

FORUM REVIEW ARTICLE

# The NAD<sup>+</sup>-Dependent Family of Sirtuins in Cerebral Ischemia and Preconditioning

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## Abstract

**Significance:** Sirtuins are an evolutionarily conserved family of NAD<sup>+</sup>-dependent lysine deacylases and ADP ribosylases. Their requirement for NAD<sup>+</sup> as a cosubstrate allows them to act as metabolic sensors that couple changes in the energy status of the cell to changes in cellular physiological processes. NAD<sup>+</sup> levels are affected by several NAD<sup>+</sup>-producing and NAD<sup>+</sup>-consuming pathways as well as by cellular respiration. Thus their intracellular levels are highly dynamic and are misregulated in a spectrum of metabolic disorders including cerebral ischemia. This, in turn, compromises several NAD<sup>+</sup>-dependent processes that may ultimately lead to cell death.

**Recent Advances:** A number of efforts have been made to replenish NAD<sup>+</sup> in cerebral ischemic injuries as well as to understand the functions of one its important mediators, the sirtuin family of proteins through the use of pharmacological modulators or genetic manipulation approaches either before or after the insult.

**Critical Issues and Future Directions:** The results of these studies have regarded the sirtuins as promising therapeutic targets for cerebral ischemia. Yet, additional efforts are needed to understand the role of some of the less characterized members and to address the sex-specific effects observed with some members. Sirtuins also exhibit cell-type-specific expression in the brain as well as distinct subcellular and regional localizations. As such, they are involved in diverse and sometimes opposing cellular processes that can either promote neuroprotection or further contribute to the injury; which also stresses the need for the development and use of sirtuin-specific pharmacological modulators. *Antioxid. Redox Signal.* 28, 691–710.

**Keywords:** sirtuins, SIRT, NAD<sup>+</sup>, cerebral ischemia, preconditioning, cerebral ischemic tolerance

## Sirtuins: Function and Background

SIRTUINS FIRST SPARKED interest in 1999, when a member of the sirtuin family known as silent information regulator 2 (Sir2) was shown to modulate lifespan in *Saccharomyces cerevisiae* (71). Cells carrying a mutant form of Sir2 had a significantly reduced lifespan compared to wild types, whereas strains overexpressing Sir2 exhibited a noticeably increased lifespan (71). It was later shown that the lifespan extension induced by caloric restriction is abolished in strains carrying a mutant Sir2 (100). Since then, there has been an explosion in the study of sirtuins in different model organisms that ultimately linked them to processes beyond aging,

including metabolic diseases such as diabetes, cancer, and neurological disorders. For a thorough discussion of these studies, the readers are guided to the following reviews among others that further elaborate on the functions of sirtuins in the context of these metabolic disorders (20, 130, 136, 137, 141, 153, 163).

In mammals, the sirtuin family of proteins consists of seven members (SIRT1–7), which share a conserved catalytic domain of 275 amino acids (16). They were originally classified as class III histone deacetylases (HDACs) owing to their ability to remove acetyl groups from lysine residues on histones proteins, yet they are currently known to act on nonhistone proteins as well as to remove additional acyl

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groups from lysine residues (151). Among the four classes of HDACs, the sirtuins are the only class that requires nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) as a co-substrate to hydrolyze acylated amino acid residues (151). Sirtuins couple the deacylation of amino acid residues to the consumption of  $\text{NAD}^+$  and as such generate two byproducts, nicotinamide (NAM) and O-acetyl-ADP ribose (OAADPr) (158) (Fig. 1). The removal of acyl groups from the lysine residues of target proteins can affect their enzymatic function, localization, or stability.

In addition, not all sirtuin members are primarily or strictly lysine deacetylates (Fig. 1). SIRT1, SIRT2, and SIRT3 show the highest deacetylase activity (4). Yet, side chains of lysine residues could be modified by additional acyl groups that contain varying number of carbon residues, have different structures, or even carry different electrical charges (29). Such acyl groups include formyl, propionyl, butyryl, crotonyl, and succinyl, among others (29). Thus, the sirtuins have a diverse array of enzymatic activities with each member having preferences for specific acyl groups (Fig. 1) (4). In addition to their diverse enzymatic functions, sirtuins also exhibit distinct subcellular localizations (Fig. 1) (4, 113). As such, sirtuins act on a variety of substrates and regulate

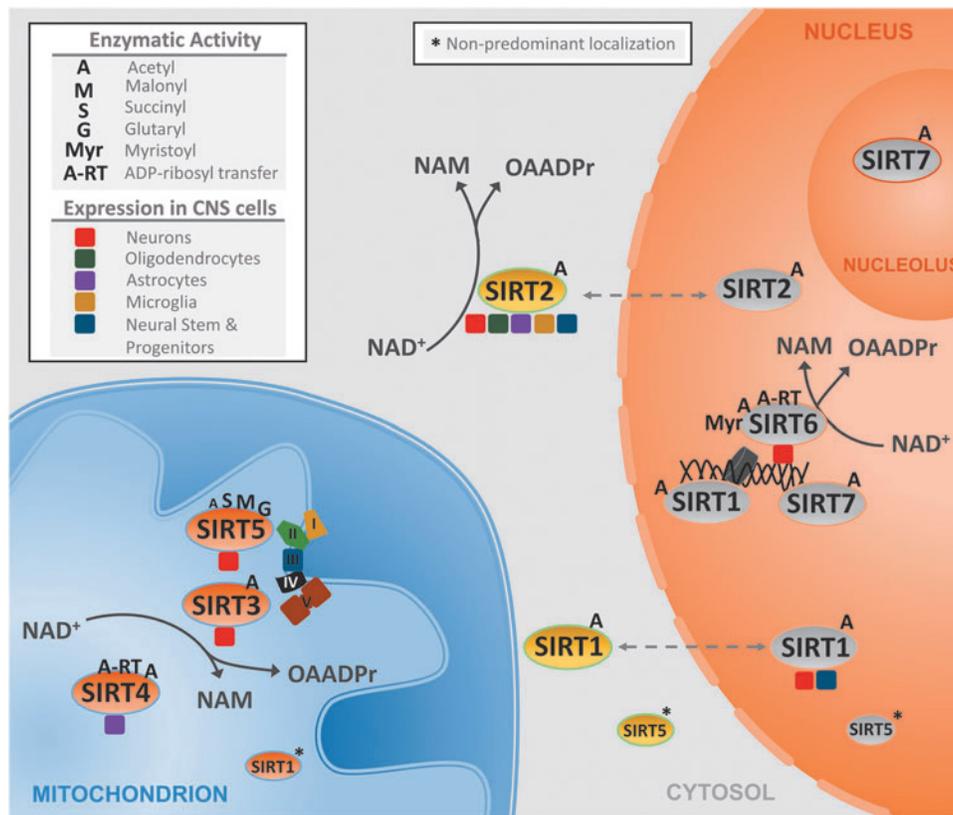
various cellular processes through their distinct enzymatic functions and subcellular localizations.

### Dynamic Regulation of Intracellular $\text{NAD}^+$

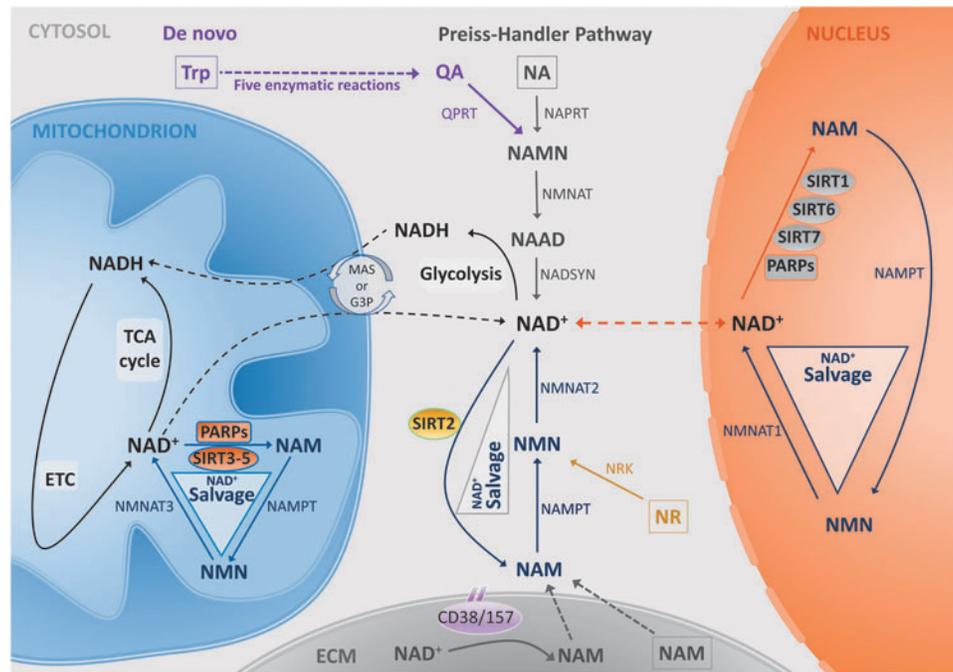
A feature common to all sirtuin members is their requirement for  $\text{NAD}^+$  as a cosubstrate (88, 151).  $\text{NAD}^+$  consists of two nucleotides joined by a phosphate group with one carrying an adenine and the other carrying nicotinamide (17). It exists in two forms, an oxidized form ( $\text{NAD}^+$ ) and a reduced form (NADH), with  $\text{NAD}^+$  being the predominant form under basal conditions (17, 163).

