Neuroprotective Mechanisms of Ischemic Tolerance Induced by Resveratrol Preconditioning

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NEUROPROTECTIVE MECHANISMS OF ISCHEMIC TOLERANCE INDUCED BY RESVERATROL PRECONDITIONING

By
Kevin B. Koronowski

A DISSERTATION

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of the University of Miami
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Ischemic stroke is a devastating neurological disorder that currently has no clinically approved neuroprotective treatments. Resveratrol is a naturally occurring polyphenol that protects against cerebral ischemic injuries when administered in a prophylactic manner. Yet, the molecular underpinnings of resveratrol-induced neuroprotection are still unclear. This is partly because the metabolic adaptations induced by a major target of resveratrol, the NAD⁺-dependent deacetylase Sirt1, have not been characterized in the brain. The goal of this study was to better understand the efficacy of resveratrol as a therapeutic agent in ischemic stroke and the metabolic pathways regulated by brain Sirt1 that promote ischemic tolerance. Evaluation of different resveratrol treatment paradigms revealed that while repetitive administration does not enhance its neuroprotective effects, desensitization to multiple exposures does not occur. Remarkably, a single application of resveratrol protected mice from focal ischemia for up to two weeks, demonstrating a novel extended window of preconditioning-induced ischemic tolerance. Further experimentation suggested that Sirt1-mediated epigenetic modifications at DNA might underlie protection in the extended window. Using inducible, neuronal-specific Sirt1 knockout mice, we found that resveratrol is unable to induce neuroprotection without intact neuronal Sirt1. Metabolomics-based analyses identified glucose metabolism as a major
metabolic pathway regulated by Sirt1 in the brain. More specifically, whereas resveratrol increased glycolytic rate in cultured neurons under basal and ischemic-penumbra-like conditions, Sirt1 inhibition blocked its metabolic effects. Together, these results provide preclinical evidence of the efficacy of resveratrol as a therapeutic agent in ischemic stroke and identify a novel mechanism of ischemic tolerance that hinges upon a Sirt1-mediated metabolic adaptation of glucose metabolism in neurons.
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Chapter 1

Introduction

1.1. Pathophysiology of Ischemic Stroke

Brain ischemia is a devastating tissue state that underlies several neurological disorders including stroke and cardiac arrest. Stroke is a leading cause of death and the leading cause of severe, long-term disability in the US\(^1\). A large portion of patients do not survive the injury while those that do suffer permanent motor, sensory and cognitive deficits. This is estimated to cost the US alone over $32.5 billion in healthcare annually\(^1\). Despite this heavy burden, there are still no clinically approved neuroprotective treatments. The stroke clinic currently encompasses treatment of ischemic stroke with the thrombolytic agent recombinant tissue plasminogen activator (rtPA), which must be administered within four hours of injury onset and is therefore extremely limited in its application\(^2\). Additionally, medications such as anti-platelets in combination with statins are recommended to prevent another ischemic episode.

Ischemic stroke, which makes up the large majority of all strokes, arises from a thrombus or embolus that occludes a blood vessel supplying an area of the brain, resulting in cell death or cerebral infarction\(^3\). The infarct is characterized by a “core” region that receives little to no blood flow (<10 ml/100 g tissue/min) and a surrounding peri-infarct area or “penumbra” that experiences a significant decrease in perfusion (<25 ml/100 g of tissue/min)\(^4\). Cells within the core perish within minutes of stroke onset, primarily as a result of excitotoxic damage that leads to necrosis. Within the penumbra however, cells are
damaged through various mechanisms incited by excitotoxicity, reactive oxygen species (ROS) and inflammation but can remain viable for hours to days following injury. It is postulated that the penumbra makes up as much as half of the total infarct volume and is therefore an attractive target for significant therapeutic intervention.

1.1.1 Brain Energy Metabolism

The brain is particularly susceptible to ischemia due to its immense metabolic demand. Despite representing ~2% of body weight, the brain accounts for an astonishing ~20% of the body’s resting metabolic rate. Whereas astrocytes account for only ~5%-15% of the total energy requirement, functional imaging studies show the largest turnover of ATP in the gray matter, which has a high density of excitatory glutamatergic synapses. Indeed, it is estimated that ~80% of ATP in the brain is consumed during pre and postsynaptic signaling, owing to the large energetic demand of neurons. To meet this demand, the brain requires continuous perfusion of energy substrates and relies heavily on mitochondrial oxidative phosphorylation (~90% of brain ATP production) with glucose as an obligatory substrate. Still, both oxidative and non-oxidative glucose degradation contribute to brain energy generation. Ultimately, tight regulation of the neurons, astrocytes, and endothelial cells that make up the neurovascular unit, couples neuronal activity with cerebral blood flow and thus meets the local energy demand.

The metabolic makeup of the brain is partially a result of the blood brain barrier (BBB), which regulates the passage of ions, energy substrates and precursors for neurotransmitter synthesis. Tight junctions between adjacent endothelial cells form the BBB, along with astrocytic processes that wrap the microvessels, forcing transit of non-
diffusible compounds to pass through these cells\textsuperscript{12}. Almost all lipid insoluble compounds and proteins require specific transport mechanisms for uptake into brain and hence a metabolic barrier exists. Concerning energy substrates, unbound fatty acids and carnitine can diffuse across the BBB\textsuperscript{13,14} and may be taken up through fatty acid transport proteins\textsuperscript{15}. Glucose transporters (GLUTs) and monocarboxylic acid transporters (MCTs)\textsuperscript{16} mediate the uptake of glucose and lactate, with cell-type specific isoforms, implicating endothelial, glial and neuronal cells in the direct uptake of these substrates. Only under conditions of long-term starvation do MCTs significantly mediate the uptake of liver-derived ketones such as β-hydroxybutyrate and acetoacetate into the adult brain\textsuperscript{17}.

It is generally accepted that glucose is the predominant energy substrate for the brain; however, it is actively debated whether glucose or its derivative lactate is mainly utilized by neurons. One theory proposes that in response to neuronal activity, astrocytes mediate the uptake and glycolytic oxidation of glucose to lactate, which is then shuttled to and taken up by neurons\textsuperscript{18}. In neurons, this astrocytic-derived lactate is then converted to pyruvate for energy production via oxidative phosphorylation. The fact that neurons have a lower glycolytic capacity from lack of 6-phosphofructo-2 kinase/fructose-2, 6-bisphosphatase 3 (PFKFB3) activity\textsuperscript{19} and that astrocytes display reduced mitochondrial oxidative capacity from lack of glutamate-aspartate transporter (GAT)\textsuperscript{20,21} and inhibited pyruvate dehydrogenase complex (PDHC)\textsuperscript{22} supports this lactate shuttle hypothesis. However, evidence also suggests that neurons directly take up glucose through GLUT3 and that it can be oxidized by glycolysis for energy production\textsuperscript{23}. In support of this notion, recent studies demonstrate that neuronal glycolysis is essential for fast axonal transport of vesicles\textsuperscript{24}, the maintenance of presynaptic function during activity\textsuperscript{25} and the generation of
synaptic ATP under energetic stress\textsuperscript{26}. It is likely that the spatial and temporal distribution of energy consuming and producing pathways are responsible for some of the experimental discrepancies in studies of brain energy metabolism.

It is debated that in addition to glucose, β-oxidation of fatty acids contributes significantly to energy production in the brain. Studies show that fatty acids can be oxidized\textsuperscript{27} once they have been taken up by the brain\textsuperscript{28}. Estimates suggest that \textasciitilde20\% of the total energy requirement of the adult brain is met by oxidation of fatty acids\textsuperscript{29}. Given the ability of the BBB to transport unbound fatty acids and carnitine from the blood to astrocytes, it is postulated that astrocytes are the compartment of β-oxidation\textsuperscript{29,30}. Despite these observations, studies also provide evidence to the contrary. Several enzymes of the β-oxidation pathway, including carnitine palmitolytransferase 1 (CPT1)\textsuperscript{31}, 3-ketoacyl-coenzyme A thiolase (HADHB)\textsuperscript{32} and acyl- or enoyl- CoA dehydrogenase (ACAD, ECAD)\textsuperscript{32}, display diminished enzymatic capacity or activity in brain. Additionally, isolated brain mitochondria display poor oxidative degradation of long-chain fatty acids, evidence by rates of CO\textsubscript{2} liberation and O\textsubscript{2} consumption from \textsuperscript{14}C-labeled fatty acids\textsuperscript{32}. Further studies are necessary to determine the physiological relevance of β-oxidation in the brain.

1.1.2 Excitotoxic Injury and Mechanisms of Reperfusion

The metabolic landscape of the brain gives rise to its susceptibility to ischemic stroke. Occlusion of a vessel supplying blood flow deprives the affected brain tissue of energy substrates, induces energetic failure and ultimately causes excitotoxic injury\textsuperscript{33}. Storage of energy in the brain is limited and may only slightly delay the onset of the initial ischemic cascade. Once on hand ATP is depleted, the creatine kinase/phosphocreatine
system, which serves as a short-term and small energy reserve\textsuperscript{34}, will be consumed rapidly. The brain does store glycogen (3-12 umol/g tissue), however in a small quantity compared to that of the liver (100 to 500 umol/g tissue)\textsuperscript{35}. The astrocytic localization of glycogen may preclude its ability to maintain neuronal ATP levels in the absence of O\textsubscript{2}, and studies in acute brain slices demonstrate that these stores delay injury onset by only a few minutes\textsuperscript{36}.

Once cellular ATP levels fall to the point of failure of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase and other ATP-dependent ion pumps, a large and rapid depolarization termed anoxic depolarization (AD) is initiated\textsuperscript{37}. Various mechanisms contribute to the initiation and propagation of these depolarizations from the ischemic core to the surrounding penumbral regions. Initial depolarization causes release of the excitatory neurotransmitter glutamate from presynaptic terminals\textsuperscript{38,39}. Glutamate binds and activates N-methyl-D-aspartic acid (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on postsynaptic terminals, resulting in the influx of Na\textsuperscript{+} and Ca\textsuperscript{2+} ions and in turn depolarization of postsynaptic cells\textsuperscript{40,41}. Simultaneously, voltage-gated Na\textsuperscript{+} and Ca\textsuperscript{2+} channels as well as non-specific cation channels mediate the influx of these ions and contribute to further depolarization\textsuperscript{37}. Eventually, glutamate reuptake transporters found on neurons and glial end feet reverse, contributing significantly to increased extracellular glutamate\textsuperscript{42}. This glutamate release and depolarization leads to a perpetuating cycle of ionic imbalance and induces what is known as excitotoxicity.

Cell death from excitotoxic injury occurs through several mechanisms and depends upon the extent of ionic compromise. In the ischemic core, massive Na\textsuperscript{+} influx causes swelling, edema and shrinkage of the extracellular space while intracellular Ca\textsuperscript{2+} overload
triggers activation of phospholipases and proteases that degrade membranes and proteins\textsuperscript{43}. Ultimately, these perturbations initiate necrotic cell death, which is prominent within hours of injury onset. In the ischemic penumbra, where cells have experienced a partial energetic impairment, apoptotic cell death occurs when ionic homeostasis cannot be restored and damage to cellular components cannot be overcome\textsuperscript{44}.

