2008). Thus, reduced catabolite washout caused an attenuation of the transmembrane H^+ gradient and a decreased activity of Na^+/H^+-exchanger and $Na^+/HCO3^-$ symport.

The protective effect of prolongation of intracellular acidosis during reperfusion has been solidly demonstrated in different models, including isolated cardiomyocytes (Schäfer et al., 2000) and intact hearts (Kitakaze et al., 1988; Ohashi et al., 1996; Preckel et al., 1998; Inserte et al., 2008). Intriguingly, there is a correlation between delayed acidosis and other cardioprotective pathways during post-conditioning such as Reperfusion Injury Salvage Kinases (RISK) (Fujita et al., 2007). According to this theory, alkalotic buffer reduced Akt and ERK phosporylation in post-conditioned myocardium.

Regarding the role of Na^+-dependent chloride transporter (NKCC) during brain post-conditioning few information are available. In a recent work it has been demonstrated that in hippocampal neurons exposed to hypoxia conditioning mimiking an *in vitro* post-conditioning, NKCC1 is strongly activated through binding with STE20/SPS1-related proline/alanine-rich kinase (SPAK) (Yang et al., 2013). This finding could be considered as starting point for future studies.

Role of Acid-sensing Cation Channels (ASIC) in Brain Conditioning

Acid-Sensing Ion Channels (ASICs) are neuronal voltage-insensitive cationic channels activated by extracellular protons. ASIC proteins are a subfamily of the ENaC/Deg superfamily of ion channels. ASICs are potential drug targets for treating a wide variety of conditions linked to both the CNS and PNS. Acid-sensing cation channels (ASIC) have been extensively studied both in physiological and in pathological conditions for their wide distribution in the nervous system and since they represent key targets in many different aspects of acidosis, a condition that occurs during ischemic stroke, as previously analyzed (Waldmann et al., 1997; Wemmie et al., 2006). Until now, six ASIC subunit proteins, encoded by four genes, have been identified: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4; among these, the subtypes 1a, 2a, and 2b are expressed in neurons of the CNS. All ASICs belong to the degenerin/epithelial Na^+ channel (DEG/ENaC) superfamily, which are Na^+- selective cation channels sensitive to amiloride (Crowell and Kaufmann, 1961; Sutherland et al., 2000). Blockade of ASIC1a and deletion of the ASIC1 gene rescued neurons from ischemic cell death. In CNS neurons, ASIC1a responds to extracellular pH reduction ranging from 6.9 to 5.0 to generate rapid depolarizing currents (Waldmann et al., 1997), and its activation enhances the probability of action potential initiation (Vukicevic and Kellenberger, 2004). ASIC1a channels are activated at the pH values associated with CNS diseases (ischemia, ∼pH 6.5–6.0; seizure, ∼pH 6.8; AD, ∼pH 6.6). In the case of homomeric ASIC1a channels, acid activation also induces $Ca2^+$ entry directly through these channels (Crowell and Kaufmann, 1961; Nedergaard et al., 1991; Dietrich and Morad, 2010). In addition, the ASIC-mediated membrane depolarization may facilitate the activation of voltage gated Ca^{2+} channels and NMDA receptor-gated channels (Gao et al., 2005), further promoting neuronal excitation and $[Ca^{2+}]_i$ accumulation.

Acidosis usually occurs during many central nervous system (CNS) diseases. Indeed, ischemic brain pH falls to 6.0 due to the accumulation of lactic acid and to the protons produced by ATP hydrolysis (Nedergaard et al., 1991; Gao et al., 2005). So the reasonable strategy to contrast pH lowering, sustained by ASICs activation, is to block its activity, since acidosis is a common feature of acute neurological conditions such as ischemic stroke, brain trauma, and epileptic seizures (Rehncrona, 1985). It is rational to suggest that restoration of acid–base balance and blockade of the down-stream pathways of acidosis may represent two promising strategies to avoid the consequences of acidosis.

In this direction ASIC involvement in neuroprotection elicited in the brain by pre- and post-conditioning has been investigated (Pignataro et al., 2007).

Indeed, Pignataro and colleagues demonstrated that ASIC1a is downregulated during both preconditioning and post-conditioning and that this downregulation was dependent on p-Akt. Particularly, p-Akt is up-regulated during ischemic pre- and post-conditioning. In fact, during harmful ischemia, Akt is transiently phosphorylated, and then activated for a short time interval after reperfusion (Beg et al., 2008), whereas after brain conditioning the phosphorylation of Akt persists longer, even 24 h later, the same time interval at which an ASIC1a downregulation occurs. Interestingly, selective p-Akt inhibition prevented ASIC1a downregulation thus reverting the conditioning-induced neuroprotection.

That p-Akt is the mediator of the downregulation of ASIC1a during pre- and post-conditioning are supported by previous papers showing that Reperfusion Injury Salvage Kinasesì (RISKs), including Akt (Nedergaard et al., 1991; Xu and Xiong, 2007; Beg et al., 2008; Pignataro et al., 2011a) are activated during conditioning. In conclusion, since reduction of ASIC1a is a key factor in the neuroprotective effect of pre- and post-conditioning, the downregulation of its expression and activity might be a potential strategy to ameliorate the ischemic damage. Besides ASIC1a, ASIC2a has also been linked to ischemic preconditioning neuroprotection. In fact, the upregulation of ASIC2a is associated to the neuroprotective effect of ischemic preconditioning on global brain ischemia (Miao et al., 2010).