#### Role of sirtuins as metabolic sensors: reliance on $\text{NAD}^+$ as a coenzyme

A crucial function for  $\text{NAD}^+$  is its role as a coenzyme in cellular redox reactions (Fig. 2) (163). In the cytosol,  $\text{NAD}^+$  is reduced to NADH during glycolysis, whereas in the mitochondria tricarboxylic acid the TCA cycle enzymes reduce additional  $\text{NAD}^+$  molecules (163, 181). In the presence of oxygen, reduced NADH molecules get oxidized by the electron transport chain (ETC) to produce the proton gradient necessary for adenosine triphosphate (ATP) synthesis



**FIG. 1. The enzymatic activities, subcellular localizations, and cell-type specific expression of sirtuins.** The figure depicts the predominant, but not exclusive, subcellular localizations of the seven sirtuin members (SIRT1–7) along with their predominant enzymatic activities. The presented subcellular localizations and enzymatic activities reported are not brain specific and represent general known activities for sirtuins studied in different cell types as well as *in vitro*. The enzymatic activities are depicted by a letter that represents the acyl group removed by the respective sirtuin. Colored boxes reflect the cell-type specific expression of sirtuins reported in the central nervous system. The enzymatic activities of sirtuins involve the coupled deacylation of amino acid residues to the consumption of  $\text{NAD}^+$  and as such generate two byproducts, NAM and OAADPr or O-acyl-ADP ribose. Asterisks\* represents some of the nonpredominant subcellular localizations of sirtuins. NAM, nicotinamide; OAADPr, O-acetyl-ADP ribose.



**FIG. 2. The dynamic regulation of intracellular NAD<sup>+</sup> levels.** Intracellular NAD<sup>+</sup> levels are highly dynamic and are regulated by NAD<sup>+</sup>-producing and NAD<sup>+</sup>-consuming pathways as well as by cellular respiration. Three NAD<sup>+</sup>-producing pathways have been identified, which are the (i) *de novo* synthesis pathway (ii) the Preiss-Handler, and (iii) the NAD<sup>+</sup>-salvage pathway. Each pathway uses different precursor molecules and enzymes for the synthesis of NAD<sup>+</sup>. The precursor molecules surrounded by boxes are obtained from the diet. Similarly, three NAD<sup>+</sup>-consuming pathways have been identified and they include the (i) sirtuins (SIRTs) (ii) the PARPs, and (iii) the cADPR synthases known as CD38 and CD157, which are reported to exist both intra- and extracellularly (only depicted as extracellularly in the figure). NAD<sup>+</sup> levels are further regulated by cellular redox reactions involved in cellular respiration, where NAD<sup>+</sup> plays a role as a coenzyme. Enzymes of glycolysis reduce NAD<sup>+</sup> in the cytoplasm, whereas the TCA cycle enzymes reduce additional NAD<sup>+</sup> molecules in the mitochondria. Reducing equivalents of cytoplasmic NAD<sup>+</sup> are then transferred to the mitochondrion through the G3P and MAS enzymes, ultimately reaching the electron transport chain complexes, where they get oxidized to generate the proton gradient necessary for ATP production. Another important factor regulating NAD<sup>+</sup> not depicted in this figure is the circadian regulation of NAD<sup>+</sup> by the circadian genes. cADPR, cyclic ADP ribose; G3P, glycerol-3-phosphate; CD38/157, ADP ribosyl cyclase/cyclic ADP ribose hydrolase 38/157; ETC, electron transport chain; MAS, malate-aspartate shuttle; NA, nicotinic acid; NAAD, nicotinic acid adenine dinucleotide; NAD<sup>+</sup>, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); NADSYN, NAD synthetase; NAM, nicotinamide; NAMN, nicotinic acid mononucleotide; NAMPT, nicotinamide phosphoribosyltransferase; NAPRT, nicotinate phosphoribosyltransferase; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide mononucleotide adenylyl transferase; NR, nicotinamide riboside; NRK, nicotinamide riboside kinase; PARPs, poly-ADP ribose transferases; QA, quinolinic acid; QPRT, quinolinate phosphoribosyl transferase; TCA cycle, tricarboxylic acid cycle; Trp, tryptophan.

(163, 181). Alternatively, in the absence of oxygen, NADH gets oxidized in the cytoplasm through the reduction of pyruvate to lactate. As such, NAD<sup>+</sup> plays a crucial role as a coenzyme necessary for ATP production using both aerobic and anaerobic respiration (163, 181).

As a result of its involvement in the redox reactions of glycolysis and oxidative phosphorylation, the ratio of oxidized NAD<sup>+</sup> to reduced NADH fluctuates with nutrient availability (Fig. 2). NAD<sup>+</sup> levels are increased in response to conditions of energetic stress, because of limited energy substrates, such as in the cases of calorie restriction, fasting, and exercise (14, 22, 81, 179). In contrast, NAD<sup>+</sup> levels decrease in response to an excess of energy substrates such as in the cases of high-fat diets and type-2 diabetes (77, 186). Since NAD<sup>+</sup> acts as a cosubstrate for the sirtuins and its relative concentration fluctuates with energy status, this allows the sirtuins to act as metabolic sensors (31). By sensing the levels of NAD<sup>+</sup>, sirtuins couple changes in the energy status of the

cell to adaptive responses, ultimately regulating metabolic efficiency (31). In addition to being regulated by NAD<sup>+</sup>, sirtuins have been reported to be negatively regulated by NAM, the byproduct of their enzymatic reaction (5, 44). NADH is thought to be an ineffective inhibitor because the concentration required should be in the millimolar range, significantly higher than its physiological levels (101, 145). Thus, the ratio of NAD<sup>+</sup>/NAM better regulates the sirtuins activity than NAD<sup>+</sup>/NADH (16).

#### *NAD<sup>+</sup>-producing and NAD<sup>+</sup>-consuming pathways*

Intracellular NAD<sup>+</sup> levels are affected not only by the cell's redox status but also by the rate of NAD<sup>+</sup> production and consumption (129). Three pathways have been characterized that synthesize NAD<sup>+</sup> (Fig. 2) (16, 163). They are known as (i) *de novo* synthesis (ii) the Preiss-Handler pathway, and (iii) the NAD<sup>+</sup>-salvage pathway (16, 163). In the *de*

*novo* pathway, tryptophan obtained from the diet is converted into quinolinic acid, which feeds into the Preiss–Handler pathway through its conversion into nicotinic acid mononucleotide (NAMN) (89). In the Preiss–Handler pathway, NAMN is converted into nicotinic acid adenine dinucleotide, which is then directly converted to  $\text{NAD}^+$ . The Preiss–Handler pathway can also utilize nicotinic acid (NA; niacin) from the diet as a precursor (16, 163). The third pathway, which is of interest in many metabolic disorders, is the  $\text{NAD}^+$ -salvage pathway (165). It recycles NAM produced by  $\text{NAD}^+$  breakdown into nicotinamide mononucleotide (NMN) through the action of nicotinamide phosphoribosyltransferase (NAMPT). NMN is then directly converted into  $\text{NAD}^+$  by NMNAT. Dietary NAM (vitamin B3) and nicotinamide riboside (NR) can feed into the  $\text{NAD}^+$ -salvage pathway (12, 165). Although dietary tryptophan, NR, NA, and NAM are all important precursors, their plasma levels are too low to maintain sufficient  $\text{NAD}^+$  production, thus it is thought that mammals rely mostly on the  $\text{NAD}^+$ -salvage pathway to preserve intracellular  $\text{NAD}^+$  (15, 51, 56, 65). To add to its dynamic regulation, intracellular levels of  $\text{NAD}^+$  oscillate in a circadian manner because of the cyclical expression of the NAMPT enzyme, which is regulated by SIRT1 along with the circadian core genes (123).

Within the cell, pools of  $\text{NAD}^+$  are found in specific subcellular compartments and regulate compartment-specific pathways (Fig. 2) (46).  $\text{NAD}^+$  can freely diffuse between the cytosol and nucleus and can cross the outer mitochondrial membrane. However, a transport mechanism is required for  $\text{NAD}^+$  export and NADH import across the inner mitochondrial membrane (16, 163). Reducing equivalents of NADH entering the mitochondrial matrix are exchanged for  $\text{NAD}^+$  leaving the matrix through the glycerol-3-phosphate and malate–aspartate shuttles (16, 163). NAM produced in each compartment is recycled back into  $\text{NAD}^+$  by compartment-specific isoforms of NMNAT and possibly NAMPT enzymes (16, 46, 163).

In addition to its role as a cosubstrate for sirtuins,  $\text{NAD}^+$  acts as a substrate for poly-ADP ribose transferases (PARPs) as well as cyclic ADP ribose (cADPR) synthases (Fig. 2) (163). The PARP family consists of 17 members in humans. Among them, PARP1 and PARP2 consume the largest amount of  $\text{NAD}^+$  as they catalyze the transfer of multiple ADP ribose units to target molecules. The remaining members are catalytically inactive or only transfer a single ADP ribose (84). PARP1 and PARP2 play a crucial role in the DNA damage repair (115). Upon DNA damage, these enzymes transfer linear or branched ADP ribose polymers to histones and proteins, which then recruit the DNA repair machinery (84). Depending on the severity of DNA damage, PARPs can either lead to DNA damage repair or to cell death caused by an energy failure because of  $\text{NAD}^+$  and subsequent ATP depletion (115). In addition to DNA repair, PARPs are involved in several other cellular processes (84, 115). The third family of  $\text{NAD}^+$ -consuming enzymes is the cADPR synthases, or ADP ribosyl cyclases that consist of two proteins CD38 and CD157 that exists both on cell membranes and intracellularly (16, 106, 163). Besides their role in signaling, these enzymes have an ADP ribosyl cyclase activity that allows them to produce cADPR from  $\text{NAD}^+$  (16, 106, 163). Molecules of cADPR then act as a second messenger to trigger the release of calcium stores from the endoplasmic reticulum (ER) (16, 106).