Mitochondrial integrity is pivotal in determining whether cells overcome pro-death signals. During ischemia, intracellular Ca\textsuperscript{2+} rises to a toxic level and eventually exhausts the Ca\textsuperscript{2+} buffering capacity of mitochondria\textsuperscript{45}. This leads to swelling of the inner mitochondrial membrane, opening of the mitochondrial permeability transition pore (mPTP), rupture of the outer membrane and collapse of the mitochondrial membrane potential ($\Delta\Psi_m$). As reperfusion sets in, dysregulation of Ca\textsuperscript{2+} and abnormally high pO\textsubscript{2} from an initial stint of hyperperfusion causes excessive production of reactive oxygen species (ROS) by electron transport chain (ETC) complexes. This excessive ROS is damaging to matrix proteins, lipids of mitochondrial membranes and mitochondrial DNA\textsuperscript{46}, in addition to extramitochondrial cellular components. Ca\textsuperscript{2+} also intercedes apoptosis from within mitochondria by activating calpains, which mediate truncation of apoptosis inducing factor (AIF) and in turn its translocation to the nucleus where fragmentation of DNA ensues\textsuperscript{47}. Additionally, opening of the mPTP releases sequestered pro-apoptotic factors into the cytoplasm, leading to formation of the apoptosome and apoptotic cell death\textsuperscript{48}.

Aside from deleterious mechanisms in neurons, other brain cell types contribute to the pathophysiology of ischemic injury. Reactive astrogliosis, the process whereby astrocytes undergo phenotypic alterations in response to CNS injury, results in formation
of the glial scar in the penumbral regions that demarcates the ischemic core\textsuperscript{49, 50}. Injury to the white matter, including oligodendrocytes, activates proteases that weaken the structural integrity of myelin sheaths\textsuperscript{3}, further compromising axonal function and the ability for neuronal repair. Resident microglia, the immune cell that constantly survey’s the CNS microenvironment, are activated within minutes of ischemia onset and infiltrate the core lesion site and surrounding tissue in the days following injury\textsuperscript{51, 52}. With breakdown of the BBB, circulating immune modulators enter the brain and contribute to the inflammatory landscape of the ischemic regions\textsuperscript{53}. As a result of cytokine, chemokine, ROS and reactive nitrogen species (NOS) release from reactive cell types\textsuperscript{54} as well as released contents from ruptured necrotic cells, inflammation can induce further apoptotic cell death depending upon the state of pro- vs anti-inflammatory mediators\textsuperscript{55}. The culmination of these deleterious mechanisms gives rise to the complex and multifaceted nature of ischemic injury in the brain.

\textbf{1.2 Strategies for Neuroprotection}

Given the unpredictable nature of stroke, treatment has been geared toward facilitating recovery. A myriad of potential post-stroke agents have seen clinical trials but none thus far have demonstrated both clinical safety and efficacy. For example, robust preclinical targets in animal studies, such as NMDA and AMPA receptor antagonists, have failed in clinical trials since they are crucial components of normal synaptic physiology and therefore affect the mental capacity of human patients\textsuperscript{56, 57}. Additionally, once the initial ischemic injury sets in motion the ischemic cascade and subsequent mechanisms of reperfusion, treatment in this therapeutic window may lose its effectiveness. Conversely, evidence demonstrating the efficacy of pre- or prophylactic treatment has been mounting
in the scientific literature. Millions at risk for stroke can be targeted by prophylaxis as a new therapeutic avenue for sizable neuroprotection. This is evidenced by preclinical studies demonstrating the efficacy of preconditioning-induced protection in cerebral ischemia.

1.2.1 Pathways of Ischemic Preconditioning (IPC)-Induced Ischemic Tolerance

Preconditioning is a type of pre-treatment that in general, refers to an intervention prior to a damaging event that can provide protection against the subsequent damaging event. This effect has been demonstrated in a variety of tissues and across pathologies. The first preconditioning studies concerning cerebral ischemia were carried out by way of ischemic preconditioning (IPC), a phenomenon where an ephemeral bout of ischemia can protect against a subsequent damaging ischemic insult. IPC elicits a protective response that is characterized by two distinct time windows. The early window of protection is observed within minutes to hours of the stimulus and relies on the immediate release of factors and post-translational modifications of existing proteins. The delayed or late window, which exhibits the most robust protection, is evident one to three days post-stimulus and has been shown to last up to seven days in one study. A large body of work demonstrates that protection in the late window hinges upon activation of signaling pathways, transcriptomic changes, epigenetic modifications and de novo protein synthesis.

A myriad of cellular and molecular targets have been identified as mediators of IPC, which together culminate in a phenotype of ischemic tolerance (reviewed in). Molecular targets include adenosine, NMDA receptors, gamma-aminobutyric acid (GABA), glutathione, signal transducer and activator of transcription (STATs), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), Toll-like receptors, protein kinase C epsilon
(PKCε), mitochondrial ATP-sensitive K⁺ channel (mitoK⁺-ATPase), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), cyclic-AMP response element-binding protein (CREB), microRNAs, nitric oxide (NO), c-Jun N-terminal kinase (JNK), p38, mitogen-activated protein kinase (MAPK) and extracellular-signal-regulated kinase (ERK), among others. The cellular pathways and deleterious processes targeted include excitotoxicity, antioxidant capacity and ROS generation, DNA repair, anti-inflammatory signaling, oxidative phosphorylation capacity, Ca²⁺ buffering of mitochondria and autophagy, to name a few. While the involvement of these mechanisms is well characterized, the impact of IPC on nicotinamide adenine dinucleotide (NAD⁺) metabolism and silent information regulator two-homologue protein (Sirtuins) function is still unclear.

NAD⁺ is a vital co-factor for redox reactions of various cellular processes, including energy production, and is a substrate for the Sirtuin family of deacylase enzymes that regulate metabolism. In hippocampal slices, IPC-induced protection is concomitant with increases in the NAD⁺/NADH ratio and silent information regulator two-homologue one (Sirt1) activity. Application of the Sirtuin inhibitor sirtinol blocks IPC-induced protection in vitro, demonstrating that Sirtuins are crucial mediators of IPC. Sirt1 activity is increased in rat hippocampus at the time of IPC-induced protection in a rat model of global ischemia. Importantly, Sirtuin inhibition with sirtinol blocked the protective effect of IPC, confirming the previous findings in vivo. From a translational aspect, there is immense therapeutic potential in pharmacological compounds that mimic the effects of IPC. Several groups including our own have demonstrated that the naturally occurring compound resveratrol mimics IPC in the brain.
1.2.2 Pathways of Resveratrol Preconditioning-Induced Ischemic Tolerance

Resveratrol, a polyphenolic small molecule that is synthesized naturally in plants as an innate defense mechanism against fungal and bacterial infections, can be traced back to herbal medicinal use in ancient times\textsuperscript{71}. However, it was not until the 1990’s that it gained much attention in the scientific literature, when it was proposed as a possible candidate for the cardio-protective effects of the “French Paradox” \textsuperscript{72}. Since then, researchers have identified anti-cancer, anti-inflammatory and anti-oxidative properties of resveratrol, which have therapeutic implications in a host of disorders; however, the notion that resveratrol can afford protection from ischemic stroke is a relatively new one.

Our lab and others have demonstrated that resveratrol acts as a preconditioning agent, mimicking the effects of IPC \textit{in vivo}\textsuperscript{70}. Several studies show that different resveratrol preconditioning (RPC) paradigms induce neuroprotection against global and focal ischemia in rodents. A single application of RPC two days prior to injury reduces infarction from middle cerebral artery occlusion (MCAo) in mice\textsuperscript{73} and asphyxial cardiac arrest (ACA, global ischemia) in rats\textsuperscript{70}. RPC treatment for seven days prior to injury is also effective in mouse\textsuperscript{74} and rat MCAo models\textsuperscript{75, 76} while one study observed protection with twenty one days of pretreatment \textsuperscript{77}. Moreover, a variety of RPC doses and routes of administration have shown efficacy in these studies. Together, these results suggest that resveratrol is a robust preconditioning agent in cerebral ischemia. Interestingly, studies also demonstrate neuroprotective effects of resveratrol administered post-injury. Therapeutic implications of pre and post-treatment are discussed in Chapter 4.

Resveratrol is a promiscuous small molecule that induces a host of cellular responses and thus its protective effects are likely the culmination of several mechanisms (Fig. 1).
During MCAo in rats, RPC reduces release of the excitatory neurotransmitters glutamate, aspartate and δ-serine while increasing release of the inhibitory neurotransmitters GABA, glycine and taurine.\(^7^6\) \textit{In vitro}, resveratrol suppresses postsynaptic glutamatergic transmission\(^7^8\) and increases astrocytic uptake of glutamate in response to oxidative stress\(^7^9\), supporting the notion that RPC attenuates excitotoxicity. In another line of evidence, RPC elicits neuroprotection through antioxidant pathways. Consistent with improved functional outcome and reduced infarct severity, RPC reduces malondialdehyde (MDA)\(^7^5\), a marker of oxidative stress, and enhances the antioxidant capacity of superoxide dismutase (SOD)\(^7^5\), heme oxygenase-1 (HO-1)\(^7^5\) and glutathione\(^7^7\). These effects may be mediated through the transcription factor Nrf2, which induces genes with antioxidant response elements (ARE) in their promoter regions\(^8^0\). RPC activates Nrf2 in cultured
astrocytes as well as increases its astrocytic expression in vivo. Whereas RPC protects wild type (WT) mice from MCAo, its protective effects and ability to upregulate antioxidant defenses are lost in Nrf2−/− mice, demonstrating a key role for this pathway. Figure 1 further illustrates the resveratrol-elicited cellular responses and targets that are linked to cerebral ischemic protection, which will be discussed in detail in the context of subsequent sections/chapters.

It is postulated that the robust, neuroprotective effects of RPC are at least in part mediated by Sirt1, since resveratrol is a Sirt1 activating compound. Several mechanisms of resveratrol-induced Sirt1 activation have been proposed. In a dose-dependent manner, resveratrol stimulates Sirt1 deacetylase activity by enhancing binding affinity for its substrates. This effect could be mediated by direct interaction of resveratrol with Sirt1, as molecular modeling experiments show that resveratrol can induce conformational changes in Sirt1 protein motifs. However, recent evidence has challenged direct activation of Sirt1 by resveratrol and alternative mechanisms of action have been proposed. Resveratrol may indirectly activate Sirt1 through CK2-mediated phosphorylation of Sirt1 at serine 659 and 661, increased binding with lamin A (aiding in nuclear matrix localization and activity of Sirt1) and increased NAD+ levels by activation of AMPK and its downstream target nicotinamide phosphoribosyl transferase (NAMPT), the rate limiting enzyme in the NAD+ salvage pathway.

1.3 The Role of Sirt1 in Brain, Cerebral Ischemia and Preconditioning

The Sirtuins are an evolutionarily conserved family of NAD+ dependent deacylase enzymes that regulate metabolic homeostasis in almost all tissues. There are seven mammalian Sirtuins (Sirt1-7), with Sirt1 being the most highly conserved member from
yeast to humans, which differ in substrate specificity and subcellular localization. However, they share the requirement for NAD\(^+\) as a cofactor for their enzymatic activity, giving rise to their “nutrient sensing” ability\(^{92,91}\). For example, under conditions of cellular energetic stress, the NAD\(^+\)/NADH ratio increases up to two-fold and activates sirtuin proteins, which in turn elicit adaptive metabolic responses\(^{93}\). Their distinct functions are determined by their preferred acyl substrate (acetyl, glutaryl, succinyl, malonyl, propionyl, butyryl, crotonyl, myristoyl, ADP-ribosyl, lipoyl) and their subcellular distribution (Sirt1, 6 and 7 – nucleus; Sirt3, 4 and 5 – mitochondrion; Sirt2 - cytosol). Although there is little overlap in terms of substrate specificity, several studies report non-primary localizations of Sirtuins and their ability to translocate within the cell\(^{94,95}\).