Role of Sodium/Calcium Exchanger (NCX) during Brain Conditioning

The sodium-calcium exchanger (Na^+/Ca^{2+} exchanger, NCX, or exchange protein) is an antiporter membrane protein that removes calcium from cells. It consists of nine transmembrane segments that can mediate Ca^{2+} and Na^+ fluxes across the plasma membrane. Depending on the intracellular concentrations of Ca^{2+}, $[Ca^{2+}]_i$, and Na^+, $[Na^+]_i$, NCX can operate either in the forward mode, coupling the uphill extrusion of Ca^{2+} to the influx of Na^+ ions, or in the reverse mode, mediating the extrusion of Na^+ and the influx of the Ca^{2+} ions (Pignataro et al., 2004). The NCX is considered one of the most important cellular mechanisms for removing $Ca2^+$. The maintenance of Na^+ and Ca^{2+} homeostasis, mediated by this exchanger, is a crucial physiological phenomenon in neuronal and non-neuronal cells. NCX countertransports four or three Na^+ ions in change of one Ca^{2+} ion (Reeves and Hale, 1984; Fujioka et al., 2000; Kang and Hilgemann, 2004); it is a high-capacity and low-affinity ionic transporter. When intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) increase this exchanger mediates the entrance of sodium ions and the extrusion of calcium ions in tight dependence of their electrochemical gradient. The way in which the antiporter works (forward or reverse) depends on several factors such as membrane potential, Na^+ and Ca^{2+} gradient. Therefore, NCX represents an important actor in the regulation of pathophysiological and physiological responses to increases of $[Ca^{2+}]_i$ and $[Na^+]_i$ (Haddad and Jiang, 1997; Dirnagl et al., 1999; Annunziato et al., 2004) In particular, three distinct NCX genes, ncx1-3, and several splicing variants have been so far described (Nicoll et al., 1990, 1996). The CNS is the only organ that expresses all three NCX gene products. NCX is particularly expressed in neurons at the level of synapses (Canitano et al., 2002) where, in the course of an action potential, Ca^{2+} crosses the plasma membrane. Calcium entry allows the fusion of synaptic vesicles with the plasmamembrane and permits the neurotransmitter release. After that, plasma membrane Ca^{2+} ATPase and NCX mediate the extrusion of the residual intracellular Ca^{2+}. In presence of $[Ca^{2+}]_i$ higher than 500 nM, NCX become the prevalent Ca^{2+} extrusion mechanism (Pignataro et al., 2014). At the same time, the deregulation of $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis may mediate the damage of neuronal and glial cells occurring after stroke and other neurodegenerative disorders.

Na^+/Ca^{2+} Exchanger in Brain Preconditioning

It is well-known that NCX may play a role in the maintenance of ionic homeostasis during ischemic conditioning (Pignataro et al., 2004, 2012; Molinaro et al., 2013). In this respect, NCX is downregulated during brain ischemia and this downregulation occurs in a manner dependent on the exchanger isoform and on the brain region interested by the damage (Pignataro et al., 2004; Boscia et al., 2006). It has been recently demonstrated that NCX1 and NCX3 isoforms represent two new effectors of the preconditioning-mediated neuroprotection (Pignataro et al., 2012). In fact, while the absence of NCX1 and NCX3 is able to partially prevent the neuroprotection mediated by ischemic preconditioning, the pro-survival factor p-Akt mediated preconditioning-induced brain protection by inducing an upregulation of NCX1 and NCX3. In this field, these results have been obtained in an experimental model of ischemic preconditioning in which preconditioning was mediated in rats by 30 min occlusion of the middle cerebral artery (MCA), followed by a subsequent harmful stimulus of 100 min of MCA occlusion, applied 72 h after the preconditioning stimulus (Pignataro et al., 2012). The correlation between NCX and p-Akt was demonstrated also by confocal fluorescence experiments, that showed a colocalization among NCX1, NCX3, and p-Akt in the temporoparietal cortex of preconditioned rats (Formisano et al., 2008; Pignataro et al., 2012). Moreover, the PI3-K inhibitor, LY294002, reverts the protective outcome elicited by ischemic preconditioning, thus giving further support to the role of p-Akt in this phenomenon (Endo et al., 2006; Pignataro et al., 2008). The overexpression p-Akt-dependent of NCX1 and NCX3 elicited by brain preconditioning may be considered a response of cells to overcome the deregulation of ionic homeostasis occurring in the brain under anoxic conditions. On the other hand, p-Akt is not the only transducer able to activate NCX1 and NCX3. Indeed, Valsecchi and colleagues underlineed that after ischemic preconditioning, hypoxia-inducible factor, HIF-1α, is strongly increased and mediated the increased expression of NCX1 expression, thus contributing to brain protection induced by ischemic preconditioning (Valsecchi et al., 2011) and suggesting that ncx1 gene represents a new HIF-1 target (Valsecchi et al., 2011). Summarizing, NCX1 and NCX3 overexpression may be considered as a mechanism that preconditioning activates in neurons and glial cellsin order to overcome the loss of ionic homeostasis caused by harmful ischemia. Interestingly, the activation of these mechanisms are long lasting, since NCX1 and NCX3 upregulation is still present 72 h after preconditioning induction, thus suggesting that these two isoforms can be included in the list of effectors able to mediate the so-called delayed preconditioning (Pignataro et al., 2013a).