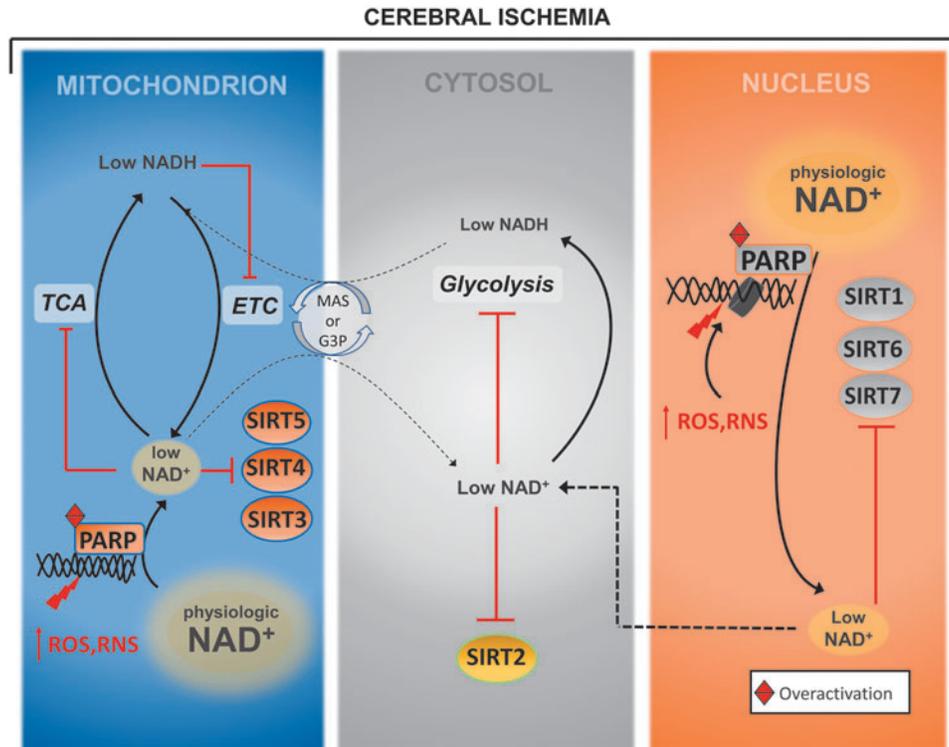
Thus,  $\text{NAD}^+$  levels within the cell are highly dynamic and are determined not only by the energy status of the cell that regulates its aerobic and anaerobic respiration rates but also by the balance of the three  $\text{NAD}^+$ -producing and three  $\text{NAD}^+$ -consuming pathways (Fig. 2) (16, 163).

### **$\text{NAD}^+$ in Cerebral Ischemia and Preconditioning**

The brain only constitutes around 2% of total body weight yet it consumes around 20% of total body energy (30). This high energy requirement makes the brain highly susceptible to ischemic injuries, which usually results from either an ischemic or hemorrhagic stroke or from cardiac arrests (30, 154, 174). Despite decades of research, cerebral ischemia remains one of the leading causes of adult disability and death in the United States and worldwide (174). Currently, recombinant tissue plasminogen activator (rtPA), a thrombolytic agent, is the sole FDA-approved treatment for ischemic stroke, yet it is administered to <5% of stroke patients (91). Numerous neuroprotective drugs have been tested in the clinic, yet nearly all have failed to promote significant recovery (76, 114). New avenues of research now aim to take advantage of other therapeutic windows as well as novel drug targets to combat cerebral ischemia (47, 75, 157).

During cerebral ischemia, the interruption of blood flow deprives cells of oxygen and glucose, which prevents them from generating sufficient ATP (154). This affects all the energy-dependent processes within the brain, specifically the  $\text{Na}^+/\text{K}^+$  ATPase, which is required to maintain the plasma membrane potential (154). The failure of the  $\text{Na}^+/\text{K}^+$  ATPase causes cells to depolarize and release excessive amounts of glutamate that activate postsynaptic AMPA and NMDA receptors, causing a massive influx of sodium and calcium (154). The massive increase in calcium initiates a complex cascade of events leading to increased reactive oxygen species (ROS) and reactive nitrogen species (RNS) that interact with and damage cellular components, including nucleic acids (104). DNA damage is then detected by the PARP enzymes, which respond by synthesizing linear and branched forms of poly-ADP ribose units from  $\text{NAD}^+$  and attach them to histones and nuclear proteins to recruit the DNA repair machinery (7, 104). When modestly activated this leads to DNA repair, but when PARPs are over-activated such as in regions experiencing severe oxidative and nitrosative stresses, they consume as much as 80% of intracellular  $\text{NAD}^+$  (7, 104). The depletion of  $\text{NAD}^+$  then impairs the generation of ATP from glycolysis and oxidative phosphorylation, causing an energy failure that could lead to cell death (78, 79, 104, 184). In addition, the reduction in  $\text{NAD}^+$  limits its availability to other  $\text{NAD}^+$ -consuming enzymes, the sirtuins and the cADPR synthases, both of which play crucial cellular functions that further contributes to the injury (Fig. 3) (16, 163).

Taking into account the importance of maintaining intracellular  $\text{NAD}^+$  levels during cerebral ischemia, many efforts have been directed toward this approach as a potential therapeutic intervention. Several studies have used PARP inhibitors or transgenic animals lacking PARP to maintain  $\text{NAD}^+$  levels to protect against the ischemia-induced injuries (7, 10, 156). The successful results of these studies allowed minocycline, an antibiotic with PARP-inhibiting activities, to reach clinical trials for the treatment of stroke (3, 80). Other approaches to maintaining  $\text{NAD}^+$  levels were made by supplementing  $\text{NAD}^+$  itself



**FIG. 3. NAD<sup>+</sup> depletion by PARP enzymes during cerebral ischemia.** During cerebral ischemia, levels of ROS, RNS increase in the brain and, in turn, these highly reactive molecules interact with and damage different cellular components including nucleic acids. The DNA damage is then detected by the PARP enzymes that respond by consuming large amounts of NAD<sup>+</sup> to synthesize linear and branched forms of poly-ADP ribose units and attach them to histones and nuclear proteins, a signal needed to recruit the DNA repair machinery. When modestly activated, this leads to DNA repair, but when PARPs are over activated such as in regions experiencing severe oxidative and nitrosative stresses, they consume as much as 80% of intracellular NAD<sup>+</sup>. The depletion of NAD<sup>+</sup> then impairs the generation of ATP from glycolysis and oxidative phosphorylation, causing an energy failure that could lead to cell death. In addition, the reduction in NAD<sup>+</sup> levels also limits its availability for other NAD<sup>+</sup>-consuming enzymes, the sirtuins and cADPR synthases, which play crucial cellular functions, thus further contributing to the injury. RNS, reactive nitrogen species; ROS, reactive oxygen species.

or its precursors (NAM and NMN) before or after the injury (6, 79, 165, 166, 170, 184, 185). Similarly, administering NAMPT activators has also shown successful outcomes in cerebral ischemic models (26, 166).

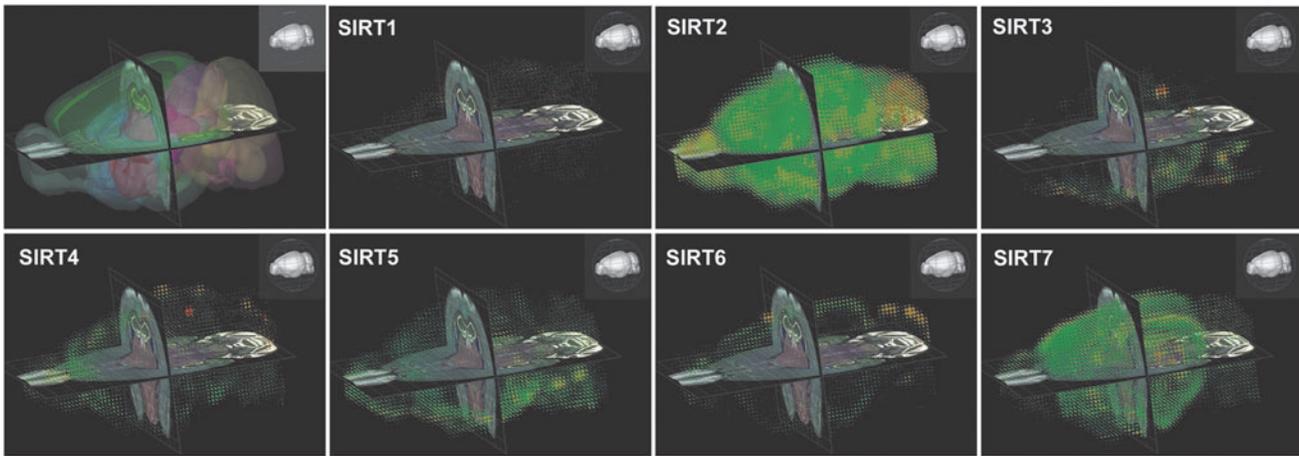
Preconditioning also constitutes a promising alternative approach that can modulate NAD<sup>+</sup> levels. Ischemic preconditioning (IPC) refers to the ability of a mild, noninjurious ischemic insult to protect against a subsequent injurious one (75). Although IPC may not be directly translatable because of the concerns and risks associated with it, IPC in the form remote limb preconditioning constitutes an alternative approach for inducing ischemic tolerance in multiple organs of the body, including the brain (109). Remote IPC is now being extensively studied in clinical trials in the context of several disorders, including cardiovascular and cerebrovascular diseases (60, 90, 96). Similarly, the administration of pharmacological agents has been shown to mimic IPC in a phenomena referred to as pharmacological preconditioning that is of immense therapeutic value as well because of its potential of being translated into the clinic (75). The endogenous mechanisms activated by preconditioning have been the focus of research by our laboratory and others with the aim of identifying novel targets and adaptations that mediate ischemic tolerance (75, 157). Preconditioning is known to increase antioxidant levels that reduce

oxidative and nitrosative stresses thus, in turn, reducing DNA damage (95, 124). This approach could be indirectly used to reduce PARP activity and thus preserve intracellular NAD<sup>+</sup> pools (156). Furthermore, our laboratory has previously shown that preconditioning stimulates NAMPT expression and increases NAD<sup>+</sup> levels in mitochondrial fractions and as such protects against cerebral ischemic injuries (116).

Thus, several studies have shown promising outcomes of NAD<sup>+</sup> supplementation in the context of cerebral ischemic injuries, yet because NAD<sup>+</sup> plays diverse roles as a coenzyme in redox reactions as well as cosubstrate for three protein families, its supplementation may affect numerous downstream pathways (16, 163). In this review, we focus on the importance of one of its downstream mediators, the sirtuin family of NAD<sup>+</sup>-consuming enzymes, and summarize the studies up to date that has explored their roles in cerebral ischemic injuries and preconditioning.