1.3.1 Metabolic Functions of Sirt1 in Brain

Sirt1, along with Sirt6 and Sirt7, are primarily localized to the nucleus\(^{96,97,98}\). Originally identified solely as a histone deacetylase, Sirt1 acts on both histone and non-histone proteins to regulate the genome and epigenome\(^{99}\). This occurs at several levels including deacetylation of lysine residues of histone tails, remodeling chromatin at specific sites, and deacetylation of transcription factors – activating or inhibiting their activity. The molecular targets of Sirt1 are vast; however, studies highlight that the functions of Sirt1 are tissue-specific and depend on the metabolic state of the organism or organ\(^{91}\). In peripheral tissues such as liver, heart and skeletal muscle, Sirt1 mediates adaptations to metabolic stress by switching cellular metabolism from anabolic to catabolic processes\(^{91}\), promoting the utilization of alternative energy substrates\(^{100,101}\) and regulating systemic glucose output\(^{102,103}\).
Sirt1 expression in the adult brain is widespread, with high levels in the hippocampus, cerebellum, cortex and hypothalamus. Most of this expression is neuronally localized, however Sirt1 is also found in neural stem cells (NSCs), neural progenitor cells (NPCs) and endothelial cells. Sirt1 has been implicated in many essential brain functions such as neurodevelopment, neurogenesis, learning and memory, synaptic plasticity and circadian rhythms, among others (reviewed in). Regarding metabolism, Sirt1 regulates systemic metabolic processes from within distinct hypothalamic nuclei. For example, in the dorsomedial (DMH) and lateral hypothalamic nuclei (LH) Sirt1 deacetylates Nk2 homeobox 1 (Nkx2-1). This in turn drives transcription of another protein orexin type 2 receptor (Ox2r), leading to neuronal activation and regulation of physiological parameters such as physical activity, O2 consumption and body temperature. In the arcuate nucleus, Sirt1 regulates systemic functions from within pro-opiomelanocortin (POMC) neurons. Lack of Sirt1 in these neurons reduces energy expenditure and sensitizes mice to diet-induced obesity. Collectively, these and other studies demonstrate the significant role Sirt1 plays in regulating metabolism at the organismal level from within the brain. Despite our knowledge of the systemic effects of Sirt1 within the hypothalamus, the cellular metabolic pathways regulated by Sirt1 in the brain have not been identified.

1.3.2 Sirt1 in Cerebral Ischemic Injury

Recent studies show that Sirt1 plays a role in the outcome of cerebral ischemic injury and may be innately regulated by ischemia. Sirt1 mice display larger infarct volumes following permanent middle cerebral artery occlusion (pMCAo), a model of ischemic stroke that does not have a reperfusion component, compared to their wild type
counterparts\textsuperscript{112}. Likewise, Sirt1-Tg mice that overexpress Sirt1 showed less hippocampal damage following bilateral common carotid artery occlusion (BCAo) than wild type mice\textsuperscript{113}. A similar protective effect was seen with Sirt1-Tg mice in a bilateral common carotid artery stenosis (BCAS) model of hypoperfusion injury\textsuperscript{114}, a common pathological occurrence following stroke. Physiological levels of Sirt1 are modulated by ischemic injury as well. For example, Sirt1 was upregulated in the peri-infarct area up to seven days following pMCAo in mice\textsuperscript{112}. On the other hand, in rats subjected to transient focal ischemia with varying periods of reperfusion, Sirt1 was found to be downregulated six hours after reperfusion compared with non-reperfused animals\textsuperscript{115}. The discrepancy between up or down-regulation could be due to the different species and ischemia models used in the two studies.

Together, these studies suggest that Sirt1 expression is inherently regulated by and might modulate the outcome of ischemic injury. However, germline Sirt1 knockout mice (Sirt1\textsuperscript{-/-}), which have provided the best evidence for a role of Sirt1 thus far, have altered development and neurological features that might confound these findings. Sirt1\textsuperscript{-/-} mice display \~50\% mortality prior to adulthood\textsuperscript{112}, and given Sirt1’s role in neurodevelopment, several neurological impairments are evident in the surviving adult mice such as deficits in several forms of memory, long-term potentiation, dendritic spine architecture, hypothalamic regulation of neuroendocrine signaling and systemic metabolism\textsuperscript{110,116,117}. Many of these impairments are recapitulated in constitutive brain specific Sirt1 knockout mice as well\textsuperscript{118,119}. Sirt1-Tg mice that constitutively overexpress Sirt1 might also display abnormal neurodevelopmental processes, however this has yet to be investigated. Lastly, given that Sirt1 expression is ubiquitous, unconditional or non-cell-type specific models
make it difficult to pinpoint the cell types of origin or termination of potential protective mechanisms. The development of conditional and inducible Sirt1 knockout mice will be paramount in circumventing these issues.

Activation or inhibition of Sirt1 with pharmacological agents affects cerebral ischemic injury as well. Treatment with a Sirt1 activator (Activator 3) at ten minutes, twenty-four hours and forty hours following pMCAo reduced infarct volume while treatment with the Sirtuin inhibitor Sirtinol increased infarct volume from vehicle levels. Several other endogenous or exogenous compounds shown to induce ischemic tolerance were also linked to activation or upregulation of Sirt1. TSG (2,3,5,4'-Tetrahydroxystilbene-2-O-ß-D-glucoside) is a naturally occurring compound with chemical similarity to resveratrol that protects against oxygen and glucose deprivation (OGD) \textit{in vitro} and MCAo \textit{in vivo}. Cultured cells exposed to TSG for three days showed increased Sirt1 protein levels as well as a partial loss of TSG-induced protection in the presence of the Sirt1 inhibitor nicotinamide. Moreover, a combination of pre- and post-treatment with melatonin reduced neurological deficit, infarct and edema twenty-four hours after MCAo. This coincided with an increase in Sirt1 expression and was linked to enhanced mitochondrial function, an effect that was blocked by the specific Sirt1 inhibitor EX-527. Furthermore, iicarin (a naturally occurring flavonoid) treatment each day for seven days following reperfusion reduced infarct volume and brain edema at seven days after MCAo. Again, Sirt1 expression was increased at this time point and Sirt1 inhibitor III ablates iicarin-induced protection from OGD \textit{in vitro}. These pharmacological experiments provide key evidence that Sirt1 is a mediator of ischemic injury and a possible target for ischemic neuroprotection.
In addition to Sirt1, several other Sirtuins have been implicated in ischemic brain injury (recently reviewed in detail in\textsuperscript{123}). We have previously demonstrated that both IPC and preconditioning with the PKC\(\varepsilon\) activator \(\Psi\varepsilon\)RACK increase desuccinylation activity in mitochondria, suggesting activation of Sirt5. Strikingly, PKC\(\varepsilon\) activation is sufficient to protect wild type mice against tMCAo, yet this protection is lost in Sirt5\(^{-/-}\) mice and thus metabolic regulation by Sirt5 contributes significantly to ischemic tolerance in brain\textsuperscript{124}. While the metabolic pathways regulated by Sirt5 in brain are still largely unknown, initial experiments show a preservation of mitochondrial complex activities following tMCAo with PKC\(\varepsilon\) preconditioning, suggesting mitochondrial bioenergetics as one plausible target. While Sirt1 and Sirt5 are demonstrated to play positive roles in ischemic injury, evidence points to detrimental ones for Sirt2 and Sirt3. Genetic ablation of Sirt2 or pharmacological inhibition with the specific inhibitor AGK2 reduces infarct volume of mice subjected to tMCAo and improves their neurological outcomes\textsuperscript{94}. Likewise, Sirt3\(^{-/-}\) mice display reduced infarct after tMCAo, an effect concomitant with reduced ceramide synthase activity and improved mitochondrial function\textsuperscript{125}. Still, other reports \textit{in vitro} and \textit{in vivo} suggest a protective role for Sirt3 and hence its role remains controversial\textsuperscript{126, 127}. As the functions of other Sirtuins in the brain are elucidated, a clearer picture of their contribution to ischemic injury and/or ischemic protection will arise.

1.3.3 Sirt1 as a Mediator of RPC

Although resveratrol and RPC elicit a myriad of responses, evidence points to a central role for Sirt1 in mediating its neuroprotective effects in ischemia. In cortical neuronal/glial co-cultures, the NAD\(^+\)/NADH ratio is increased two days after RPC treatment\textsuperscript{90}, suggesting that it may activate Sirtuins. Indeed, RPC increases Sirt1 activity.
30 minutes\textsuperscript{69} and 1 hour\textsuperscript{70} after treatment in hippocampal slices \textit{in vitro} and hippocampus tissue \textit{in vivo}, respectively. In both cases, administration of the pan-Sirtuin inhibitor sirtinol blocked RPC-induced protection, demonstrating a key role for Sirtuins. Concerning Sirt1 specifically, RPC modulates Sirt1 target proteins in a manner consistent with increased Sirt1 activity. At the time of robust RPC-induced ischemic tolerance, mitochondrial uncoupling protein 2 (UCP2), an inner membrane proton leak channel and gene target of Sirt1 promoter binding, was decreased\textsuperscript{70}. This effect was concomitant with enhanced mitochondrial respiration and protection from global ischemia in rats.

While the aforementioned studies demonstrate RPC and Sirt1 effects in brain, systemic mechanisms of ischemic tolerance are also evident. Sirt1 regulates systemic processes and non-neuronal pathways that may contribute significantly to the extent of protection in ischemic injury. Sirt1 promotes angiogenesis through the migration and sprouting of endothelial cells\textsuperscript{128} and can maintain cerebral blood flow through deacetylation of endothelial eNOS\textsuperscript{114}. Mice overexpressing Sirt1 are resistant to bilateral common carotid artery occlusion, a model of global ischemia, by retaining cerebral perfusion up to 45-50\% of baseline compared to just 20-25\% of baseline in control animals\textsuperscript{113}. Moreover, resveratrol elicits pro-angiogenic effects in brain endothelial cells through mitogen-activated protein kinase (MAPK) / extracellular signal-regulated kinase (ERK) signaling and upregulation of eNOS\textsuperscript{129}.

Despite this evidence, resveratrol protects isolated neurons in culture\textsuperscript{130} as well as organotypic cultures\textsuperscript{69} that have supporting cell types but lack circulation, suggesting a neuronal or non-systemic mechanism of neuroprotection. As noted previously, resveratrol induces a host of responses across tissues and may activate other Sirtuins as well\textsuperscript{131}. It
remains to be empirically tested whether Sirt1 is necessary for RPC-induced ischemic tolerance. Another important question is then which brain cell types are targeted by RPC and Sirt1, since evidence suggests endothelial, neuronal and systemic processes may all play a role. Identifying the sites of origin and/or termination of RPC-induced neuroprotection, as well as the Sirt1-regulated pathways that induce ischemic tolerance is crucial for the development and translation of new therapies.

1.4 Concluding Remarks and Hypothesis

The overall aim of this project is to examine the neuroprotective potential of RPC in ischemic stroke and determine the role of Sirt1 in mediating its neuroprotective effects. We hypothesized that RPC induces a lasting state of ischemic tolerance through neuronal Sirt1-triggered metabolic adaptations. Using a transient MCAo model of ischemia-reperfusion injury, we evaluated the robustness and duration of different RPC treatment paradigms. Next, through an inducible and conditional genetic approach to deleting Sirt1 in adult brain, we determined the involvement of neuronal Sirt1 in RPC-induced ischemic tolerance. Lastly, metabolomics methodology allowed us to investigate the metabolic pathways regulated by Sirt1 in the brain and examine their contribution to neuroprotection in RPC. Elucidating the long-term effects of pharmacological preconditioning agents will provide a framework for the development of novel clinical applications in the future. Furthermore, our results improve our understanding of sirtuin biology in the brain, a field with therapeutic implications across neurological disorders.
Chapter 2. Resveratrol Preconditioning Induces a Lasting State of Ischemic Tolerance

2.1. Summary

Previous work demonstrates that RPC protects against cardiac arrest in rats and ischemic stroke in mice when administered two days prior to injury; however, the frequency and duration of RPC treatment that can afford the most robust protection has yet to be identified. In this chapter, different treatment paradigms were evaluated in an effort to determine the effects of repetitive and intermittent RPC on its ability to provide neuroprotection from ischemic stroke. We found that repetitive RPC treatment did not enhance the effects of a single treatment nor desensitize mice to its neuroprotective effects. Intermittent RPC treatment, distributed over a fourteen-day period, induced protection that lasted two weeks following the treatment regimen and remarkably, a single RPC treatment induced protection that also lasted for a two-week period. Furthermore, in cortex two weeks following a single RPC treatment, Sirt1 protein and promoter binding to two of its targets, BDNF and UCP2, were increased. This coincided with an increase in BDNF and a decrease in synaptic UCP2 protein. For the first time, we report a long-lasting state of ischemic tolerance resulting from a single dose of a pharmacological preconditioning agent.