Na^+/Ca^{2+} Exchanger in Brain Post-conditioning

Regarding NCX role during brain post-conditioning, NCX3 has been confirmed as a molecular effector involved in the neuroprotection (Pignataro et al., 2011b). In particular, interesting results have been obtained in an experimental model of ischemic post-conditioning, performed in the laboratory of Pignataro and colleagues by subjecting adult male rats to 10 min of MCAO 10 min after 100 min of MCAO. Results showed that p-Akt expression after post-conditioning increased and timely mirrors the increase in NCX3 expression. On the other hand, NCX3 silencing reverted the post-conditioning-mediated neuroprotection and the selective inhibition of p-Akt prevented NCX3 upregulation and the neuroprotection (Pignataro et al., 2011b). NCX3 overexpression during post-conditioning may contribute to counteract the ionic homeostasis dysregulation. By contrast NCX1 silencing did not revert the post-conditioning-induced neuroprotection, thus showing that NCX1 does not play a relevant role during post-conditioning. This result can be explained by the observation that unlike NCX1 and NCX2, the isoform NCX3 may be still operative during ATP depletion (Linck et al., 1998; Secondo et al., 2007). In addition, the

differences in NCX1 and NCX3 promoters render NCX3 a better target for the prosurvival kinase cAMP response element-binding protein (CREB), an Akt downstream player (Gabellini et al., 2003). After post-conditioning, the phosphorylation of Akt is present longer (Pignataro et al., 2008) and timely parallels the intervals of NCX3 upregulation. Furthermore, confocal experiments in the temporoparietal confirmed that the increased expression of these two proteins after post-conditioning occurs in the same cells. These considerations, beside showing that p-Akt palys a key role in neuroprotection, more important suggest NCX3 as one of the additional p-Akt effectors involved in the neuroprotective effect of ischemic post-conditioning.

Role of Plasma Membrane Ca^{2+} ATPase (PMCA) in Brain Conditioning

The plasma membrane $Ca2^{+}$ ATPase (PMCA) is a transport protein in the plasma membrane of cells that functions removing calcium ($Ca2^{+}$) from the cell; so it is also an important regulator of the calcium concentration in the extracellular space. This protein is the only plasmamembrane high affinity Ca^{2+} transporting system. It removes Ca^{2+} from the cytosol in all eukaryotic cells, and for most of them it is the only Ca^{2+} exporting system together with Na^{+}/Ca^{2+} exchanger which represent the principal Ca^{2+} exchanger in heart cells and neurons (Jensen et al., 1993). The high Ca^{2+} affinity of the pump, however renders it the only system capable of fine-tuning its exchanges with the external ambient (Brini et al., 2013).

Conversely to other plasma membrane buffering systems, such as NCX and ATPases, plasma membrane Ca^{2+} -ATPase may export intracellular Ca^{2+} even with relatively lower transport capacity during prolonged membrane hyperpolarization and during conditions of increased intracellular Na^{+} concentrations, as occurred under ischemic conditions. Ohta et al. (1996) suggested that ischemia-tolerant CA1 neurons, in a model of bilateral common carotid arteries occlusion in gerbils, export more efficiently a larger amount of Ca^{2+} (Kato et al., 2005).

Moreover, in gerbils, ischemic preconditioning by pre-exposure to 2-min ischemia is extremely effective in protecting against ischemic cell death since there is a modification of the electrophysiological properties of CA1 neurons (Kawai et al., 1998; Shimazaki et al., 2000). In this experimental conditions, expression of PMCA1 is increased in the hippocampus following 2-min ischemia (Kato et al., 2005). The same authors evaluated PMCA1 expression also in a model of tolerance induced by a low dose treatment with 3-Nitro Propionic Acid (3-NPA). They observed an increase in the expression of Bcl-2 in the hippocampus in both the models of preconditioning. However, PMCA1 increased just in the 2-min ischemia model and not in the 3-NPA model, thus suggesting that it plays an important but not essential role in the enhancement of ischemic tolerance (Kato et al., 2005).

Conclusions

The identification of the players involved in the control of ionic homeostasis during pre and post-conditioning should provide more direct opportunities for translational neuroprotection trials. Indeed, the present review, summarizing the specific effect of the plasmamembrane proteins involved in the maintenance of cellular ionic concentration, would provide information of which transporters needed to be activated or inhibited in order to protect the brain by the ischemic injury.

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Novel cellular mechanisms for neuroprotection in ischemic preconditioning: a view from inside organelles

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Ischemic preconditioning represents an important adaptation mechanism of CNS, which results in its increased tolerance to the lethal cerebral ischemia. The molecular mechanisms responsible for the induction and maintenance of ischemic tolerance in the brain are complex and not yet completely clarified. In the last 10 years, great attention has been devoted to unravel the intracellular pathways activated by preconditioning and responsible for the establishing of the tolerant phenotype. Indeed, recent papers have been published supporting the hypothesis that mitochondria might act as master regulators of preconditioning-triggered endogenous neuroprotection due to their ability to control cytosolic calcium homeostasis. More interestingly, the demonstration that functional alterations in the ability of mitochondria and endoplasmic reticulum (ER) managing calcium homeostasis during ischemia, opened a new line of research focused to the role played by mitochondria and ER cross-talk in the pathogenesis of cerebral ischemia in order to identify new molecular mechanisms involved in the ischemic tolerance. In line with these findings and considering that the expression of the three isoforms of the sodium calcium exchanger (NCX), NCX1, NCX2, and NCX3, mainly responsible for the regulation of Ca^{2+} homeostasis, was reduced during cerebral ischemia, it was investigated whether these proteins might play a role in neuroprotection induced by ischemic tolerance. In this review, evidence supporting the involvement of ER and mitochondria interaction within the preconditioning paradigm will be provided. In particular, the key role played by NCXs in the regulation of Ca^{2+}-homeostasis at the different subcellular compartments will be discussed as new molecular mechanism proposed for the establishing of ischemic tolerant phenotype.

Keywords: ischemic preconditioning, sodium calcium exchanger, mitochondria, neurons, calcium

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Introduction

Cerebral ischemia is a multifactorial and complex disease (1, 2). Indeed, the intracellular events activated by the loss of perfusion of the brain and responsible for neuronal damage range from impairment of intracellular homeostasis to mitochondrial dysfunction and free radical production (3–5). The complexity of these events explains the great discrepancy between the frequency of cerebral ischemic accidents and the lack of effective treatments able to inhibit or slow neuronal

demise following the ischemic insult. Hence, the urgent need to identify new potential targets for the development of innovative therapeutic strategies able to defend the ischemic brain.