### Sirtuins in Cerebral Ischemia and Preconditioning

All members of the sirtuin family are known to be expressed in the brain, and they display distinct regional, cellular, and subcellular localizations (Figs. 1 and 4). Cerebral ischemia and preconditioning differentially regulate the



**FIG. 4. The mRNA distribution of sirtuins in a mouse brain reveals region-specific gene expression.** Images obtained from the Allen Mouse Brain Atlas-Brain Explorer<sup>®</sup> 2 based on an ISH experiment targeting sirtuins. Gene-specific antisense probes were used to detect the mRNA distribution of the seven sirtuin members (SIRT1–7) in the brain of a 56-day-old male mouse using sagittal brain sections. The *top left* image represents the three-dimensional anatomical structures of the mouse brain. Original ISH sagittal images can be viewed on the Allen Brain Atlas website along with the ISH probe sequences used for each experiment. ISH, *in situ* hybridization.

expression and activity of different sirtuins and, in turn, they play significant roles in determining the outcome of these insults (Fig. 5 and Table 1).

#### Nuclear sirtuins

**SIRT1.** SIRT1 is the most conserved sirtuin across species and so far the best studied (144). Its predominant enzymatic activity is deacetylation although it hydrolyzes other acyl groups as well (4, 8, 120). SIRT1 plays a role in transcriptional repression by deacetylating histone residues such as H3K9, H3K56, and H4K16. SIRT1 also acts on nonhistone proteins to regulate DNA repair, glycolysis, mitochondrial biogenesis, inflammation, oxidative stress, and apoptosis (21, 34, 118, 162, 169). The systemic overexpression of SIRT1 mimics the effects of caloric restriction and increases the lifespan of several model organisms, yet in mice, although its systemic overexpression does not increase lifespan, it can still promote several health benefits (13, 59). In addition, numerous studies have suggested that the health benefits promoted by calorie restriction in different organs of the body including the brain using various models of ischemia and neurodegeneration are mediated at least, in part, through the activity of SIRT1 (66, 87, 138, 139, 150).

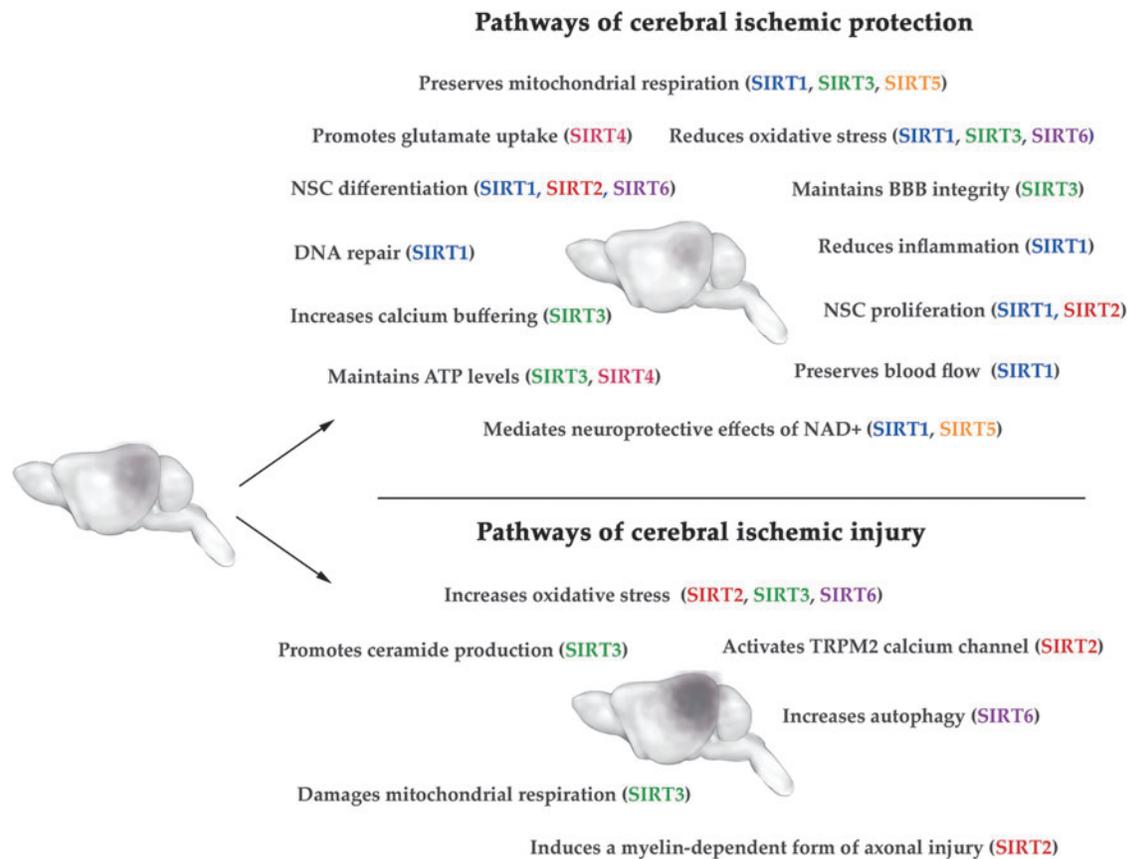
In the brain, SIRT1 is predominately localized in neuronal nuclei, yet has been detected in neural stem cells, astrocytes, and microglia *in vitro*, and in glial cells of postmortem human brains (83). It regulates important processes in the brain including genomic stability, neurogenesis, neurite outgrowth, synaptic plasticity, and cognition (126). Interestingly, SIRT1 in the hypothalamus can regulate systemic metabolic activities, and when overexpressed in the hypothalamus, it increases the lifespan of mice (143).

In the context of cerebral ischemia, most but not all studies support a neuroprotective role for SIRT1 (83). These studies support a role for SIRT1 in preserving mitochondrial function, mediating the neuroprotective effects of NAD<sup>+</sup>, reduc-

ing oxidative stress, preserving blood flow, and reducing inflammation among other pathways (36, 54, 83, 166, 171). For a detailed discussion of these and other studies, the readers are directed to a recent review that focuses solely on the role of SIRT1 in cerebral ischemia (83). In this review, we discuss the most recent and compelling studies.

SIRT1 levels are regulated by cerebral ischemic injuries, as levels fall in the ischemic brain and continue to decline with tissue reperfusion (72). Interestingly, SIRT1 increases in peri-infarct areas of the cortex up to 7 days after permanent middle cerebral artery occlusion (pMCAo) (58). In turn, SIRT1 activity modulates the outcome of cerebral ischemic injuries. *Sirt1*<sup>-/-</sup> mice display larger infarct volumes than wild-type mice (58), whereas mice overexpressing SIRT1 are more resistant to the injury (53, 54).

Both preconditioning and postconditioning approaches can tap into the neuroprotective properties of SIRT1. IPC reduced hippocampal cell death and improved outcome after asphyxial cardiac arrest (ACA), a rodent model of global cerebral ischemia (36). Intracerebroventricular (ICV) administration of the sirtuin inhibitor sirtinol blocked these protective effects. It should be noted that sirtinol is a multi-sirtuin inhibitor and although these studies may stress the importance of SIRT1, other sirtuins may be contributing to the observed results. Another preconditioning intervention, hyperbaric oxygen exposure, also reduced infarct and improved the neurobehavioral score from transient middle cerebral artery occlusion (tMCAo), effects that were abolished with ICV administration of *Sirt1*-targeted siRNA (177). Pharmacological preconditioning mimetics are of potential therapeutic value and have shown efficacy in several studies. For example, introducing resveratrol, a SIRT1 activator, 2 days before ACA mimics the effects of IPC and protects rats against ACA in a sirtuin-dependent manner (36). Curcumin, another naturally occurring compound with SIRT1-activating properties, when administered for 5 days before the injury, protects against a tMCAo but not in the



**FIG. 5. Pathways of cerebral ischemic protection and injury regulated by sirtuins.** The figure represents the main pathways regulated by the seven sirtuin members (SIRT1–7) in the context of cerebral ischemic injuries. These pathways were shown to be modulated upon the pharmacological or genetic manipulation of sirtuins using *in vitro* and *in vivo* models of cerebral ischemia and, in turn, either promote ischemic protection against the insult or further contribute to it.

presence of sirtinol (110). Targeting SIRT1 postinjury is also efficacious. Melatonin, the indoleamine produced in the body and found in foods, is another SIRT1-modulating compound. Administration of melatonin at ischemia and reperfusion onset reduced infarct, brain edema, and improved neurological score against tMCAo in an SIRT1-dependent manner (180). Many studies highlight the ability of resveratrol to induce ischemic tolerance in rodent models when administered hours or even days after injury (41, 57, 155, 168). Nevertheless, in addition to SIRT1 activation, resveratrol was shown to regulate SIRT3 and SIRT5 as well as nonsirtuin proteins, thus its protective effects should be carefully interpreted in a context-dependent manner as it may not be solely dependent on SIRT1 (48). In addition, it should be noted that resveratrol has been tested in clinical trials in the context of several disorders, including stroke wherein it was administered along with tPA and have shown promising outcomes (23). Resveratrol has also been tested in other cardiovascular disorders (1, 105, 188) as well as neurodegenerative disorders such as Alzheimer's disease (160). These studies could in the future facilitate its translation into clinical trials to be tested against cerebral ischemic insults in the form of preconditioning as well as postconditioning.

Intriguingly, both brain-derived and peripherally derived mechanisms of SIRT1-mediated ischemic tolerance are evident. In favor of brain-derived protection, preconditioning

and pharmacological agents are effective at protecting primary neuronal and organotypic cultures *in vitro* (45, 140). In addition, *in vivo* studies have shown that ICV administration of SIRT1 or sirtuin-inhibiting compounds or direct injections of siRNA abolishes the protection despite the fact that peripheral SIRT1 remains active (36, 177).