2.2. Introductory Remarks

Work from our laboratory shows that one application of preconditioning stimuli such as sub-lethal ischemia, resveratrol \(^{70}\) or the protein kinase C epsilon (PKC\(\varepsilon\)) activator \(\Psi\varepsilon\text{RACK} \^{124}\), protects against cerebral ischemia \textit{in vivo} when administered prior to injury. Evidence from other studies suggests that altering the frequency or duration of
preconditioning stimuli may enhance its protective effects. This is evident with resveratrol, as various resveratrol paradigms, which differ in timing and frequency of treatment, can protect against MCAo. When considering a prophylactic treatment, it is also important to determine if daily administration desensitizes the targeted pathway. Additionally, intermittent bouts of hypoxia (with varying O² levels) induces protection from MCAo for up to two months, demonstrating that intermittent exposure to a preconditioning stimulus can induce a long-lasting state of ischemic tolerance. It remains to be tested if a long-lasting state of tolerance is achievable with a pharmacological preconditioning agent. Given that resveratrol activates Sirt1, an epigenetic regulator with the potential to mediate chronic adaptations to environmental stimuli, repetitive RPC may have lasting neuroprotective effects. In this chapter, we investigated the conditions of RPC that most robustly induce neuroprotection against focal cerebral ischemia in mice and the potential epigenetic mechanisms of Sirt1.

2.3. Methods

Animals and Pharmacological Treatments

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Miami and were in accordance with institutional regulations. Eight-twelve week-old C57Bl/6J male mice obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) were randomly assigned to different treatment groups. Mice were housed at 25°C in a supervised facility of the division of veterinary resources 2-5/cage (only after injury were they housed singly for 24 hours) on a 12 hour light/dark cycle with ad libitum access to standard chow and water. They were transported to the laboratory only for experimental procedures. Mice were injected intraperitoneal (IP)
with 10 mg/kg *trans*-resveratrol (3,4′,5-Trihydroxy-*trans*-stilbene, Sigma, St. Louis, MO, USA) in 1.5% dimethyl sulfoxide (DMSO, Sigma) or the vehicle control (1.5% DMSO).

**Transient Middle Cerebral Artery Occlusion (tMCAo)**

Right-side tMCAo was produced as previously described\(^{133}\). Mice were anesthetized with 2% isoflurane (Piramal Healthcare, Andhra Pradesh, India) in 100% O\(_2\), and maintained at 1 – 1.5% throughout the surgery for 60 minute occlusion time experiments. For longer-term recovery experiments, 45 minutes of occlusion time was used, and mice were not under anesthesia during the occlusion. Rectal and temporalis muscle temperatures were constantly monitored and maintained at 37.0 °C using heating pads (Harvard Apparatus, Holliston, MA, USA). The right common carotid artery was exposed through a midline neck incision and dissected free from surrounding nerves and fascia. Next, the occipital artery branches of the external carotid artery were dissected and coagulated. tMCAo was induced by inserting a silicone-coated 8-0 monofilament nylon surgical suture (Doccol, Sharon, MA, USA) into the internal carotid artery and circle of Willis via the proximal ECA. After 60 or 45 minutes of tMCAo, the intraluminal suture was carefully removed, the neck incision was closed, and the animal was kept in a humidified neonatal incubator set at 32 °C for one hour (60 minute occlusion mice) or for 7 days (45 minute occlusion mice) before being returned to its home cage. Cerebral blood flow was monitored prior to, during and immediately following tMCAo using a Laser-Doppler probe (Perimed Systems, Stockholm, Sweden) over the skull above the occlusion site. Animals that did not display a ≥ 70% reduction in cerebral blood flow were excluded.
**Open Field**

Mice were placed in a 10.75 inches L x 10.75 inches W x 8 inches H rodent open field arena (Med Associates INC, Fairfax, VT, USA), to which they were not previously habituated. Mice were allowed to explore for 20 minutes while their activity was tracked with EthoVision XT software (Noldus, Leesburg, VA, USA). This activity was evaluated between 13:00 and 17:00 hours for all animals. Distance travelled was measured in meters for two ten-minute periods.

**Pole Test**

The pole test was conducted as previously described\(^\text{134}\). Mice were placed at a vertical orientation on a medical tape covered (for grip purposes) metal pole (diameter 8 mm, height 55 cm). An observer recorded the “time to turn” response of the animal, defined as the time in seconds from placement on the pole until reorientation into a completely downward facing position. Three trials, each 1 minute apart, were recorded and the best time for each mouse was used for analysis. The observer was blind to the treatment or genotype of the mice.

**Calculation of Neurological Score**

Twenty-four hours after tMCAo, animals were scored based on a neurobehavioral battery (focal neurological score: movements such as body symmetry, gait, climbing, circling behavior, forelimb symmetry, compulsory circling, and whisker response) as previously described\(^\text{133}\). Focal score ranged from 0 – 28, where 0 is considered normal and 28 indicates severe neurological deficits.
**Quantification of Infarct Volume**

Mice were anesthetized with 2% isoflurane and transcardially perfused with heparinized ice-cold saline. Animals were then decapitated and the brain was rapidly removed, sliced and stained with triphenyl tetrazolium chloride (TTC, Sigma)\(^{135}\). Brain slices were fixed in 10% formaldehyde (Merck Millipore, Billerica, MA, USA) and imaged using a flat-bed image scanner. Images were corrected for auto color intensity and contrast. Infarct volume was measured using Image J (NIH, Bathesda, MD, USA), where infarct volume = (contralateral hemisphere volume – volume of noninfarcted tissue of the ipsilateral hemisphere) / (volume of contralateral hemisphere) × 100.

**Chromatin Immunoprecipitation and Subsequent qPCR**

Mouse brain cortices were dissected, frozen, pulverized in liquid nitrogen and resuspended in cross-linking solution containing 1% formaldehyde in PBS for 10 minutes at room temperature. The reaction was stopped by adding glycine to a final concentration of 125 mM and placing the samples on ice for 5 minutes. After two washes in cold PBS, the pellet was incubated in cell lysis buffer (20 mM Tris-HCl, pH 8.0, 85 mM KCl, 0.5% NP-40) supplemented with protease inhibitors (1 mM PMSF and Mini Complete Protease Inhibitor Cocktail Tablet (Roche, Branchburg, NJ, USA) on ice for 10 minutes. After centrifugation, the supernatant was removed and the nuclei were resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS supplemented with protease inhibitors) and incubated for 10 minutes on ice. Next, chromatin was sonicated to 200-500 bp fragment size, centrifuged at maximum speed for 20 minutes and diluted in ChIP dilution buffer (0.01% SDS, 1.1% Triton-X100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, supplemented with protease inhibitors). Input aliquots were saved at
this point. The sonicated chromatin was pre-cleared by incubation with Protein G Magnetic Beads (Thermo Fisher Scientific, Waltham, MA, USA) and salmon sperm DNA for 1 hour rotating at 4°C. The cleared supernatant was set up for chromatin immunoprecipitations with 5 µg of anti-Sirt1 antibody (Merck Millipore) or control IgG overnight at 4°C rotating. The next day, salmon sperm DNA-blocked beads were added to the immunoprecipitation reactions and samples were incubated rotating for an additional hour. Beads were washed sequentially for 5 minutes at 4°C on the rotator with 1 ml each of: low salt wash (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl supplemented with protease inhibitors), high salt wash (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), LiCl wash (0.25 M LiCl, 1% NP-40, 1% Na deoxycholate, 1 mM EDTA, 20 mM Tris-HCl, pH 8.), and TE wash (10 mM Tris- HCl, pH 8.0, 1 mM EDTA). Beads were resuspended in 250 µl of elution buffer (1% SDS, 0.1 M NaHCO3) and incubated at room temperature for 15 minutes with frequent agitation. Eluted samples were re-eluted with another 250 µl of elution buffer. Cross-linking was reversed by adding NaCl to a final concentration of 0.2 M and incubating for 4 hours at 65°C. Samples were treated with proteinase K (Sigma) for 1 hour at 45°C. The DNA was purified by phenol-chloroform extraction and EtOH precipitation. Quantitative real-time PCR was performed in triplicate using SYBR Green reagent (Roche) in the LightCycler® 480 II (Roche Applied Science) machine and analyzed with the ΔΔCT method. DNA-relative enrichment was determined by normalizing to an input genomic DNA and pre-serum IgG as background control. Primer sequences are found in Table 1.
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Isolation of Synaptosomes

A Percoll gradient fractionation protocol was used to isolate synaptosomes according to Dunkley and colleagues with slight modifications. Mice were decapitated under 2% isoflurane anesthesia and their cortices immediately immersed in isolation medium at 4°C (250 mM sucrose, 1.0 mM ethylenediaminetetra-acetic acid, and 0.25 mM dithiothreitol at pH 7.4). Tissue was chopped with scissors, rinsed thoroughly in isolation medium and homogenized in a glass Teflon homogenizer (Wheaton, Millville, NJ, USA) with 7 up-and-down strokes. The homogenate was diluted to give 10% w/v and centrifuged at 500 × g for 5 minutes (Sorvall RC5 centrifuge, Newton, CT, USA). The supernatant was layered on a Percoll (Sigma) gradient (prepared in 12.0 ml polycarbonate tubes consisting of 2.0 ml each of 23, 15, 10 and 3% v/v Percoll). The sample layered gradient was centrifuged at 32,500 × g for 5 minutes. Synaptosomes were then collected between the 23-15% and 15-10% Percoll junctions, washed once with isolation medium by centrifugation at 15,000 × g for 10 minutes and once with 0.25 M sucrose at the same speed and duration. The final pellet here was resuspended in RIPA buffer (pH 8.0 consisting of 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, supplemented with 1% protease inhibitor cocktail (Sigma), 1% phosphatase inhibitor (PhosphoStop, Roche)) and processed for protein analysis according to the Western blot protocol below.

Western Blot Analysis

Mice were decapitated under 2% isoflurane anesthesia, brains were removed and cortices dissected. This tissue was homogenized in RIPA buffer (see above) and incubated on ice for 30 minutes. Debris was then pelleted by centrifugation at 12,000 × g for 20
minutes at 4°C. Protein concentration was measured by BioRad Protein Assay (BioRad, Hercules, CA, USA) and equal amounts of protein were run on 12% SDS-polyacrylamide gels (BioRad). The separated proteins were then transferred to a nitro-cellulose membrane (BioRad) and the blot was blocked with 5% blotting grade blocker (BioRad) in TBST (0.1% Tween-20) for 2 hours at room temperature. Probing with primary antibodies was achieved by overnight incubation at 4°C (see Table 1). Next, blots were incubated with species specific HRP-conjugated secondary antibodies (1:5000, GE healthcare, Little Chalfont, UK) for 1 hour at room temperature. Protein bands were developed by addition of ECL reagents (Thermo Fisher Scientific) and detected using x-ray film (Denville Scientific, Holliston, MA, USA). Exposed films were digitalized and blots were analyzed densitometrically using Image J software. Two normalized values (for the same sample) from different blots were averaged to give the values presented.