On these premises, in the recent years, the attention of the researchers focused on ischemic tolerance a phenomenon, also known as ischemic preconditioning (IPC), which consists of a sub-lethal anoxic insult that makes the tissue in which it occurs more resistant to a subsequent and potentially lethal ischemia (6–11). The relevance of this phenomenon is to correlate to the study of the endogenous mechanisms activated in neurons to allow cell survival after a sub-lethal ischemic stimulus. By this way, it is possible to identify new molecular targets useful to develop alternative therapeutic strategies to treat the ischemic disease. The great interest in the cerebral IPC and in the tolerance evoked by itself also comes from the similarity of this phenomenon with those clinical situations occurring in the human brain. Indeed, it is well known that transient ischemic attacks (TIAs) do not cause structural damage but appear to protect brain against a subsequent "stroke" (12, 13).

Therefore, IPC or ischemic tolerance of the brain lie in a natural adaptive process that can be mimicked by a variety of sub-lethal insults, such as transient hypoxia, spreading depression, oxidative stress, hyperthermia, or heat shock, and that increases the tissue tolerance to a subsequent, potentially lethal ischemia. This adaptive cytoprotection is a fundamental property of living cells, which allows them to survive to the exposure to potentially recurrent stressors. This phenomenon was clearly identified in the heart by Murry et al. (14) as preconditioning, or subsequently as ischemic tolerance, and in 1990 it was described also in the brain by Kitagawa et al. (15). Since then, it immediately attracted the interest of clinical and basic neuroscientists for several reasons. First, this biological process became widely recognized as a pertinent and effective experimental instrument to understand how the brain protects itself against ischemia, thereby providing an innovative approach for the discovery of novel neuroprotective strategies. Second, retrospective case-control studies showed a clinical correlate of the phenomenon discovered experimentally.

Molecular Mechanisms Involved in Ischemic Preconditioning

Among the molecular mechanisms underlining ischemic tolerance, the substantial changes in gene expression play a crucial role, suggesting that preconditioning is able to stimulate a genomic reprograming of cells which in turn is responsible for the cytoprotection and cellular survival described in those tissues interested by the ischemic event (16, 17). The effects in the cell genome, occurring after IPC, represent the signature of the complex interplay of multiple signaling pathways. It is interesting to underline that they are highly specialized pathways in different cell types of the brain and concur to the cellular and systemic response activated in the tissue to combat the noxious stimulus. Indeed, it has been reported that hundreds of genes undergo upregulation or downregulation in response to IPC stimuli (18–20), and that these changes in gene expression differ between the harmful ischemic insult and the sub-lethal IPC. In particular, preconditioning seems to attenuate the response to ischemia (19), and the tolerance established modifies the expression of genes involved in the suppression of metabolic pathways, in the control of immune responses, in the modulation of ion-channel activity, and in the changes of blood coagulation parameters (19).

As recently reported, three phases, temporally consecutive of the preconditioning phenomenon have been identified as induction, transduction, and tolerance.

Induction and transduction mechanisms of tolerance

The induction of tolerance requires that the preconditioning stimulus has to be recognized by molecular sensors as a sign of a more severe phenomenon that will occur. This implicated the identification of numerous types of sensors, including neurotransmitters, neuromodulators, cytokines, and toll-like receptors (21) as well as ion channels and redox-sensitive enzymes (22–24) which in turn will activate subsequent transduction pathways responsible for initiating the adaptive response. The activation of the transduction phase also implies the involvement of different transducers depending, at least in part, on the nature of the preconditioning stimulus. They include members of mitogen-activated protein kinases (MAPKs) and their phosphorylated Ras, Raf, MEK, and ERK subfamilies (7, 22, 24, 25); mitochondrial ATP-sensitive K+ (KATP) channels (26, 27) Akt (28–31); and the protein kinase C-ε isoform (32). The possibility that the nitric oxide (NO)-based adaptive response to hypoxia in *Drosophila* (33) is evolutionarily conserved suggests that this multifunctional modulator might be a logical choice as an autocrine and paracrine mediator of preconditioning stress. Indeed, pharmacological and genetic evidence supporting the involvement of NO in the transduction process is continuing to mount. As matter of fact, it has been reported that NO can exert a neuroprotective role during preconditioning (7, 34), and we have recently proposed that NO-induced neuroprotection is associated to preservation of mitochondrial function (24). On the other hand, preconditioning positively affects the integrity of mitochondrial oxidative phosphorylation after cerebral ischemia, an effect restricted to the delayed window of preconditioning (35). Moreover, preconditioning prevents mitochondrial swelling, preserves membrane integrity fluidity, and protects mitochondrial energy metabolism during cerebral ischemia avoiding ATP consumption (36).

Given the redox sensitivity of many of those above-mentioned kinases and transcription factors, reactive oxygen species might also be contemplated within transducers (37–39). Furthermore, adenosine, another prototypical paracrine mediator and "retaliatory metabolite," whose production is linked to ATP degradation, might be considered a transducer responsible for tolerance induction, in some *in vitro* and *in vivo* models (26, 27, 40, 41).

It is worth of note that although in general, mitochondria have been considered important mediators of endogenous neuroprotection, the mechanisms by which they might "integrate" cytoprotective signaling of preconditioning remain to be fully elucidated.