Furthermore, several studies support SIRT1-mediated mechanisms that arise in the periphery or in non-neuronal cell types. Bilateral common carotid artery occlusion, a model of global ischemia, reduced cerebral blood flow to 20–25% of baseline in wild-type mice but only to 45–50% in SIRT1 overexpressing mice, suggesting that SIRT1 can preserve cerebral flow and in turn reduce the severity of ischemia (54). Also, SIRT1 overexpression maintained cerebral blood flow to near baseline levels hours and days after bilateral common carotid artery stenosis by preventing endothelial nitric oxide synthase acetylation, promoting its activation, and preserving of cerebral flow (53). These effects were abolished by sirtinol administration and were not attributed to the generation of collateral vasculature that may circumvent anterior and posterior circulation of the brain (53). Angiogenesis is another process by which enhanced cerebral blood flow can improve outcome after ischemic injury. SIRT1 is expressed in endothelial cells in the brain and is known to mediate their migration, sprouting, and production of erythropoietin (135). SIRT1 deacetylates hypoxia-inducible factor 2 alpha

TABLE 1. SUMMARY OF *IN VIVO* STUDIES INVOLVING THE GENETIC OR PHARMACOLOGICAL MANIPULATION OF SIRTUINS IN THE CONTEXT OF CEREBRAL ISCHEMIA

<i>Sirtuin modulating compound</i>	<i>Treatment</i>	<i>Injury</i>	<i>Method of sirtuin inhibition</i>	<i>Evaluation</i>	<i>Outcome</i>	<i>Targets</i>	<i>Ref</i>
Multisirtuin modulation Resveratrol (10 mg/kg), IPC (2 min BCAo w/HT)	Once 2 days prior, once 2 days prior	ACA	Sirtinol (10 $\mu$ l of 1 mM) ICV	7 days post	Reduced hippocampal cell death, improved neurological score	UCP2, mitochondrial function	(36)
Curcumin (50 mg/kg)	Once/day for 5 days prior	2 h tMCAo	Sirtinol (15 mg/kg) IP	24 h post	Reduced infarct, brain edema, improved neurological score	TNF $\alpha$ , IL-6, mitochondrial function, Bcl-2, p53, Bax	(110)
Sirt1 Hyperbaric oxygen preconditioning (1 h 100% oxygen) Melatonin (10 mg/kg)	Once/day for 5 days prior	2 h tMCAo	siRNA (10 $\mu$ l of 50 nM), ICV	7 days post	Reduced infarct volume ratio, improved neurobehavioral deficit	Nrf2, HO-1, SOD1	(177)
NAD <sup>+</sup> -lentiviral NAMPT overexpression (0.5 $\mu$ l per site of $1 \times 10^9$ TU/ml) Activator 3 (10 mg/kg)	Once at ischemia onset, once at reperfusion onset 4 sites (cortex and hippocampus) 3 weeks prior 10 min, 24 h, 40 h after	30 min tMCAo	EX-527 (10 $\mu$ g) 3x/day for 2 days prior, ICV	24 h post	Reduced infarct, brain edema, improved neurological score	Bcl2, Bax, mitochondrial function	(180)
Sirt1 overexpression	N/A	2 h tMCAo	Sirt1 <sup>+/-</sup> mice	24 h post	Reduced infarct volume, improved neurological deficit	AMPK, LKB1	(166)
Sirt1 overexpression	N/A	pMCAo	Sirt1 <sup>-/-</sup> , Sirtinol (10 mg/kg) 10 min, 24 h, 40 h after IP	48 h post	Reduced infarct volume	p53, NF- $\kappa$ B	(58)
Sirt2 Sirt2 knockout	immediately after	BCAS	N/A	28 days post	Improved histopathology, spatial working memory	eNOS, cerebral blood flow	(53)
N/A	30 min after	10 min BCAo	Sirtinol (1 mg/kg), IV	7 days post	less hippocampal damage	Cerebral blood flow	(54)
Sirt2 Sirt2 knockout	immediately after	45 or 1 h tMCAo	Sirt2 <sup>-/-</sup> , AGK2 (0.764 mg/kg), 24 h post superficial cervical vein	24 h post	Reduced infarct, improved neurological deficit	N/A	(176)
N/A	30 min after	8 min CA/CPR	AGK2, 30 min after (10 $\mu$ g/kg), IV	3 days, 7 days or 30 days after	Reduced CA1 injury in males	TRPM2, long- term potentiation	(148)
Sirt2 knockout	N/A	45 min or 15 min of tMCAo	Sirt2 <sup>-/-</sup>	48 h, 7 days	Improved neurological deficit, no change in stroke volume	N/A	(85)
Sirt3 Sirt3 knockout	N/A	1 h tMCAo	Sirt3 <sup>-/-</sup>	24 h	Reduced infarct	Ceramide synthases, mitochondrial function, ROS	(128)
Sirt4 None							
Sirt5 Sirt5 knockout	$\Psi$ 8RACK (PKC $\epsilon$ activator, 0.75 mg/kg)	85 min tMCAo	Sirt5 <sup>-/-</sup>	24 h	Reduced infarct	NAMPT, AMPK, mitochondrial function	(117)
Sirt6 None							
Sirt7 None							

ACA, asphyxial cardiac arrest; BCAS, bilateral common carotid artery stenosis; BCAo, bilateral common carotid artery occlusion; eNOS, endothelial nitric oxide synthase; ICV, intracerebroventricular; N/A, not available; PKC $\epsilon$ , protein kinase C epsilon; ROS, reactive oxygen species; tMCAo, transient middle cerebral artery occlusion; TU, transduction units.

(HIF-2 $\alpha$ ), increasing its transcription and ultimately the erythropoietin-derived adaptation of red blood cells to low oxygen (37). This effect is seen with IPC and sufficient to induce neuroprotection in hippocampal neurons (189). Inflammation is another SIRT1-regulated pathway that could culminate in neuroprotection. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) is a proinflammatory mediator that contributes to ischemic injury during the reperfusion phase (133). SIRT1 deacetylates NF- $\kappa$ B, thus decreasing its activity (182). Resveratrol inhibits NF- $\kappa$ B by deacetylation, which is associated with increased SIRT1 activity (61). Together these studies demonstrate several non-neuronal-derived mechanisms of SIRT1-mediated ischemic tolerance.

Although most studies support a neuroprotective role for SIRT1, a few lines of evidence suggest that SIRT1 does not protect against ischemia-like insults or may even be detrimental. One interesting study found that overexpressing SIRT1 protected against low potassium-induced cell death in cerebellar granule neurons (CGNs); however, this protection was not abolished by the sirtuin inhibitor NAM or sirtinol, suggesting deacetylase-independent mechanisms of neuroprotection (132). Overexpressing a mutant SIRT1 that lacks deacetylase activity was reported to afford protection, leading to the hypothesis that SIRT1 may have chaperone-like activity that mediates neuroprotection (132). Moreover, some studies have postulated that SIRT1 may contribute to the rapid decline of NAD<sup>+</sup> seen after ischemia (2, 115). NAM, which inhibits sirtuins, protected against excitotoxic cell death by preserving NAD<sup>+</sup> levels (102). More studies are needed to tease apart these intricate aspects of SIRT1 function in ischemia and to refine the involvement of SIRT1 in preconditioning and postischemia neuroprotection.

**SIRT6.** SIRT6 possesses a HDAC, ADP ribosylase, and a demyristoylase activity (69, 107, 111). It acts on acetylated lysine residues such as H3K9ac and H3K56ac to repress gene expression and promote genome stability, particularly important in telomere maintenance (111, 112). SIRT6 also plays a role in DNA damage repair through the ADP ribosylation and thus activation of PARP1 (107). Furthermore, SIRT6 has been reported to possess anti-inflammatory functions and to play a role in maintaining metabolic homeostasis and preventing aging and senescence (19, 175, 191). Mice lacking *Sirt6* gene have a shortened lifespan, exhibit aging-like degenerative process, are susceptible to DNA damage, and show a severe hypoglycemic phenotype because of increased glucose uptake and glycolysis (119, 191). Yet mice overexpressing *Sirt6* are resistant to the metabolic damages caused by high-fat diets and show extended lifespan in male but not female mice (73, 74).

In the context of cerebral ischemic injuries, few studies have explored the functions of SIRT6. During ischemic/reperfusion (I/R) injuries, levels of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) are elevated and contribute to the oxidative stress-induced injury (32). Shao *et al.* showed that in response to H<sub>2</sub>O<sub>2</sub>, SIRT6 is reduced in SH-SY5Y neuronal cells (146). The overexpression of SIRT6 further exacerbated the necrotic cell death and ROS production. In these cells, SIRT6 induced autophagy by inhibiting the AKT signaling pathway, whereas the inhibition of either SIRT6 or autophagy was able

to rescue them from H<sub>2</sub>O<sub>2</sub>-induced cell death (146). Cardinale *et al.* showed that SIRT6 is highly expressed in the cortex and hippocampus of mice and is specifically abundant in the nucleus and synaptosomal fractions (18). When cultured *in vitro*, cortical and hippocampal neurons showed decreased SIRT6 expression with increased maturation. The overexpression of SIRT6 decreased the viability of these cells in response to H<sub>2</sub>O<sub>2</sub> treatment (18).

Yet, other reports support a protective role for SIRT6. A study by Hu *et al.* showed that pretreating brain endothelial cell line (bEnd.3) with Na<sub>2</sub>S (an exogenous donor of H<sub>2</sub>S) protected the cells from an oxygen and glucose deprivation (OGD)-induced cell death (63). This correlated with decreased ROS production and increased activities of antioxidant genes superoxide dismutase (SOD) and CAT (catalase). Treatment with Na<sub>2</sub>S also increased the expression and activity of SIRT6 which was reduced by OGD. Knocking down SIRT6 reduced the activities of SOD and CAT and abolished the Na<sub>2</sub>S-mediated protection against an OGD in endothelial cells (63). As mentioned above, a study by Zhao *et al.* showed that the overexpression of NAMPT increased the survival of mice and their functional recovery when subjected to an MCAo which correlated with increased neurogenesis (190). The knockdown of SIRT1, 2, or 6 prevented the differentiation of neural stem cells *in vitro* in response to NAD<sup>+</sup> or NMN supplementation. Although the study supports a positive role for SIRT6 in promoting the differentiation of neural stem cells (NSC), its importance in cerebral ischemia was not experimentally validated (190). Another study which potentially hints toward a protective role for SIRT6 in the brain was performed by Lee *et al.* (93). The authors report SIRT6 to be predominately found in neuronal cells. In response to an *in vitro* and *in vivo* models of cerebral ischemia, SIRT6 is reduced and this correlated with the translocation of high mobility group box-1 protein (HMGB1) from the nucleus to the cytoplasm and its extracellular release, a process known to promote inflammation in the brain. The knockdown of SIRT6 in SH-SY5Y cells did not affect OGD-induced cell death but increased the nuclear translocation of HMGB1 and its extracellular secretion. The study supports a role for SIRT6 in preventing the HMGB1 release which might potentially reduce inflammation in the brain although not directly shown (93).