**BDNF ELISA Assay**

BDNF concentration was quantified using a ChemiKineTM BDNF Sandwich ELISA Kit (Merck Millipore) according to the manufacturer’s instructions. Samples were processed via the Western blot protocol above. Each sample was first normalized to 5 µg/µl protein then diluted with sample dilution buffer (see kit) to give 2.5 µg/µl and 1.25 µg/µl. These two concentrations were used in duplicate to calculate BDNF concentration in pg/ml.

**Statistical Analysis**

Mice were randomly assigned treatment groups. All data acquisition and analyses were done in a blinded manner. Statistical analysis was performed in Prism6 software (GraphPad, San Diego, CA). Data are presented as mean ± SEM. Two-sample, unpaired
student $t$–test and one-way ANOVA with Bonferroni post-hoc tests were used as indicated in the text and figure legends. Significance is denoted in each figure. Sample size was selected for each experiment depending upon prior experiments conducted by the lab and power analysis in order to detect an effect size of 0.8 using G*power 3.1 software.

2.4 Results

2.4.1 RPC Promotes Functional Recovery Post-Injury

Our previous work demonstrates that RPC reduces infarct volume following tMCAo in mice 24 hours following injury\textsuperscript{73}. It is important to distinguish whether this protection is simply prolonging the therapeutic window, that is, prolonging or delaying cell death for another more permanent neuroprotective intervention, or if cells in the core and or penumbral regions are being spared from cell death long term following the injury. To answer this question, we aimed to determine the neuroprotective effect of RPC 7 days

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  \item Student $t$–test and one-way ANOVA with Bonferroni post-hoc tests were used as indicated in the text and figure legends. Significance is denoted in each figure. Sample size was selected for each experiment depending upon prior experiments conducted by the lab and power analysis in order to detect an effect size of 0.8 using G*power 3.1 software.
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  \item **2.4.1 RPC Promotes Functional Recovery Post-Injury**
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\end{itemize}
following injury by evaluating motor function (Fig. 2 A). Mice were subjected to the open field, a gross measure of locomotor activity and to the pole test, a fine motor coordination task that requires striatal and cortical function. Seven days after injury, sham and tMCAo mice traveled similar distances in the open field and were unaffected by RPC or Veh treatment (p>0.05, one-way ANOVA, sham-n=3-4, RPC/Veh-n=8-9), indicating no difference in basal locomotor activity (Fig. 2 B). However, tMCAo induced a deficit in the pole test in Veh but not RPC treated mice (p<0.05, one-way ANOVA, sham-n=3-4, RPC/Veh-n=8-9), demonstrating preservation of motor coordination in RPC mice (Fig. 2 C). These results demonstrate that RPC improves motor function recovery 7 days following ischemic stroke.

2.4.2 Repetitive RPC Treatment is Efficacious

Given that a single RPC treatment 2 days prior to injury protects against tMCAo in mice, we sought to determine whether repetitive RPC treatment causes tachyphylaxis, diminishing the neuroprotective effect of RPC, or actually enhances this effect, inducing a more robust state of neuroprotection. To this end, mice were treated with RPC or Veh every other day for 14 days prior to tMCAo, mimicking the standard preconditioning paradigm repeated 7 times (Fig. 3 A). Compared to Veh, RPC retained its ability to induce protection and reduced infarct volume by 20% (p<0.01, Student’s t-test, n=12) (Fig. 3 B). Based on this finding, we postulated that increasing the frequency of RPC treatment might enhance this protection. Thus, mice were treated with RPC or Veh once per day for 14 days prior to tMCAo (Fig. 3 C). Similar to every other day treatment, everyday treatment reduced infarct volume by 27% compared to Veh (p<0.01, Student’s t-test, Veh n=12, RPC n=14) (Fig. 3
D), suggesting that more frequent treatment does not confer more robust protection.

2.4.3 RPC Induces an Extended Window of Ischemic Tolerance

Since the degree of protection by RPC was not improved with additional applications over time, we rationalized that perhaps less frequent RPC treatment is more efficacious. Our previous studies show that IPC induces ischemic tolerance that lasts for at least 4 days in organotypic slice cultures\textsuperscript{69} and others report preconditioning-induced protection lasting up to 7 days\textsuperscript{64,65}. Given that epigenetic modifications may mediate these longer-term, protective windows of preconditioning\textsuperscript{137}, we hypothesized that intermittent RPC treatment might induce a long-lasting state of ischemic tolerance. To test this hypothesis, we used a geometrical growth pattern of intermittent RPC consisting of 4
applications at intervals of 1, 2, 4 and 8 days with 14 days between the last treatment and tMCAo (Fig. 4 A). Strikingly, compared to Veh, intermittent RPC reduced infarct volume by 24% (p<0.01, Student’s t-test, Veh n=15, RPC n=16) (Fig. 4 B). An important distinction to make here is whether the culmination of 4 applications of RPC intermittently, give rise to this long-lasting ischemic tolerance or if simply one application of RPC can induce ischemic tolerance for this extended period of time. To test this, mice were treated with RPC or Veh once, 14 days prior to tMCAo (Fig. 4 C). Remarkably, one application of RPC 14 days prior to injury reduced infarct volume by 33% compared to Veh (p<0.01, Student’s t-test, Veh n=9, RPC n=10) (Fig. 4 D). To confirm that this reduction in infarct volume was accompanied by improved neurological performances, we calculated neurological score 24 hours following tMCAo and found that compared to Veh, RPC

Figure 4. A single dose of RPC induces ischemic tolerance for at least 2 weeks in vivo. (A and C) Schematics of experimental paradigm. Arrow indicates application of treatment. (B and D) Left - representative TTC-stained brain sections at 24 hrs following tMCAo. Right – quantification of infarct volume shown as % infarct volume of total ipsilateral hemisphere. (B) Intermittent RPC treatment significantly (students t-test, ** = p<0.01, RPC – n=16, Veh – n=15) reduced infarct volume compared to Veh. (D) A single RPC treatment 2 wks prior to injury significantly (students t-test, ** = p<0.01, RPC – n=10, Veh – n=9) reduced infarct volume compared to Veh. Neurological score was calculated from a battery of sensory and motor tasks (see methods) and was significantly improved by RPC treatment (students t-test, * = p<0.05).
improved this functional outcome measure by 28% (p<0.05, Student’s t-test, Veh n=9, RPC n=10) (Fig. 4 D). These results demonstrate that a single application of RPC induces a novel, extended window of ischemic tolerance that lasts for at least 14 days in vivo.

2.4.4 Sirt1-Linked Adaptations in the Extended Window

Sirt1 is a well-established target of resveratrol and our previous studies show that blockade of Sirtuins ablates RPC-induced ischemic tolerance\textsuperscript{69,70}. Hence, we investigated whether changes in Sirt1 expression occurred in this extended window of ischemic tolerance. Fourteen days following a single application of RPC, cortical Sirt1 protein levels were increased 1.5-fold of Veh (p<0.01, Student’s t-test, n=10) (Fig. 5 A). To assess whether this increase had functional implications, we turned our attention to putative chromatin binding sites for Sirt1. Previous work found that RPC-induced ischemic

![Figure 5. Sirt1-linked adaptive responses of RPC-induced chronic ischemic tolerance. (A-D) Analysis of Sirt1-linked targets in cortex 14 days following a single application of RPC. (A) Western blot analysis revealed an increase in Sirt1 protein compared to Veh. // = non-adjacent bands from the same blot (students t-test, ** = p<0.01, n=10). (B) ChIP-qPCR analysis showed increased Sirt1 binding to BDNF and UCP2 promoter regions in RPC treated mice compared to Veh (students t-test, ** = p<0.01, * = p<0.05, n=3). (C) BDNF levels were increased by RPC treatment compared to Veh, expressed as pg/mg of cortical protein (students t-test, * = p<0.05, n=7). Synaptic UCP2 protein levels were decreased by RPC treatment compared to Veh, normalized to the mitochondrial marker VDAC. The UCP2 functional dimer (70 kDa band) was used for quantification, no 35 kDa monomer band was detected (students t-test, * = p<0.05, n=6).
tolerance was associated with a reduction in UCP2, a proton leak channel of the inner mitochondrial membrane\textsuperscript{70}, and enhanced mitochondrial function. An additional target of Sirt1, BDNF\textsuperscript{138}, is a growth factor that promotes neuronal survival and has a known neuroprotective role in preconditioning. Thus, we evaluated Sirt1 binding to promoter regions within the BDNF and UCP2 genes. Chromatin immunoprecipitation and subsequent qPCR fourteen days following a single application of RPC revealed that Sirt1 binding to both the BDNF and UCP2 promoters was enhanced, 2.66-fold and 1.14-fold, respectively, compared to Veh (BDNF $p<0.01$, UCP2 $p<0.05$, Student’s t-test, $n=3$) (Fig. 5 B). Concomitantly, cortical BDNF levels were increased by 27% ($p<0.05$, Student’s t-test, $n=7$) and synaptic UCP2 levels were decreased by 23% ($p<0.05$, Student’s t-test, $n=6$) compared to Veh (Fig 5 C and D). These data suggest that Sirt1-mediated modulation of BDNF and UCP2 expression contribute to ischemic tolerance in the extended window.
Chapter 3. Neuronal Sirt1 Mediates RPC-Induced Ischemic Tolerance

3.1. Summary

Previous work shows that Sirtuins are necessary for RPC-induced ischemic tolerance\textsuperscript{70} yet it remains unclear if activation of Sirt1 specifically is essential for ischemic tolerance and if so, in which cell types since both resveratrol and Sirt1 regulate brain and systemic processes alike. Additionally, the metabolic pathways regulated by Sirt1 in the brain at the cellular level are not known. In this chapter, the role of neuronal Sirt1 in RPC-induced ischemic tolerance against ischemic stroke and brain metabolism was investigated. It was determined that RPC-induced ischemic tolerance requires the deacetylase activity of neuronal Sirt1. Metabolomics analysis revealed alterations in glucose metabolism upon neuronal Sirt1 deletion, which were concomitant with transcriptional changes in glucose transport and glycolytic machinery genes. Furthermore, deletion of neuronal Sirt1 impaired glycolytic ATP production and the ability to use glucose to delay anoxic depolarization under anoxia. Finally, we found that RPC increases glycolytic rate under basal and energetic stress conditions in a Sirt1-dependent manner, an effect that was evident in ischemic penumbra-like conditions. These results demonstrate that neuronal Sirt1 is a crucial mediator of RPC-induced protection and identify a novel role for Sirt1 in the regulation of glycolytic function in the brain.

3.2. Introductory Remarks

While resveratrol induces a host of cellular responses, it is postulated that activation of Sirt1 is at least in part responsible for its neuroprotective properties. Sirt1 is expressed in several brain cell types including neurons, neural stem cells, neural progenitor cells and
endothelial cells \(^{108}\). Endothelial Sirt1 regulates angiogenesis \(^{128}\) and cerebral perfusion \(^{113}\), two systemic processes that contribute significantly to the extent of ischemic injury. Likewise, resveratrol elicits pro-angiogenic effects in brain endothelial cells through upregulation of endothelial nitric oxide synthase (eNOS) \(^{129}\), a target of Sirt1 deacetylation \(^{114}\). Yet, resveratrol also protects isolated neurons in culture \(^{130}\) as well as organotypic cultures \(^{69}\) that have supporting cell types but lack circulation, from ischemia \textit{in vitro}, suggesting a neuronal or non-systemic mechanism of neuroprotection. Identifying the sites of origin and/or termination of RPC-induced neuroprotection, as well as the pathways that induce ischemic tolerance, are important distinctions to uncover. Concerning metabolism, Sirt1-mediated adaptations are extensively characterized in peripheral tissues; however, the brain may lack this metabolic plasticity given its tight regulation of energy metabolism which is essential for continuous neuronal activity. It is still unclear how Sirt1 regulates metabolism at the cellular level in the brain and particularly neurons. This regulation is likely important for the maintenance of neuronal function in normal physiology and under pathological conditions. Next, we investigate the contribution of neuronal Sirt1 to RPC-induced ischemic tolerance and identify the potential metabolic pathways through which Sirt1 exerts neuroprotection at the cellular level in the brain.