Role of mitochondria in ischemic tolerance

It is well known that mitochondria are also able to sense and shape cytosolic Ca^{2+} signals by taking up and subsequently releasing

Ca^{2+} ions during physiological and pathological Ca^{2+} elevations (42). Mitochondrial function is strictly dependent on maintaining of mitochondrial calcium homeostasis. In fact, thanks to the activity of Ca^{2+}-sensitive mitochondrial dehydrogenases these organelles can regulate oxidative phosphorylation and ATP synthesis during conditions of high cellular demand (43). Therefore, to explore the relationship between mitochondrial oxidative capacity and calcium buffering activity during IPC might represent an attractive strategy mediating neuroprotection in ischemic conditions. In particular, considering that the influence of Ca^{2+} in the regulation of mitochondrial function is highly dependent on the spatiotemporal distribution of $[Ca^{2+}]_i$ (44, 45), the assumption that during IPC an interaction between mitochondria and ER might affect mitochondrial metabolic properties, and in turn neuronal survival has been also investigated (31). This hypothesis is supported by the concept that microdomains of high $[Ca^{2+}]_i$ have been identified near Ca^{2+} channels on the plasma membrane and ER (46).

Cellular Ionic Homeostasis and Energy Metabolism During Ischemic Preconditioning

It has been widely described that IPC activates intracellular biological responses prior to a potential lethal insult (17, 47–49), and that these events make the tissue in which they occur more resistant to the subsequent severe ischemia (17, 47, 50–52). In this regard, it is possible to speculate that an increase of energy metabolism or a latency in anoxic depolarization following the onset of ischemic insult might represent one of the mechanisms by which tissues strengthen their tolerance when exposed to a sub-lethal insult. Indeed, it has been reported that a reduction in energy demand and in the activity of ion channels represents determinant factors for ischemic tolerance establishment (19, 53, 54). To this aim, *in vitro* experiments performed in cortical neurons demonstrated that the exposure to brief non-injurious oxygen and glucose deprivation (OGD) causes an impairment in voltage-gated potassium channels (55). Similarly, the impairment of Na^+/K^+-ATPase activity occurring in rat hippocampal and cortical neurons exposed to global forebrain ischemia is counteracted by IPC (56). Interestingly, in the last years, it has been shown that some integral plasma membrane proteins, involved in the control of Ca^{2+} and Na^+ ion influx or efflux, the sodium calcium exchangers (NCXs) might function as crucial players in the pathogenesis of brain ischemic damage (57). Therefore, these proteins, by regulating Na^+ and Ca^{2+} homeostasis, may represent more suitable molecular targets for therapeutic intervention in ischemic stroke. As matter of fact, *in vivo* experiments performed in gerbils demonstrated that during IPC, an increase in Ca^{2+}-ATPase activity and an enhancement in mitochondrial calcium sequestration occur in CA1 hippocampal neurons (47). In line with this findings, intracellular calcium measurements in hippocampal neurons of preconditioned gerbils showed that the increase in $[Ca^{2+}]_i$ occurring after anoxic and aglycemic episodes was markedly reduced in the ischemic tolerant animals (58). However, the molecular mechanisms responsible for this effect are still matter of debate and not completely clarified. As recently proposed by Kato et al. (59), a possible explanation could be related to the increased expression of the Ca^{2+}-ATPase isoform 1 [plasma membrane calcium ATPase 1 (PMCA-1)]. Moreover, considering that mitochondria play a crucial role in the regulation of intracellular Ca^{2+} homeostasis due to their high capacity to sequestrate Ca^{2+} both in physiological and in pathological conditions, they have been included among the possible intracellular mechanisms triggered by preconditioning stimuli. Indeed, although it is widely reported that the excessive amount of Ca^{2+}, as it occurs during ischemia, causes an impairment of mitochondrial function with consequent massive uncoupling of oxidative phosphorylation, mitochondrial depolarization and mitochondrial permeability transitional pore (MPTP) opening, reversal of the action of ATP synthase and cell death, and studies performed both in the heart and in the brain demonstrated that the inhibition of MPTP opening and its signaling cascade represent crucial events responsible for cytoprotection observed in IPC (53, 60). What are the molecular mechanisms underlining these effects is still object of investigation. Among them, nitrite and protein kinases have been proposed as possible MPTP regulators (61–64) **Table 1**.

More interestingly, the hypothesis that a modulation of the expression and activity of the NCXs might have a role in the regulation of calcium and sodium homeostasis during ischemic tolerance has been recently suggested (31, 50, 71). This might be relating to the ability of Na^+/Ca^{2+} exchanger to work in concert with selective ion channels and ATP-dependent pumps, in maintaining the physiological cytosolic concentrations of these two ions (72).

NCXs functional properties in ischemic brain

In the brain, unlike other tissues, NCX is present in three different gene products, named NCX1, NCX2, and NCX3, with a

TABLE 1 | Mitochondrial effectors of preconditioning-induced neuroprotection.

Type of study	Species	Stimulus	Mechanism	Reference
In vivo	Rat	Ischemic	Energy metabolism and mitochondrial function	(35) (65)
In vitro	Hippocampal neurons	Ischemia	Mitochondrial ATP-dependent potassium channel	(66)
In vivo	Rat			
In vivo	Mouse	Hypoxia	GLUT-1	(18, 63)
	Rat			(67)
In vivo	Rat	Normobaric Hypoxia	Phosphofructokinase and LDH	(67)
In vivo	Rat	Ischemia	Calcium/calmodulin-dependent protein kinase II	(68, 69)
In vivo	Gerbil	Ischemia	Akt/protein kinase B	(28)
In vivo	Rat	Ischemia	mitogen-activated protein	(70)
In vitro	Cortical neurons	Hypoxia	NCX3	(31)
In vitro	Cortical neurons	Hypoxia	MnSOD	(24)
In vivo	Rat	Ischemia		(23)