The study by Pfister *et al.* showed that in CGNs, the overexpression of SIRT6 induced apoptosis in healthy neurons. Yet in a neuroblastoma cell line (HT22), SIRT6 overexpression was protective against homocysteic acid-induced apoptosis (132).

Dong *et al.* while assessing the association between genetic variants in the sirtuin members and carotid plaque presence and abundance found an association between a single nucleotide polymorphism (SNP) (rs107251) and two haplotypes in *SIRT6* gene to correlate with an increased risk for carotid plaque presence as well as plaque abundance (39). In a follow-up study, the authors showed that the SNP rs107251 also significantly correlated with total plaque area. Since the accumulation of carotid plaque increases the risk of clinical atherosclerosis, an underlying cause of ischemic strokes, this study suggests a potential role for *SIRT6* in increasing the risk ischemic strokes (38).

Furthermore, SIRT6 was reported to be post-translationally modified by nitrogen species under conditions

of nitrosative stresses (62). Using primary human retinal microvascular endothelial cells, Hu *et al.* showed that the treatment with a peroxynitrite donor induces the nitration of SIRT6 which reduces its deacetylase activity. Similarly, using an *in vivo* model of LPS-induced retinal inflammation, SIRT6 was modified by nitration and showed reduced activity. This study shows the importance of nitrosative stresses in regulating SIRT6 activity (62).

Thus the limited studies assessing the role of SIRT6 in cerebral ischemic injuries are somehow contradictory with evidence supporting both a detrimental and a protective role for SIRT6.

**SIRT7.** SIRT7 is mainly found in the nucleolus where it plays a positive role in the regulation of ribosomal RNA transcription through the deacetylation of RNA polymerase I subunit (24, 43, 159). SIRT7 also regulates tRNA transcription through the indirect activation of RNA pol III (159). Both events are required for protein translation. Moreover, SIRT7 acts as a HDAC acting on the H3K18ac mark (9). The increased activity of SIRT7 associated with hypoacetylation of H3K18 has been linked to tumor progression, the reason why SIRT7 is considered a drug target for cancer therapy (9, 144). Furthermore, SIRT7 plays a positive role in the regulation of nuclear-encoded mitochondrial genes through its deacetylation of GABP $\beta$ 1 (142). Additionally, SIRT7 exhibits a histone desuccinylase activity (97). In response to DNA damage SIRT7 desuccinylates H3K122 and promotes chromatin condensation and DNA damage repair (97). Mice lacking *Sirt7* have reduced lifespan, exhibit inflammatory cardiomyopathies, and fatty liver diseases among others (4, 149, 161).

The role of SIRT7 in cerebral ischemic injuries has not been studied. A single study that aimed at identifying blood biomarkers for cardioembolic stroke has revealed *SIRT7* to be among the stroke-associated genes (173). Following a systems biology approach, the authors combined publically available microarray data from human blood samples taken at three different timepoints after a cardioembolic stroke with protein-protein interaction information obtained from a publically available database, to identify protein biomarkers with significant stroke relevance. The authors revealed six genes that show high association with time after a stroke, among them is *SIRT7*. Based on pathway analysis and previously known functions, the authors predicted SIRT7 to play a protective role in cardioembolic stroke, yet this was not validated experimentally (173).

#### Cytoplasmic sirtuins

**SIRT2.** SIRT2 is predominately localized in the cytoplasm of cells but is also known to translocate to the nucleus (4). Among the known functions of SIRT2, is the deacetylation of the microtubule protein  $\alpha$ -tubulin in the cytoplasm and the deacetylation histone 4 lysine 16 in the nucleus, which is necessary for chromosome condensation (52). SIRT2 regulates both processes to control the entry and exit of the cells from mitosis (52). SIRT2 is highly abundant in the brain (Fig. 4) and was thought to be predominately expressed in oligodendrocytes; yet several studies have reported SIRT2 expression in microglia, astrocytes, neurons and neural stem cells (98, 99, 172, 176, 190). In the brain, SIRT2 controls

different processes including the inhibition of oligodendrocyte differentiation by microtubule deacetylation, pro- and anti-inflammatory functions in microglia, induction of cell death in neurons, and inhibition of neurite outgrowth (52, 98, 99, 127, 187).

Contrary to the overgeneralization that activating sirtuins promotes beneficial outcomes in the brain, SIRT2 seems to play an unconventional detrimental role. In the context of Parkinson's and Huntington's disease as well as in cerebral ischemia, the pharmacological inhibition of SIRT2 or its genetic ablation promotes beneficial outcomes (25, 28, 131, 148, 176). A recent study by Xie *et al.* showed that down-regulating SIRT2 in the brain was neuroprotective against cerebral ischemic injuries (176). In the study, SIRT2 was reported to be highly expressed in the cytoplasm of neurons and absent in microglia and astrocytes. In response to *in vitro* and *in vivo* models of cerebral ischemia, SIRT2 translocated to the nucleus of neurons and increased in abundance. The authors reported the same observation in the brains of post-mortems ischemic stroke patients. The administration of AGK2, a SIRT2 inhibitor, immediately after the injury or ablating *Sirt2* genetically significantly reduced infarct volume of mice subjected to tMCAo and improved their neurological impairments (176). Yet the exact nuclear functions of SIRT2 were not determined by the study. Although the authors show an increase in the H4K16 acetylation mark in *Sirt2*<sup>-/-</sup> mice compared to wild types in response to the tMCAo, the genomic targets of this modification were not identified. Other potential nuclear targets suggested by the authors but not experimentally addressed are the FOXO transcription factors. Under cellular stresses, SIRT2 is known to deacetylate FOXO1 and FOXO3a, which in turn, induces an increase in autophagy and mitochondrial superoxide dismutase (MnSOD) expression, respectively (176). Another study by Shimizu *et al.* showed that SIRT2 mediates a male-specific form of injury in the brain of mice subjected to a global cerebral ischemia (148). In response to cerebral ischemia, SIRT2 gets activated and, in turn, produces the OAADPr as a byproduct of its enzymatic activity. The OAADPr then activates the calcium-permeable transient receptor potential channel M2 (TRPM2), which is known to induce cell death when activated for a prolonged time (49, 68, 148). Although all sirtuins can produce OAADPr as a byproduct of their enzymatic activities, this study may suggest that the subcellular localization of OAADPr can regulate different subcellular signaling pathways. Introducing AGK-2 was able to reduce the cerebral injury and alleviate the functional deficits in wild-type mice but not in TRPM2 knockout mice (148). Interestingly, AGK-2 was not able to protect female mice from the same ischemic injury (68, 148). The authors partly attribute this sex-specific difference to the dynamic activation of SIRT2. In females, SIRT2 activity quickly returns to baseline after the injury, which the authors believe was not sufficient to activate the TRPM2 receptor, whereas in males it remains elevated for 24h after. The possible mechanism behind this sex-specific differential activation of SIRT2 was not addressed in the study, yet the authors believe their results support the oxidative stress-mediated male-specific mechanisms of cell death because of the involvement of the TRPM2 receptor, which is known to be predominately activated in males by oxidative stress (148). Krey *et al.* also showed that the knockout of SIRT2

was able to prevent neurological deficits in male mice subjected to two models of MCAo although no reductions in infarct volume or inflammatory cell count were observed (85). In ischemic brains, SIRT2 was reported to be upregulated and enriched in myelin-rich regions, whereas its expression was not detected in astrocytes, microglia, or neurons. Owing to the role of SIRT2 in regulating myelination and to the absence of inflammatory changes or infarct volume reductions, the authors concluded that SIRT2 induces a myelin-dependent form of axonal dysfunction in neurons, which was alleviated by its inhibition (85). In addition, an *in vitro* study by Nie *et al.* showed that in response to oxidative stress, SIRT2 was upregulated and promoted apoptosis in a differentiated PC12 cell line, a neuronal-like cellular model (127). The inhibition of SIRT2 with AGK2 or its knockdown reduced the cell death induced by hydrogen peroxide by reducing ROS production, the results of which support a role for SIRT2 in mediating oxidative stress-induced apoptosis (127). Furthermore, a study by Pfister *et al.* showed that the mere overexpression of SIRT2 in healthy CGNs and in HT22 cells was able to induce apoptosis (132).

In contrast, few studies have demonstrated a neutral or potentially neuroprotective role for SIRT2. A study by Chen *et al.* showed that the inhibition of SIRT2 by a different inhibitor AK7 was not able to reduce the infarct volumes or prevent neurological deficits of mice (25). Yet in this study, the authors pooled animals exposed to different treatments in their analysis that may have masked any protective effect of the drug (25). In 2015, Zhao *et al.* showed that transgenic mice overexpressing NAMPT had increased the number of neural stem cells, which correlated with enhanced functional recovery and survival after MCAo (190). The authors then knocked down all the sirtuin members in NSC cultured *in vitro* to identify downstream mediators of NAD<sup>+</sup> and showed that the knockdown of SIRT2 limited the cell's proliferation and prevented their differentiation. This study supports a positive role for SIRT2 in promoting NSC proliferation and differentiation, yet its direct role in cerebral ischemic injury was not shown experimentally (190).

Thus so far the majority of studies support a detrimental role for SIRT2 in cerebral ischemic injuries, although through different proposed mechanisms (85, 148, 176). The study by Shimizu *et al.* raises the important point that these effects are sex specific, a concept that warrants further scrutiny when targeting sirtuins in cerebral ischemia (148).