3.3. Methods

\textit{Animals and Pharmacological Treatments}

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Miami and were in accordance with institutional regulations. Eight-12 week-old male mice (\(~20-24\) g) used throughout the study and
randomly assigned treatment groups. Mice were housed at 25°C in a supervised facility of the division of veterinary resources 2-5/cage (only after injury were they housed singly for 24 hrs) on a 12 hr light/dark cycle with ad libitum access to standard chow and water. They were transported to the laboratory only for experimental procedures. Mice were injected intraperitoneal (IP) with 10 mg/kg trans-resveratrol (3,4’,5-Trihydroxy-trans-stilbene, Sigma) in 1.5% dimethyl sulfoxide (DMSO, Sigma) or the vehicle control (1.5% DMSO) 2 days prior to experimentation. For induction, tamoxifen (trans-2-[4-(1,2-Diphenyl-1-butanyl)phenoxy]-N,N-dimethylethylamine, Sigma) was administered IP at 0.13 mg/g in 10% ethyl alcohol (200 proof) and 90% corn oil (Sigma) once per day for 5 consecutive days. This period was followed by 5 days of rest for any residual protein to be completely removed from the animal prior to experimentation.

*Generation of inducible, neuron-specific Sirt1 knockout mice (Sirt1<sup><sub>neu⁻/⁻</sub></sub>)*

SLICK-H mice express the creER<sup>T2</sup> fusion protein (cre recombinase fused to a triple mutant human estrogen receptor that only binds the metabolite of tamoxifen, 4-hydroxytamoxifen) and EYFP under back-to-back copies of the *Thy1.2* promoter. Sirt1<sup>flox/flox</sup> mice harbor loxP sites flaking exon 4 (catalytic domain) of Sirt1. These two mouse strains were purchased from The Jackson Laboratory (SLICK-H, stock no: 012708, Sirt1<sup>flox/flox</sup>, stock no: 008041) and backcrossed into the C57BL/6J genetic background. Crossings of these two strains generated our Thy1.2-creER<sup>T2</sup>; Sirt1<sup>flox/flox</sup> (Sirt1<sup>neu⁻/⁻</sup>) mice. Sirt1<sup>flox/flox</sup> littermates were used as controls.
**Immunofluorescence**

Immunofluorescence was conducted as described previously<sup>139</sup>, with modifications. Briefly, mice were anesthetized with 2% isoflurane and perfused with cold saline followed by 4% paraformaldehyde in PBS (pH 7.4). The brain was dissected and placed in 4% paraformaldehyde overnight at 4°C. Twenty-four hours later, the brain was placed in 20% sucrose solution for up to 2 days. Coronal 30 µM sections were cut with a CM-1850 cryostat (Leica, Wetzlar, Germany), mounted and blocked in 3% donkey serum (PBS-T, 1% tween-20, PBS). Sections were incubated with primary antibodies (see Table 1) overnight at 4°C and secondary antibodies (Alexa Fluor 488, 568 or 647, 1:500, Thermo Fisher Scientific) for 2 hours at room temperature. Sections were imaged on an Olympus BX50 confocal microscope (Olympus, Center Valley, PA) and rendered on Imaris software (Bitplane, Concord, MA).

**Real-Time qPCR**

Real-time qPCR was conducted as described previously<sup>124</sup>. In short, TRIzol (Thermo Fisher Scientific) homogenized hippocampi were used for RNA extraction by RNeasy Mini Kit (Qiagen, Hilden, Germany). One µg of RNA was used as the template for cDNA synthesis by qScript cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA). Real-time qPCR was carried out in triplicate using the LightCycler® 480 II (Roche Applied Science). Results were analyzed by the ΔΔCT method and normalized as fold change of control. Primer sequences are found in Table 1.

**Western Blot Analysis**
Western blotting was performed as previously described\textsuperscript{140}. Mice were decapitated under 2\% isoflurane anesthesia, brains were removed and cortices homogenized in RIPA buffer. Protein concentration was measured by Bradford assay (BioRad) and equal amounts of protein were run SDS-polyacrylamide gels (RioRad). Eight-percent gels resolved WT and mutant Sirt1 proteins. The separated proteins were then transferred to a nitro-cellulose membrane (BioRad) and the blot was blocked with 5\% blotting grade blocker (BioRad) in TBST (0.1\% Tween-20) for 2 hours at room temperature. Primary antibody incubation was achieved overnight at 4\°C (see Table 1). Following addition of HRP-conjugated secondary antibodies (1:5000, GE healthcare) for 1 hour, blots were developed with ECL reagents (Thermo Fisher Scientific) and visualized with X-ray film (Denville Scientific).

\textit{Transient Middle Cerebral Artery Occlusion (tMCAo)}

Right-side tMCAo was produced as previously described\textsuperscript{133}. Mice were anesthetized with 2\% isoflurane (Piramal Healthcare, Andhra Pradesh, India) in 100\% O\textsubscript{2}, and maintained at 1 – 1.5\% throughout the surgery. Rectal and temporalis muscle temperatures were constantly monitored and maintained at 37.0 °C using heating pads (Harvard Apparatus). The right common carotid artery was exposed through a midline neck incision and dissected free from surrounding nerves and fascia. Next, the occipital artery branches of the external carotid artery were dissected and coagulated. tMCAo was induced by inserting a silicone-coated 8-0 monofilament nylon surgical suture (Doccol) into the internal carotid artery and circle of Willis via the proximal ECA. After 60 minutes of tMCAo, the intraluminal suture was carefully removed, the neck incision was closed, and the animal was kept in a humidified neonatal incubator set at 32 °C for one hour before being returned to its home cage. Cerebral blood flow was monitored prior to, during and
immediately following tMCAo using a Laser-Doppler probe (Perimed Systems) over the skull above the occlusion site. Animals that did not display a $\geq 70\%$ reduction in cerebral blood flow were excluded.

**Calculation of Neurological Score**

Twenty-four hours after MCAo, animals were scored based on a neurobehavioral battery (focal neurological score: movements such as body symmetry, gait, climbing, circling behavior, forelimb symmetry, compulsory circling, and whisker response) as previously described\textsuperscript{133}. Focal score ranged from 0 – 28, where 0 is considered normal and 28 indicates severe neurological deficits. The investigator scoring neurological deficits was blinded to the experimental conditions.

**Quantification of Infarct Volume**

Mice were anesthetized with 2\% isoflurane and transcardially perfused with heparinized ice-cold saline. Animals were then decapitated and the brain was rapidly removed, sliced and stained with triphenyl tetrazolum chloride (TTC, Sigma)\textsuperscript{135}. Brain slices were fixed in 10\% formaldehyde (Merck Millipore) and imaged using a flat-bed image scanner. Images were corrected for auto color intensity and contrast. Infarct volume was measured using Image J (NIH), where infarct was calculated as a percentage of the total ipsilateral hemisphere.

**Non-Targeted Primary Metabolomics**

Sample preparation, data acquisition and data processing was performed by the West Coast Metabolomics Center (UC-Davis) as previously described\textsuperscript{141}. Briefly, this non-targeted primary metabolism platform was achieved by gas chromatography – time of
flight – mass spectrometry (GC-TOF-MS). Mass spectrometer (Leco Pegasus IV, St. Joseph, MI) settings: unit-mass resolution at 17 spectra s\(^{-1}\) from 80-500 Da at -70 eV ionization energy and 1800 V detector voltage with 230\(^{0}\)C transfer line and 250\(^{0}\)C ion source. Data processing: ChromaTOF vs. 2.32 without smoothing, a 3 s peak width, baseline subtraction above noise level, automatic mass spectral deconvolution and peak detection at 5:1 (signal/noise).

**Metabolomics Statistical and Biochemical Analysis**

Peak intensity values normalized to tissue weight were used for statistical analysis in MetaboAnalyst 3.0\(^{142}\). Unknown metabolites (BinBase identifiers are given) were analyzed separately from structurally annotated metabolites. Values were log transformed and auto scaled. No filtering was applied given the relatively small number of peaks identified. T-test with false discovery rate correction was used to determine significance between groups (p<0.05, FDR<0.23, n=8). MetaMapR\(^{143}\) was used to generate networks that display structural or spectral relationships between metabolites. This package references PubChem substructure fingerprints to calculate structural similarity. To incorporate unknown metabolites, spectral likeness is calculated from mass spectra filtered by retention time. Networks were plotted and visualized in Cytoscape\(^{144}\) where nodes and edges were assigned certain features to highlight the differences between genotypes or treatment conditions.

**Acute Brain Slice Preparation**

Mice were anesthetized with 2% isoflurane. Their brains were rapidly removed and cut down the midline into two cubed blocks for sagittal sectioning. Slices of 300 \(\mu\)M
thickness were cut with a Leica VT1000S microtome in cold artificial cerebrospinal fluid (aCSF) solution of the following constitution: 4.5 mM KCl; 2 mM MgSO4; 1.25 mM Na2HPO4; 126 mM NaCl; 2 mM CaCl2; 26 mM NaHCO3; 10 mM Glucose; bubbled with 95% O2, 5% CO2; osmolality approximately 302-306. Midbrain of the slices was removed. Slices containing hippocampus and surrounding cortex were transferred immediately to aCSF at room temperature for 1 hour prior to use. Slices were transferred to an interface recording chamber for 30 minutes prior to experimentation where they were superfused at a rate of 2 ml/min with cCSF (32°C ± 1 °C, bubbled with 95% O2, 5% CO2) and oxygenated with humidified 95% O2, 5% CO2.

**Extracellular Field Recordings and Anoxic Depolarization**

Extracellular field recording was achieved as previously described[48]. Field population spikes (fPSs) and field excitatory post-synaptic potentials (fEPSPs) were recorded with NaCl-filled (150 mM) glass micropipettes in the pyramidal cell body layer and dendrites of CA1 hippocampus, respectively, using a SUPER-Z head-stage attached to a BMA-931 AC/DC Bioamplifier, Digidata1200 and pClamp 9.0 recording software. Schaffer collateral axons were electrically stimulated by 0.3 ms constant current pulses with bipolar tungsten electrodes using a S48 square pulse stimulator. Slices were stimulated at half-maximal response from input-output curves, where amplitude and 80-20 rise slope were calculated using Clampfit software (Molecular Devices, Sunnyvale, CA, USA). Slices were excluded if they displayed double fSpike properties, indicative of poor slice health. For AD experiments, extracellular recording monitored the onset of a DC-shift while Schaffer collaterals were stimulated every 15 seconds to monitor fPSs. Baseline was recorded for 5 minutes in normal aCSF prior to experimental conditions: aCSF no glucose
(4.5 mM KCl; 2 mM MgSO$_4$; 1.25 mM Na$_2$HPO$_4$; 126 mM NaCl; 2 mM CaCl$_2$; 26 mM NaHCO$_3$; 7 mM Sucrose; bubbled with 95% O$_2$, 5% CO$_2$; osmolality 302-306); aCSF with glucose (4.5 mM KCl; 2 mM MgSO$_4$; 1.25 mM Na$_2$HPO$_4$; 121 mM NaCl; 2 mM CaCl$_2$; 26 mM NaHCO$_3$; 20 mM Glucose; bubbled with 95% O$_2$, 5% CO$_2$; osmolality 302-306).