distinct distribution pattern in different brain regions (73). Under physiological conditions, NCX works primary extruding Ca^{2+} in response to a depolarization or to an increase in intracellular Ca^{2+} concentrations coupled to receptor stimulation (74). However, during hypoxic conditions, when the dysfunction of the two plasma membrane ATP-dependent pumps Na^+/K^+ ATPase and Ca^{2+} ATPase occurs, NCX assumes a relevant role in controlling the intracellular homeostasis of these two cations, since it is able to operate promoting Na^+ ions extrusion and Ca^{2+} influx (74, 75). Although this reverse mode of operation in the early phase of anoxia does undoubtedly elicit an increase in $[Ca^{2+}]_i$, its effect could be beneficial for neurons because it contributes to decrease $[Na^+]_i$ overload, a phenomenon which would otherwise lead to cell swelling and thus to sudden necrotic neuronal death. Conversely, in the later phase of neuronal anoxia, when $[Ca^{2+}]_i$ overload takes place, NCX working in the forward mode of operation can contribute to the lowering of Ca^{2+} concentrations, and thus it can protect neurons from intracellular Ca^{2+} overload, neurotoxicity, and subsequent cell death (74). As we recently demonstrated, NCX1, NCX2, and NCX3 may exert different roles during *in vitro* and *in vivo* anoxic conditions leading to a new paradigm in the pathogenesis of ischemic damage. Indeed, we provided evidences that in cells singly and stably transfected with NCX3, this isoform contributes more significantly to the maintenance of $[Ca^{2+}]_i$ homeostasis during experimental conditions mimicking ischemia, thereby preventing mitochondrial $\Delta\Psi$ collapse and cell death (76). This is due to a different sensitivity of the three NCX isoforms to the changes in ATP content occurring during anoxia. Indeed, NCX3 results more resistant to ATP changes compared to NCX1 and NCX2 (76). In addition, in *in vivo* experiments, the selective knocking down of NCX1 and NCX3, but not of NCX2, by antisense oligodeoxynucleotide strategy (57) or the disruption of the *ncx3* gene, renders the brain more susceptible to the ischemic insult (77). Moreover, the induction of permanent middle cerebral artery occlusion in rats correlates with NCX1 mRNA upregulation in the peri-infarct area thus suggesting the possibility that this isoform could be a new druggable target for the treatment of cerebral ischemia. In line with this hypothesis, we recently demonstrated that NCX1 transcript and protein were upregulated by ischemic cerebral preconditioning. This effect was mediated by the transcriptional factor HIF1α and was accompanied by a relevant neuroprotection (71).

Role of NCX1 and NCX3 in the ischemic preconditioning

Recent findings demonstrated that the two isoforms of the Na^+/Ca^{2+} exchanger, NCX1 and NCX3, which are involved in several pathophysiological aspects of cerebral ischemia, can be included among the members of the growing family of the mediators involved in the ischemic brain tolerance due to their ability to regulate neuronal calcium homeostasis (**Figure 1**). In particular, we provide evidence that neuroprotection observed in preconditioned neurons exposed to OGD/reoxygenation is correlated to the increase in NCX1 and NCX3 protein expression. Indeed, the treatment with siRNA against NCX1 and NCX3 prevents this effect. These data are in accordance with results recently observed *in vivo* in an animal model of IPC (50). Consistently with these results, the upregulation of NCX1 and NCX3 protein expression in neurons exposed to IPC was dependent on PI3K/Akt activation, since the treatment with LY294002 was able to abolish this increase. Interestingly, we demonstrated that NO plays a key role in the triggering PI3K/Akt pathway as the increase in the phosphorylated form of Akt observed within 30 min after IPC was completely abolished by the treatment with L-NAME. More importantly, the treatment with L-NAME was able to inhibit NCX3 but did not affect NCX1 protein expression (31).

Calcium Homeostasis in ER and Mitochondria During Ischemic Preconditioning

It has recently demonstrated that in neurons NCX3, apart its localization on the plasma membrane, is also expressed on the outer mitochondrial membrane where it contributes to the extrusion of calcium from mitochondria (78). It is well known that mitochondria, in addition to the generation of cellular energy, play an important role in regulating cellular calcium homeostasis (79–82) in concert with the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), the plasma membrane Ca^{2+}-ATPase, and Na^+/Ca^{2+} exchanger (83). On the other hand, the maintenance of mitochondrial calcium homeostasis is an important requirement ensuring mitochondrial function. In fact, Ca^{2+}-sensitive dehydrogenases can regulate oxidative phosphorylation and ATP synthesis during times of high cellular demand (43). Therefore, it is possible to hypothesize that the increased expression of NCX3, we observed within 48 h from the sub-lethal insult, might exert neuroprotective effects regulating calcium handling and improving mitochondrial oxidative capacity. This finding is in line with the results previously obtained in an *in vitro* model of IPC and demonstrating that the increase in nNOS expression and $NO^{\bullet}$ production through the activation of Ras/ERK1/2 pathway stimulated mitochondrial MnSOD. These effects associate with a reduction in free radical production and cytocrome *c* release from mitochondria to cytosol, and in turn with an improvement of neuronal survival (24). The demonstration that NCX3 might represent a target of IPC-induced neuroprotection (31) adds new insight into the molecular mechanisms involved in the ischemic brain preconditioning. Indeed, the over-expression of NCX3 might help mitochondria to preserve their energetic capacity making them less vulnerable to the subsequent lethal insult represented by OGD/reoxygenation. This hypothesis was strongly supported by the results obtained measuring the activity of NCX during IPC. In fact, we demonstrated that IPC induced an increase of NCX activity in the reverse mode of operation that was still observed in preconditioned neurons exposed to OGD/Reoxygenation. This is an early event since it occurred within 30 min after IPC stimulus was due to the contribution of NCX1 and NCX3 isoforms and was promoted by NO because it was abolished by the treatment with L-NAME. Intriguingly, this effect was associated with an increase in ER calcium content. Consistently with data previously published in our Laboratory (84), we speculated that NCX1, working in the reverse mode of operation, plays a key role in