#### Mitochondrial sirtuins

**SIRT3.** SIRT3 is known as the main mitochondrial lysine deacetylase (130). It plays a crucial role in maintaining mitochondrial homeostasis by acting as a stress response protein. SIRT3 regulates mitochondrial energy metabolism and ROS levels in response to reduced nutrient availability and increased oxidative stress (48, 130). It deacetylates and activates several protein targets that play a role in the TCA cycle, oxidative phosphorylation, fatty acid oxidation, amino acid metabolism, urea cycle, and ROS detoxification (48, 130). Interestingly, the presence of an active enhancer in the *SIRT3* gene has been linked to increased longevity in humans (11).

In the context of cerebral ischemic injuries, several studies have explored the potential functions of SIRT3. Using an

*in vitro* model of neuronal cultures subjected to excitotoxic stress, Kim *et al.* showed that SIRT3 promotes neuroprotection against NMDA-mediated toxicity (78). Treating neuronal cultures with NMDA depleted cytosolic but not mitochondrial NAD<sup>+</sup> levels through PARP activation and only activated SIRT3 among the mitochondrial sirtuins. Inhibiting PARP-1 reduced SIRT3 activation, and transfecting cells with an enzyme that consumes cytosolic but not mitochondrial NAD<sup>+</sup> was sufficient to activate SIRT3. Furthermore, the overexpression of SIRT3 reduced cell death and lowered ROS levels in response to NMDA, whereas its knockdown exacerbated it. Thus, the study revealed a neuroprotective role of SIRT3 against excitotoxic injuries (78). Using a similar model, a study by Cheng *et al.* showed that SIRT3 mediates adaptive responses in neurons to excitotoxic and bioenergetics stresses (27). The knockout of SIRT3 increased the susceptibility of neurons to oxidative and mitochondrial stresses, while its rescue restored their resistance. This protection was proposed to be through the suppression of mitochondrial ROS levels. Notably, the stimulation of SIRT3 seen in hippocampal neurons by running on a wheel was required to mediate the neuroprotective effects of running (27).

Using *in vitro* models of primary neuronal cultures or neuronal cell lines subject to oxidative stress by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), SIRT3 was also reported to be neuroprotective. In 2013, Wang *et al.* showed that H<sub>2</sub>O<sub>2</sub> treatment reduced SIRT3 levels in motor neuron-like cells NSC34 (164). Pretreatment with valproic acid or lithium alleviated this toxicity. The induced protection correlated with increased SIRT3 levels, yet the authors did not experimentally show whether the protection is SIRT3 dependent (164). Dai *et al.* showed that treating mouse hippocampal cells HT22 with H<sub>2</sub>O<sub>2</sub> increased SIRT3 levels in a time- and dose-dependent manner. Similarly, the knockdown of SIRT3 worsened the injury, whereas its overexpression provided protection that correlated with reduced ROS and lipid peroxidation, yet with no changes in antioxidant enzyme activities (32). SIRT3 also reduced apoptosis and reversed the H<sub>2</sub>O<sub>2</sub>-mediated damage to the respiratory chain complexes, calcium buffering capacity, ATP production, and mitochondrial swelling (32). The same authors reported the same observation in rat neuronal cultures wherein they additionally observed increased antioxidant enzyme activity in response to SIRT3 overexpression, which correlated with reduced ROS (33).

Using an *in vitro* model of ischemia that is the OGD, SIRT3 was also reported to be neuroprotective. Shulyakova *et al.* showed that overexpressing SIRT3 in neuronally differentiated PC12 cells was protective against an OGD and reversed the OGD-induced increase in ROS (152). A similar study by Wang *et al.* also showed that the knockdown of SIRT3 in PC12 cells worsened the OGD-induced injury, whereas a recombinant form of SIRT3 rescued the cells and activated the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ) and MnSOD, both of which reduce oxidative stress. The knockdown of both genes abolished the SIRT3-mediated neuroprotection (167).

The role of SIRT3 in cerebral ischemia has also been explored using *in vivo* animal models. Yin *et al.* showed that ketones reduced the infarct volume and improved the neurological deficits of mice when introduced directly after a tMCAo (183). The protection correlated with increased

NAD<sup>+</sup>/NADH, SIRT3 levels, and SIRT3 downstream antioxidant genes forkhead box O3a (FoxO3a) and superoxide dismutase 2 (SOD2). A reduction in protein oxidation and preservation of mitochondrial function were also reported. Overexpressing SIRT3 in primary neurons increased their resistance to rotenone, an inhibitor of mitochondrial complex I, whereas its knockdown abolished the protection and decreased the levels of FoxO3a and SOD2. Thus, the study supports an SIRT-3-dependent neuroprotective role of ketones through the activation antioxidant genes, which, in turn, preserve mitochondrial function (183). In another study, Huang *et al.* showed that in response to a subarachnoid hemorrhage (SAH), SIRT3 levels are reduced (64). SIRT3 was detected in both neurons and endothelial cells and by measuring SOD2 levels, an SIRT3 downstream target; the authors reported a time-dependent increase in its expression after the SAH, which they interpreted as an increase in ROS production. Because of the downregulation in SIRT3 that correlated with increased cell death, the authors concluded that SIRT3 is required for the neuroprotection, yet this was not shown experimentally (64). Another study conducted *in vivo* by Yang *et al.* assessed the role of SIRT3 in endothelial cells against hypoxic injuries (178). Using *in vitro* and *in vivo* models, minocycline protected the blood brain barrier (BBB) against hypoxic injuries by inhibiting the expression of HIF-1 $\alpha$  (hypoxia-inducible factor 1 alpha) and its nuclear translocation. This correlated with reduced expression of its downstream targets that increase BBB permeability the matrix metalloproteinase -2 and -9 (MMP-2, MMP-9), and an increased expression of tight junction genes. In response to hypoxia, prolyl hydroxylase domain-containing protein 2, which targets HIF-1 $\alpha$  for degradation, was reduced concurrently with SIRT3, yet minocycline was able to upregulate both genes under hypoxic conditions. Interestingly, the knockdown of SIRT-3 *in vivo* abolished the protective effects of minocycline on BBB integrity and increased HIF-1 $\alpha$  expression, along with its downstream targets (178).

Yet contrary to previous studies, some studies have reported detrimental roles for SIRT3. A recent study by Novgorodov *et al.* showed that SIRT3 contributes to the cerebral ischemic injury by increasing ceramide production (128). Ceramide is found in the plasma, ER, and mitochondrial membranes where it plays important physiological functions, yet during cerebral ischemic injuries, its levels increase, which induces defects in the mitochondrial respiratory complexes and increases ROS production (128, 192). The study showed that I/R injury activates SIRT3, which, in turn, deacetylates the mitochondrial ceramide synthase, leading to its increased enzymatic activity and ceramide abundance. SIRT3 KO mice had reduced ceramide synthases activity in response to I/R that correlated with reduced mitochondrial ceramide, and as such had preserved mitochondrial respiration, reduced oxidative damage, and reduced infarct volume in response to a tMCAo (128). Furthermore, a study by Pfister *et al.* showed that in CGNs and in HT22 cells, the mere overexpression of SIRT3 was able to induce apoptosis (132).

In the context of preconditioning, a recent work from our laboratory by Morris-Blanco *et al.* showed that the ischemic tolerance mediated by protein kinase C epsilon (PKC $\epsilon$ ) preconditioning is independent of SIRT3 activity but relies on the activity of the mitochondrial SIRT5 (117). Our laboratory had previously shown that PKC $\epsilon$  increases the mitochondrial

NAD<sup>+</sup>/NADH ratio. In a follow-up study, the increase in mitochondrial NAD<sup>+</sup>/NADH ratio was shown to only affect the activity of mitochondrial SIRT5 with no effects on that of SIRT3, suggesting that the ischemic tolerance mediated by PKC $\epsilon$  preconditioning is independent of SIRT3 activity (116, 117).

Although several studies support a neuroprotective role for SIRT3 in cerebral ischemic injuries, additional studies would be required to better resolve its role because some studies have reported detrimental functions for SIRT3.

**SIRT4.** SIRT4 has a low deacetylase activity, yet possesses an ADP ribosyl transferase activity as well as a lipamidase activity (130). In nutrient-replete conditions, SIRT4 inhibits fatty acid oxidation in skeletal muscles and promotes lipogenesis in white adipose tissues through the deacetylation and repression of the malonyl CoA decarboxylase. Thus *Sirt4* KO mice are resistant to diet-induced obesity (92). During nutrient-sufficient conditions, SIRT4 in the pancreas reduces the activity of the glutamate dehydrogenase (GDH) by ADP ribosylation, and thus reduces insulin secretion by  $\beta$  cells. While during calorie restriction, SIRT4 activity is downregulated in the pancreas and allows  $\beta$  cells to release insulin in response to amino acids (50). In liver and muscle cells, the knockdown of SIRT4 has been shown to increase fatty acid oxidation (125). Based on these studies, the pharmacological inhibition of SIRT4 is considered a potential therapeutic strategy against metabolic diseases (48, 125).

In the brain, very little is known about the functions of SIRT4 or its enzymatic targets. A study in 2013 reported SIRT4 to be highly expressed in astrocytes and radial glial cells in the postnatal brain specifically in the mitochondria and to be reduced in expression during development (82). Using a radial glial cell line, CTX8, the overexpression of SIRT4 reduced its astrocytic development *in vitro* possibly by inhibiting the SIRT4 downstream target GDH1, thus supporting a role for SIRT4 in regulating gliogenesis (82).