Anoxia was induced by bubbling with 95% N, 5% CO$_2$ and de-oxygenating slices with humidified 95% N, 5% CO$_2$. Latency until AD = time from anoxia onset to AD onset, defined as rate of DC-shift exceeding 10 V/s.

**Primary Neuronal-Enriched Cultures**

Primary neuronal culture was carried out as previously described$^{124}$, with some modifications. Pregnant female Sprague-Dawley rats (E18-19) were acquired from Charles River (Wilmington, MA, USA). After being anesthetized with 2% isoflurane, 6-12 pups were harvested from pregnant females and their brains harvested into dissection media (HBSS (Ca$^{2+}$ and Mg$^{2+}$ free), 1 mM sodium pyruvate, 20 mM glucose, and 10 mM HEPES, pH 7.4). After removal of the cerebellum, hippocampus and olfactory bulbs, meninges were dissected away and cortical tissue was dissociated in 1x trypsin and DNase at 37$^\circ$C for 15 minutes with mixing every 5 minutes. Trypsin and DNase were removed by 3 washes with neuronal media (MEM, 1% glutamax, 10% FBS and 20 mM glucose). Tissue was then homogenized and passed through a 70 µm filter to remove debris and cells were counted with a hemocytometer. For Seahorse XFp experiments, neurons were plated at a density of 80,000 cells per well in neuronal media. Cultures underwent half media changes every 3$^{rd}$ day for 10 days prior to experimentation.
**Glycolytic Rate Measurements**

Glycolytic rate was measured using the Seahorse XFp Analyzer (Agilent) according to manufacturer’s instructions and published standards[50]. As a result of glycolysis, protons are extruded into the media, changing its pH, which is detected by solid state sensor probe measurements, which are taken every few seconds. These measurements are then averaged over 2-5 minutes and extracellular acidification rate (ECAR) is calculated. Neurons were treated with resveratrol (100 µM, 1.5% DMSO, 0.9% saline), vehicle (1.5% DMSO, 0.9% saline) and/or the Sirt1 specific-inhibitor EX-527 (Sigma, 1µM, 10µM, 0.9% saline) 2 days prior to measurements. On the day of measurements, neurons were washed 3x with seahorse media (XF Base Medium Minimum DMEM, 20 mM glucose, 1 mM sodium pyruvate, 2 mM glutamine) and then incubated in seahorse media in a CO2-free incubator at 37°C for 1 hour prior to loading into the Seahorse Analyzer. Extracellular flux cartridges were loaded with the mitochondria-uncoupling agent FCCP (Sigma, 2µM) and the mitochondrial complex V (ATP synthase) inhibitor oligomycin (Sigma, 1µM). ECAR was normalized to cell count in a reference well and is expressed as fold change of Veh.

**Statistical Analysis and Blinding Procedures**

Mice were randomly assigned treatment groups throughout. All data acquisition and analyses were done in a blinded manner. Statistical analysis was performed in Prism6 software (GraphPad, San Diego, CA). Data are presented as mean ± SEM. Two-sample, unpaired student t –test, one- and two-way ANOVA with Bonferroni post-hoc tests and
repeated measures two-way ANOVA with Bonferroni post-hoc tests were used as indicated in the text and figure legends. Significance is denoted in each figure. Sample size was selected for each experiment depending upon prior experiments conducted by the lab and power analysis in order to detect an effect size of 0.8 using G*power 3.1 software. Kolmogorov-Smirnov tests measured distribution differences between data in cumulative fraction plots.

3.4 Results

3.4.1 Establishing a Viable Sirt1 Knockout Mouse Model

In order to circumvent the developmental defects of germline Sirt1 knockout mice and specifically investigate post-developmental neuronal Sirt1 function, we generated tamoxifen-inducible, neuronal-specific Sirt1 knockout mice (Fig. 6 and 7). This was achieved by crossing SLICK-H mice, where creERT2 and enhanced yellow fluorescent protein (EYFP) are under control of the pan-neuronal promoter Thy1.2, with Sirt1 flox/flox mice that harbor loxP sites flanking the catalytic domain (exon 4) of Sirt1, on the C57BL/6J background (Fig. 6 A). In these mice, tamoxifen treatment results in deletion of exon 4 from Sirt1 exclusively in neurons, rendering a mutant protein that lacks deacetylase activity. We refer to the induced Sirt1 flox/flox, Thy1.2-creERT2 mice as Sirt1 neu-/- and used Sirt1 flox/flox littermates as controls. Sirt1 neu-/- exhibit strong EYFP fluorescence throughout major brain regions (Fig. 6 B) as described previously. Importantly, EYFP did not show robust expression in the hypothalamus (Fig. 6 C) and body weight did not change one week after induction (data not shown), suggesting that Sirt1 regulation of systemic metabolism from the hypothalamus is not overtly altered. Immunostaining revealed co-localization of EYFP and the neuronal marker NeuN (Fig. 6 D) but not the
glial marker GFAP (Fig. 6 E), demonstrating neuronal specificity of deletion. In non-

Figure 6. Generation of inducible, neuronal-specific Sirt1 knockout mice (Sirt1\textsuperscript{\textasciitilde neu/-}). (A) Sirt1\textsuperscript{\textasciitilde neu/-} mice were generated by crossing SLICK-H (EYFP reporter for inducible cre expression, left) with Sirt1\textsuperscript{flox/flox} (right). (B-E) Confocal z-stack images (10-25 \textmu m total) of 2 \textmu m planes from tissue sections of non-induced Sirt1\textsuperscript{\textasciitilde neu/-} mouse brains. (B) Immunostaining for EYFP and Sirt1 exhibit neuronal-like expression patterns (Hipp – hippocampus; Ctx – cortex; scale bar = 400 \textmu m). (C) Dashed line outlines hypothalamus (Hypo) where little EYFP expression is observed (scale bar = 400 \textmu m). (D) EYFP expression is evident in striatum (Str – striatum, scale bar = 70 \textmu m). (E) Neither EYFP nor Sirt1 co-localize with the glial marker GFAP (Ctx – cortex, scale bar = 20 \textmu m).

induced mice, Sirt1 displays a primarily neuronal expression pattern, co-localizing with EYFP and NeuN but not GFAP (Fig. 6 B and 6 E). Efficiency of tamoxifen-induced Sirt1 deletion (Fig. 7 A) was assessed by western blot, where in controls full-length wild-type (WT) Sirt1 protein is observed as two bands at 110 kDa. In Sirt1\textsuperscript{\textasciitilde neu/-}, these bands were observed at a lower molecular weight, indicating truncation of the Sirt1 protein caused by deletion of exon 4 (Fig. 7 B). No mutant Sirt1 or EYFP protein was found in other tissues
such as the heart (Fig. 7 C and D). Background CreERT² activity was not observed, as non-induced Sirt1neu⁻/⁻ mice do not express any mutant Sirt1 protein (Fig. 7 E). Deletion was also confirmed at the RNA level using primers specific for WT Sirt1 mRNA (p<0.001, Student’s t-test, n=4) (Fig. 7 F). After induction of Sirt1neu⁻/⁻ mice, no overt phenotype was observed. Together, this evidence demonstrates efficient and specific deletion of neuronal Sirt1 in Sirt1neu⁻/⁻ mice.

### 3.4.2 Deletion of Neuronal Sirt1 Renders RPC Ineffective

Next, we sought to determine if neuronal Sirt1 is required for RPC-induced ischemic tolerance. Control and Sirt1neu⁻/⁻ mice were treated with RPC (resveratrol 10 mg/kg; IP) or Veh (1.5% DMSO) 2 days prior to transient middle cerebral artery occlusion (tMCAo, ischemic stroke) for 60 min (Fig. 8 A). Control mice received tamoxifen to
control for any confounding effects of this compound. Twenty-four hours following tMCAo, functional outcomes were scored from a battery of sensory-motor tasks\textsuperscript{14} and infarct size was quantified by triphenyl tetrazolium chloride (TTC) staining. Mortality from tMCAo did not differ between genotypes or treatments (data not shown). RPC significantly reduced infarct size (45.2%, $p<0.05$, two-way ANOVA, Bonferroni post-hoc, $n=5-9$) and improved functional outcome (25.9%, $p<0.05$, two-way ANOVA, Bonferroni post-hoc, $n=5-9$) in control but not Sirt1neu-/- mice (Fig. 8 B-D). Cerebral blood flow measurements during ischemia or at the onset of reperfusion did not differ between groups (Fig. 8 E), suggesting that these results were not due to

Figure 8. RPC-induced ischemic tolerance is lost without neuronal Sirt1. (A) Experimental timeline of mouse induction, treatment, stroke and post-stroke analysis. (B) Neurological scoring, where a lower value indicates better function, was improved by RPC in control but not Sirt1neu-/- mice (Control-Veh [n=8], Control-RPC [n=6], Sirt1neu-/-Veh [n=9], Sirt1neu-/-RPC [n=5], * = $p<0.05$, ns = not significant, two-way ANOVA – Bonferroni post-test). (C) Representative TTC-stained brain sections following tMCAo. (D) Infarct, quantified as a percentage of the total ipsilateral hemisphere, was reduced by RPC in control but not Sirt1neu-/- mice (* = $p<0.05$, ns = not significant, two-way ANOVA – Bonferroni post-test). (E) Cerebral blood flow measured by laser doppler flowmetry was unchanged between groups (ns = not significant, two-way ANOVA – Bonferroni post-test).
changes in cerebral perfusion. These data demonstrate that RPC-induced ischemic tolerance requires neuronal Sirt1.

3.4.3 Metabolomics Analysis of Neuronal Sirt1 Knockout Mice

It is well established that Sirt1 regulates various metabolic pathways within peripheral tissues and at the organismal level in the hypothalamus. However, the cellular metabolic pathways downstream of Sirt1 in neurons remain largely unknown. To identify these pathways, which likely play a role in RPC-induced ischemic tolerance, non-targeted primary metabolite profiles were generated from hippocampi of tamoxifen treated control and Sirt1<sup>-neu<sup>–</sup></sup> mice (Fig. 9 A). The hippocampus was selected because it exhibited the most efficient deletion of Sirt1, which was confirmed in samples used for metabolomics (Fig. 9 B). In total, 227 peaks were captured, 115 of which were structurally annotated metabolites.
### Table 2. Significantly Altered Annotated Metabolites in Sirt1neu-/- Hippocampus*  

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<th>Metabolite</th>
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*Data from non-targeted primary metabolomics analysis of control and Sirt1neu-/- hippocampus. Fold change is with respect to control.