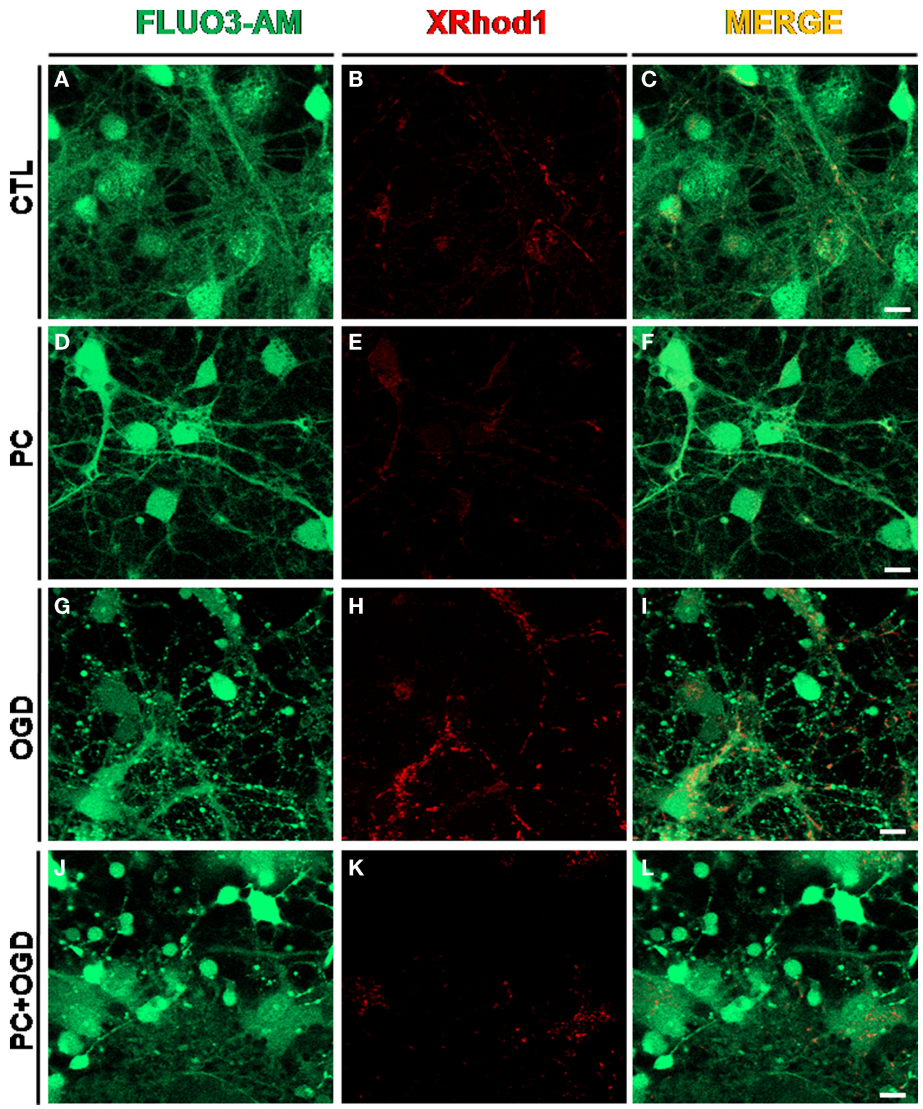

FIGURE 1 | Effect of ischemic preconditioning on cytosolic and mitochondrial calcium content in cortical neurons exposed to OGD. Confocal images showing in red mitochondrial Ca^{2+} content measured by XRhod-1 probe (200 nM, 15 min at RT) and in green cytosolic calcium concentration measured by Fluo 3 AM probe (5 μM, 30 min at RT). **(A–C)**: cortical neurons under control conditions (CTL); **(D–F)**: cortical neurons exposed to 30 min of oxygen and glucose deprivation (OGD), preconditioning stimulus (PC); **(G–I)**: cortical neurons exposed to 3 h OGD; **(J-L)**: preconditioned neurons exposed to OGD (PC + OGD). Scale bars: 1 μm.

the regulation of ER calcium refilling in the early phase of IPC (**Figure 2**). Indeed, the treatment with siNCX1, but not siNCX3, was able to prevent this phenomenon. The novel aspect of the study is the demonstration that NO promoted NCX1-induced ER refilling that was hampered by L-NAME pretreatment. This finding is in accordance with data recently published by Secondo et al. and demonstrating that NO was able to stimulate NCX1 to work in the reverse mode of operation, whereas NO did not affect NCX3 activity (85). The possibility that NCX1 activation in the early phase of IPC could affect mitochondrial calcium content promoting mitochondrial calcium uptake could not be excluded. However, in the late phase of IPC mitochondrial calcium handling is mainly regulated by NCX3 that is able to promote mitochondrial calcium extrusion (**Figure 2**). These data are supported by the finding that NCX3 expression increased 48 h after the IPC insult (31). We have previously demonstrated that NCX3 is distributed also at mitochondrial level; therefore, it is possible to speculate that during IPC, the increased expression of NCX3 on mitochondria might contribute to the efflux of calcium from the organelle thus protecting neurons by the subsequent mitochondrial calcium overload induced by lethal OGD/reoxygenation exposure. The finding that the treatment with CGP and siNCX3 counteracted the effect of IPC on mitochondrial calcium content, leading to the lack of IPC-neuroprotection, further supports this hypothesis (31). Collectively, we can conclude that a functional interplay between NCX1 and NCX3 occurs during IPC. This phenomenon is tightly dependent on NO and Akt activation, and by contributing to the modulation of intracellular ionic homeostasis, could represent one