In the context of cerebral ischemia, the role of SIRT4 has been largely unexplored, with only one study assessing its role in excitotoxic injuries (147). In this study, SIRT4 levels were reported to be upregulated in the hippocampus of mice after kainic acid (KA) treatment. SIRT4 knockout mice showed increased cell death and increased seizure phenotypes in response to KA exposure. In addition, using *in vitro* cultures of either astrocytes or mixed neuronal-astrocytic cultures, SIRT4 was reported to be predominately expressed in astrocytes and present at lower levels in neurons. Mice lacking SIRT4 showed significantly reduced rates of GLT-1-dependent glutamate uptake compared with wild types under baseline as well as in response to KA. Supporting this observation, hippocampal lysates lacking SIRT4 showed significantly reduced levels of GLT-1 at the cell surface yet with no differences observed in total cell lysates. This study supports a protective role for SIRT4 against excitotoxic injuries in astrocytes through the increased surface expression of GLT-1. In the same study, the authors showed that neuronal cultures lacking SIRT4 had lower baseline ATP levels and a dose-dependent decrease in ATP levels in response to KA. These data support a role for SIRT4 in energy production in neurons, which may be important in their protection against excitotoxic injuries (147). Thus the study supports a

neuroprotective role for SIRT4 in the brain by reducing excitotoxicity, yet the overall functions of SIRT4 in the brain are still largely unexplored specifically in the context of ischemic injuries.

**SIRT5.** SIRT5 possesses several enzymatic activities including demalonylation, deglutarylation, desuccinylation, and a low deacetylation activity (86). SIRT5 regulates several metabolic processes, including glycolysis, respiration, fatty acid oxidation, apoptosis, and ROS detoxification (130, 144). One of the well-studied functions of SIRT5 is its regulation of the urea cycle in the liver. The urea cycle detoxifies ammonia derived from the deamination of amino acids through its conversion into urea, a less toxic molecule (121, 122). In the liver, SIRT5 within the mitochondrial matrix deacetylates and activates the rate-limiting enzyme of the urea cycle called carbamoyl phosphate synthetase 1 (CPS1) (121, 122). During fasting and calorie restriction, the regulation of CPS1 by SIRT5 is increased to detoxify the excess ammonia released from amino acids metabolism (121, 122). In nonliver cells, SIRT5 can also regulate the urea cycle through the desuccinylation of glutaminase (134).

In the brain, SIRT5 has not been extensively studied. A study by Liu *et al.* supports a neuroprotective role for SIRT5 in Parkinson's disease. (103). In addition, Li *et al.* showed that the absence of SIRT5 in mice exacerbates the KA-induced seizures and increases the neuronal degeneration in the hippocampus (94). KA increased SIRT5 levels in the surviving mitochondria of the hippocampus, which the authors interpreted as a reflection of its neuroprotective role.

A study by Pfister *et al.* assessed the role of all the sirtuin genes on the survival of neuronal cells *in vitro*. In CGNs induced to undergo apoptosis, SIRT5 is protective when localized in the nucleus and cytoplasm, but when localized in the mitochondria it induces apoptosis. While using HT22 cells, SIRT5 overexpression, which localized to the mitochondria, induced apoptosis. Thus it is possible that the subcellular localization of SIRT5 determines its outcome on the survival of neurons (132).

In the context of cerebral ischemic injuries, a study by Morris-Blanco *et al.* showed that SIRT5 is required for the neuroprotection afforded by PKC $\epsilon$  and IPC against cerebral ischemic insults (117). Using mixed neuronal-astrocytic cultures, PKC $\epsilon$  and IPC increased both the activity and expression of mitochondrial SIRT5 but not SIRT3, which correlated with a reduction in mitochondrial succinylation but not acetylation. Consistently, SIRT5 knockout mice showed increased levels of mitochondrial lysine succinylation in the cortex, which correlated with a reduction in the oxygen consumption rate of mitochondrial respiratory complexes. Also, PKC $\epsilon$  preconditioning enhanced mitochondrial respiration in wild-type mice but not in SIRT5 knockouts. Furthermore, SIRT5 knockout mice lose the neuroprotection mediated by PKC $\epsilon$  preconditioning against cerebral ischemic insults as revealed by increased necrotic cell death and larger infarct volumes. Thus, this study supports a role for SIRT5 as a mediator of cerebral ischemic tolerance afforded by preconditioning possibly through the regulation of mitochondrial respiration (117).

Dong *et al.* in 2011 evaluated the association between genetic variants in the sirtuin genes and presence or number

of carotid plaques (39). The study revealed two SNPs and a 4-SNP haplotype in the *SIRT5* gene to be associated with increased number of carotid plaques. The study also revealed an association between smoking, hypertension, and diabetes and *SIRT5* SNPs with number and abundance of carotid plaques. Since the accumulation of carotid plaques increases the risk of clinical atherosclerosis, an underlying cause of ischemic strokes, this study suggests a potential role for *SIRT5* in increasing the risk of ischemic strokes. (39).

Thus although some reports support a neuroprotective role of SIRT5 in cerebral ischemic injuries, its functions are still largely unexplored and await further studies.

## Conclusion

In conclusion, numerous studies have supported the importance of targeting the sirtuin family members either before or after a cerebral ischemic injury (Fig. 5 and Table 1). Both approaches would be of significant interest in clinical settings for the therapeutic targeting of cerebral ischemic injuries. Preconditioning offers an appealing approach to target the sirtuin family because it induces a coordinated response of multiple rather than single sirtuin members, which, in turn, integrates many cellular physiological processes to create the ischemic tolerant phenotype. The processes regulated by sirtuins in the context of cerebral ischemic injuries as reviewed above involve DNA repair, mitochondrial respiration, oxidative stress regulation, calcium buffering, and glutamate uptake among several others (Fig. 5). Yet there is still a need to better understand the functions of the less characterized sirtuin members, especially that many of them are highly abundant in the brain and display distinct regional and cellular localization (Figs. 1 and 4). Also, there is a need for the use of sirtuin-specific pharmacological activators or inhibitors as opposed to multisirtuin modulators, which were used in the initial studies mainly because of the opposing functions reported by different sirtuin members.

Most studies discussed above have used male model organism in their studies, whereas female model organisms have been underrepresented. Taking into account the sex-specific difference seen with some members of the sirtuin family as well as with the use of PARP modulators, this stresses the need to better understand the sex-specific mechanisms of sirtuins in a preclinical setting before translating some of these findings into the clinic. Moreover, since sirtuins rely on NAD<sup>+</sup> to conduct their enzymatic functions, future studies can potentially address combinatorial approaches that regulate both NAD<sup>+</sup> levels and sirtuins' activity to combat cerebral ischemic injuries and promote ischemic tolerance or recovery.

Moreover, interesting findings from a recent study have revealed that chronic exposure to hypoxia can actually compensate for defects in mitochondrial complex respiration through the activation of the endogenous hypoxia response (67). Findings from this study can have promising applications in the context of cerebral ischemia, which is known to induce defects in the mitochondrial complexes. Future studies can also determine whether members of the sirtuin might be playing a role in this hypoxic response as has been suggested recently by some studies (40, 70).

In addition, taking into account the recent findings that mitochondria can be transferred between cells of the central

nervous system which can potentially mediate protective effects and that many of the cellular physiological processes that are regulated by the sirtuins converge into mitochondrial regulated processes, future studies should address this avenue to determine whether sirtuins themselves are part of the exchange or whether they play a role in a cell nonautonomous manner through mitochondrial exchange (35, 55). Studies of mitochondrial transfer are now being assessed in clinical trials using cardiac tissues, which could potentially open the door for future trials that could introduce them into the brain (42, 108).

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### Abbreviations Used

ACA = asphyxial cardiac arrest  
 BBB = blood brain barrier  
 BCAo = bilateral common carotid artery occlusion  
 BCAS = bilateral common carotid artery stenosis  
 bEnd.3 = brain endothelial cell line  
 cADPR = cyclic ADP ribose  
 CAT = catalase  
 CGNs = cerebellar granule neurons  
 CPS1 = carbamoyl phosphate synthetase 1  
 eNOS = endothelial nitric oxide synthase  
 ER = endoplasmic reticulum  
 ETC = electron transport chain  
 FoxO3a = forkhead box O3a  
 GDH = glutamate dehydrogenase  
 H<sub>2</sub>O<sub>2</sub> = hydrogen peroxide  
 HDACs = histone deacetylases  
 HIF-1 $\alpha$  = hypoxia-inducible factor 1 alpha  
 HIF-2 $\alpha$  = hypoxia-inducible factor 2 alpha  
 HMGB1 = high mobility group box-1 protein  
 HT22 = immortalized mouse hippocampal neuronal cell line  
 I/R = ischemic/reperfusion  
 ICV = intracerebroventricular  
 IPC = ischemic preconditioning  
 KA = kainic acid  
 MMP-2 = matrix metalloproteinase 2  
 MMP-9 = matrix metalloproteinase 9  
 MnSOD = mitochondrial superoxide dismutase  
 NA = nicotinic acid  
 Na<sub>2</sub>S = sodium sulfide  
 NAAD = nicotinic acid adenine dinucleotide  
 NAADP = nicotinic acid adenine dinucleotide phosphate  
 NAD<sup>+</sup> = nicotinamide adenine dinucleotide  
 NAM = nicotinamide  
 NAMN = nicotinic acid mononucleotide  
 NAMPT = nicotinamide phosphoribosyltransferase  
 NF- $\kappa$ B = nuclear factor kappa-light-chain-enhancer of activated B cells  
 NMN = nicotinamide mononucleotide  
 NMNATs = nicotinamide mononucleotide adenylyl transferases  
 NR = nicotinamide riboside  
 NRK = nicotinamide riboside kinase  
 OAADPr = O-acetyl-ADP ribose  
 OGD = oxygen and glucose deprivation  
 PARPs = poly-ADP ribose transferases  
 PGC-1 $\alpha$  = PPAR- $\gamma$  coactivator 1- $\alpha$   
 PKC $\epsilon$  = protein kinase C epsilon  
 RNS = reactive nitrogen species  
 ROS = reactive oxygen species  
 rtPA = recombinant tissue plasminogen activator  
 SAH = subarachnoid hemorrhage  
 Sir2 = silent information regulator 2  
 SIRT = sirtuin  
 SNP = single nucleotide polymorphism  
 SOD = superoxide dismutase  
 SOD2 = superoxide dismutase 2  
 TCA cycle = tricarboxylic acid cycle  
 tMCAo = transient middle cerebral artery occlusion  
 TRPM2 = calcium permeable transient receptor potential channel M2