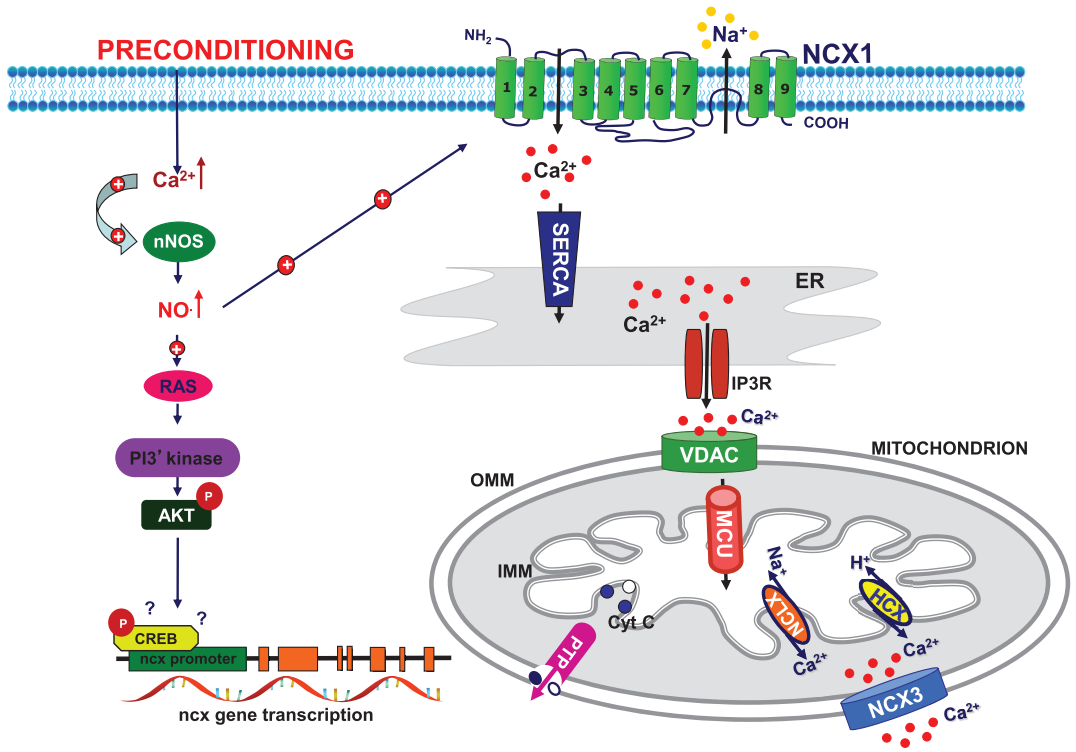

FIGURE 2 | Schematic model of preconditioning-induced neuroprotection. Preconditioning stimulus activates neuronal nitric oxide synthase (nNOS) to produce NO which in turn stimulates plasma membrane NCX1 activity in the reverse mode of operation and promotes endoplasmic reticulum (ER) refilling in the early phase of PC. NO activate PI3K/Akt pathway, which increases the expression and activity of plasma membrane and mitochondrial NCX3, thus promoting mitochondrial calcium efflux in the late phase of preconditioning. These mechanisms working in concert promote neuronal survival. *PI3K:* phosphatidylinositol-3-kinases; *CREB:* cAMP response element-binding protein; *SERCA:* sarco/ER Ca^{2+}-ATPase; *IP3R:* inositol trisphosphate receptor; *MCU:* mitochondrial calcium uniporter; *VDAC:* voltage-dependent anionic channel; *NCLX:* Na^{+}/Ca^{2+} exchanger Li-dependent; HCX: Ca^{2+}/H^{+} exchanger; *PTP:* permeability transition pore; *OMM*: outer mitochondrial membrane; *IMM:* inner mitochondrial membrane.

of the mechanisms responsible for neuroprotection induced by IPC (**Figure 2**).

Conclusion

The experimental data described in this review support the hypothesis that mitochondria play a pivotal role in neuroprotective events provided by preconditioning. Indeed, both the mitochondrial antioxidant enzyme MnSOD and the proteins involved in the regulation of mitochondrial calcium homeostasis, such as mNCX3, represent new targets and mediators involved in preconditioning-induced neuroprotective responses. This is in line with the results reported in the literature that "mitochondrial preconditioning" has many neuroprotective effects, both during and following neurotoxic insults, including an improvement of neuronal viability, the attenuation of the intracellular Ca^{2+} influx, the suppression of ROS generation, the inhibition of apoptosis, and the maintenance of ATP levels (86). Therefore, understanding the precise mitochondrial mechanisms involved in preconditioning will provide important information necessary to develop new and more effective therapeutic strategies for neurodegeneration occurring in brain ischemia. In this regards, the identification of NCX3 as new player in the regulation of mitochondrial calcium efflux and the demonstration that an increase in its expression occurs during preconditioning, promoting mitochondrial calcium homeostasis, might help to draw new therapeutic strategy potentially able to delay neuronal loss occurring in ischemia. More interestingly, the demonstration that an increase in NCX1 and NCX3 activity is responsible for Ca^{2+} cycling from ER and mitochondria, and proof that it activates intracellular events leading to neuroprotection observed in IPC support this hypothesis (31). In this scenario, the identification of a compound able to selectively stimulate the activity of NCX1 and to prevent neuronal degeneration in *in vitro* and *in vivo* models of ischemia has been recently synthesized (77). Moreover, compounds able to stimulate NCX3 activity are in progress of development in our Laboratory.

Therefore, based on this early observation, these studies have potentially revealed new molecular targets in cerebral ischemia and neurodegenerative diseases pathogenesis, which ultimately may open up alternative avenues for future therapeutic intervention.

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