### Table 3. Significantly Altered Unknown Metabolites in Sirt1neu-/- Hippocampus*  

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*Data from non-targeted primary metabolomics analysis of control and Sirt1neu-/- hippocampus. BinBase identifiers are given for metabolite name. Fold change is with respect to control.
Statistical analysis using MetaboAnalyst\textsuperscript{26} found 23 metabolites significantly altered in Sirt1\textsuperscript{neu-/-} compared to tamoxifen treated controls (p<0.05, False Discovery Rate (FDR)<0.23, Student’s t-test, n=7-8) (Fig. 9 C and Table 2). Of the structurally unknown metabolites, 14 were significantly altered and are listed in Table 3 along with their BinBase identifiers for further reference. Further biochemical analysis of structural similarity networks generated in MetaMapR\textsuperscript{18} revealed that the majority were carbohydrates or purines related to glucose metabolism (Fig. 10 A). Interestingly, no tricarboxylic acid (TCA) cycle metabolites measured were significantly altered despite a known role for Sirt1
in mitochondrial function and biogenesis\textsuperscript{27}. Metabolites associated with ATP consuming processes of glucose and its derivatives were more abundant, suggesting more flux through these pathways. These included glycolysis (early steps – glucose, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate), pentose phosphate pathway and purine synthesis (ribitol, inosine monophosphate [IMP], inosine, guanosine, adenine, xanthine) and complex carbohydrate synthesis (mannose) \textsuperscript{(Fig. 10 B-D)}. Interestingly, 3-phosphoglycerate, a metabolite associated with the first ATP producing step in glycolysis, and serine, which can be interconverted to 3-phosphoglycerate, were less abundant. This suggests that glucose may be diverted to branch point pathways and exit

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure11}
\caption{Transcriptional changes in glucose metabolism machinery in Sirt1\textsuperscript{neu-/-}. (A-E) Gene expression (mRNA) from control and Sirt1\textsuperscript{neu-/-} hippocampi shown as fold change of control (GLUT1 – solute carrier family 2 facilitated glucose transporter member 1, GLUT3 – solute carrier family 2 facilitated glucose transporter member 3, Hk1 – hexokinase 1, Hk2 – hexokinase 2, GPI – glucose phosphate isomerase, PKF – phosphofructokinase M, TPI – triosephosphate isomerase, GAPDH – glyceraldehyde 3-phosphate dehydrogenase, PGK – phosphoglycerate kinase, PGAM – phosphoglycerate mutase, PyrK – pyruvate kinase, G6PD – glucose 6-phosphate dehydrogenase, HIF1\textalpha – hypoxia inducible factor 1 alpha, HIF2\textalpha – hypoxia inducible factor 2 alpha; * = p<0.05, ** = p<0.01, \textit{t}-test, n=5-9).}
\end{figure}
glycolysis prior to this step. Together, these data suggest that neuronal Sirt1 regulates glucose and purine metabolism in the brain.

To determine if these changes may be due to transcriptional regulation of glucose metabolism machinery, we measured expression of glucose transport proteins, enzymes and transcription factors in hippocampus of Sirt1<sup>neu/-</sup> and control mice. GLUT1, the main glucose transporter found on both astrocytes and neurons, but not the neuronal-specific GLUT3<sup>28</sup>, was increased (38.1%, p<0.05, Student’s t-test, n=5-9) in Sirt1<sup>neu/-</sup>, possibly accounting for the observed increase in glucose abundance (Fig. 11 A). Of the glycolysis enzymes, only the two ATP consuming enzymes, hexokinase (Hk – 86.2%, p<0.05, Student’s t-test, n=5-9) and phosphofructokinase (PFK – 36.9%, p<0.05, Student’s t-test, n=5-9) were increased (Fig. 11 B), concordant with the observed metabolite changes. The neuronal-enriched Hk2<sup>29</sup> but not astrocytic-enriched Hk1, was increased, supporting the notion that the changes in metabolite abundance may reflect those found in neurons. The rate-limiting enzyme controlling glucose entry into the pentose phosphate pathway, glucose 6-phosphate dehydrogenase (G6PD), was increased (38.7%, p<0.05, Student’s t-test, n=5-9) in Sirt1<sup>neu/-</sup> (Fig. 11 C), which could account for the observed increase in purine nucleotides and the lesser abundance of 3-phosphoglycerate further downstream in glycolysis. Many of these proteins are regulated by hypoxia-inducible factor-1 alpha (HIF-1α), a transcription factor that mediates adaptive responses to hypoxia and interacts with Sirt1<sup>30</sup>. HIF-1α (41.7%, p<0.01, Student’s t-test, n=5-9) but not HIF-2 α expression was increased in Sirt1<sup>neu/-</sup> (Fig. 11 D). Two other Sirtuins known to regulate glycolysis in peripheral tissues, Sirt2 and Sirt6, had similar levels of expression in control and Sirt1<sup>neu/-</sup>
These data suggest that Sirt1 may regulate glucose metabolism through transcriptional control of key pathway mediators.

**3.4.4 Deletion of Neuronal Sirt1 Alters Glucose Metabolism**

The initial steps of glycolysis, purine synthesis and synthesis of complex carbohydrates are all ATP consuming processes, which displayed a higher abundance of associated metabolites in Sirt1<sup>neu-/-</sup> mouse brain. We hypothesized that perhaps glucose is being preferentially diverted to these ATP consuming pathways rather than being fully oxidized to pyruvate as an energy substrate by glycolysis. To test glycolytic ATP production, we measured latency until anoxic depolarization (AD), a large and rapid depolarization of neurons that occurs under anoxic conditions once cellular ATP levels fall.
to the point of Na\(^+/\)K\(^+\)-ATPase failure and ultimately ion gradient collapse\(^{31}\). Studies show that when mitochondria are inhibited by anoxia, glucose can extend latency to AD through glycolytic ATP production\(^{32}\). We measured latency until AD by extracellular field recording in the hippocampal CA1 region of acute brain slices (Fig. 12 A, Fig. 13 A). Gross synaptic transmission did not differ between control and Sirt\(^{1}\) neu-/- slices, evidenced by similar evoked field population spike (fPS) amplitude (p>0.05, repeated measures ANOVA, n=3-4), evoked field excitatory post-synaptic potential (fEPSP) slope (p>0.05, repeated measures ANOVA, n=3-4) and expression of Na\(^+\)/K\(^+\) ATPase subunits (p>0.05, Student’s t-test, n=5) (Fig. 12 B-D). Latency to AD under anoxia and perfusion of artificial cerebrospinal fluid (aCSF) with no glucose was similar between control and Sirt\(^{1}\) neu-/- (control – 3.60 min, Sirt\(^{1}\) neu-/- - 3.36 min, p>0.05, two-way ANOVA, Bonferroni post-hoc.

**Figure 13. Glycolytic ATP production is impaired in Sirt\(^{1}\) neu-/-:** (A) Schematic of experimental manipulations in CA1 hippocampus of acute brain slices. Under conditions of anoxia and perfusion of artificial cerebrospinal fluid (aCSF) containing glucose, latency to anoxic depolarization (AD) is a readout of glycolytic function (B) Examples traces of the AD event under different experimental conditions (IAA = iodoacetate, glycolysis inhibitor). (E) Quantification of latency to AD. Under anoxia, glucose was able to extend latency to AD in control but not Sirt\(^{1}\) neu-/- slices (*** = p<0.001, two-way ANOVA, Bonferroni post-test, n=3-4; n/m = not measured, IAA = iodoacetate).
n=3-4) (Fig. 13 B and C). In controls, perfusion with 20 mmol/l glucose aCSF under anoxia significantly extended latency to AD (8.31 min, \(p<0.001\), two-way ANOVA, Bonferroni post-hoc, \(n=3-4\)), suggesting normal glycolytic ATP production that contributes substantially to AD latency under these conditions. The glycolysis inhibitor iodoacetate (IAA) abolished the extended latency to AD with 20 mmol/l glucose in control (1.74 min, \(p<0.001\), two-way ANOVA, Bonferroni post-hoc, \(n=3-4\)), confirming that this effect is mediated through glycolysis. In Sirt1\(^{neu-/-}\), however, perfusion with 20 mmol/l glucose aCSF failed to extend latency to AD (\(p>0.05\), two-way ANOVA, Bonferroni post-hoc).
n=3-4), indicating impaired glycolytic ATP production compared to control (Fig. 13 B and C). Together, these data suggest that neuronal Sirt1 promotes glycolytic ATP production.

3.4.5 RPC Increase Glycolytic Rate in Neurons

Sirt1 inhibits glycolysis in liver and skeletal muscle through activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and repression of HIF-1α to promote utilization of alternative energy substrates such as fatty acids for oxidative metabolism. Given that the brain preferentially utilizes glucose as an energy substrate and that glycolytic ATP production was impaired upon deletion of neuronal Sirt1, we hypothesized that Sirt1 may positively regulate glycolysis as an energy pathway. To test this hypothesis in neurons, we measured glycolytic rate in rat primary neuronal-enriched cultures using the Seahorse XFp (Fig. 14 A, Fig. 15 A) and inhibited Sirt1 pharmacologically given the low deletion efficiency of inducible cre/lox systems in vitro and to avoid any confounding effects of tamoxifen treatment. Under basal conditions,

Figure 15. RPC increases glycolytic rate under penumbra-like conditions. (A) Schematic of measurement of glycolytic rate (ECAR – extracellular acidification rate) by Seahorse XFp in primary neuronal-enriched cultures under ischemic penumbral-like conditions (energetic stress (mitochondrial uncoupling with FCCP [2 µM], Complex V inhibition with oligomycin [1 µM]) 25% of normal glucose). (B-D) Glycolytic rate is expressed as fold change of Veh over time in RPC or Veh treated cells, where arrow indicates application of mitochondrial stressing compounds. Notice the maintenance of a higher glycolytic rate in RPC treated neurons than Veh for at least 40 min (*** = p<0.001, ns = not significant, t-test, n=4).
inhibition of Sirt1 with the specific inhibitor EX-527 (10 µM) 2 days prior to measurements modestly reduced glycolytic rate (12.1%, p<0.01, one-way ANOVA, Bonferroni post-hoc, n=5) (Fig. 14 B and C). EX-527 also inhibited maximal glycolytic rate (7.3%, p<0.05, one-way ANOVA, Bonferroni post-hoc, n=5) in response to energetic stress, elicited by application of the mitochondrial uncoupler FCCP (2 µM) and complex V (ATP synthase) inhibitor oligomycin (1 µM) (Fig. 14 B and D). Activation of Sirt1 with RPC treatment (100 µM resveratrol, a concentration previously shown to protect neurons from ischemia in vitro\(^9\)) 2 days prior to measurements increased basal (20.4%, p<0.001 one-way ANOVA, Bonferroni post-hoc, n=5) and maximal (29.2%, p<0.001, one-way ANOVA, Bonferroni post-hoc, n=5) glycolytic rate compared to Veh (Fig. 14 E-G). Inhibition of Sirt1 at the time of RPC treatment significantly reduced these increases (basal – 12.1%, p<0.05, maximal – 10.1%, p<0.01, one-way ANOVA, Bonferroni post-hoc, n=5), demonstrating the Sirt1-dependence of these effects (Fig 14 E-G). Enhanced glycolytic function could be important in ischemic penumbra, where energy substrates are significantly decreased but still present. Thus, we tested whether these RPC-induced changes in glycolytic rate occur under conditions of low glucose (Fig 15). Indeed, when glycolytic rate was measured in cells exposed to 25% of normal glucose (5 mmol/l glucose instead of 20 mmol/l), maximal glycolytic rate was increased in RPC neurons compare to Veh (16.6%, p<0.001, Student’s t-test, n=4) (Fig. 15 B-D). Together these data demonstrate that RPC increases glycolytic rate in neurons through Sirt1.
Chapter 4. Discussion

4.1 Overview

There is enormous therapeutic potential in pharmacological compounds that mimic IPC, such as resveratrol. Preconditioning with resveratrol protects against global and focal ischemia in rodent models yet evaluation of its translational potential and identification of its robust neuroprotective mechanisms is in its infancy. Likewise, the metabolic functions of Sirt1, a molecular target of resveratrol, are poorly understood intracellularly in the brain. The goal of this work was to evaluate the efficacy of prophylactic resveratrol treatment (RPC) in the context of ischemic stroke and to improve our understanding of the protective mechanisms of Sirt1-mediated metabolic regulation in neurons. In Chapter 2, it was determined that resveratrol is an efficacious prophylactic agent in a rodent model of ischemic stroke. Strikingly, a single RPC treatment induced a long-lasting state of ischemic tolerance that may depend on Sirt1-mediated regulation of proteins that enhance neuronal and mitochondrial function. In Chapter 3, neuronal Sirt1 was identified as an essential mediated or RPC-induced ischemic tolerance, irrespective of cerebral perfusion. Neuronal deletion of Sirt1 affected brain glucose metabolism and impaired glycolytic function. RPC increased glycolytic rate in neurons in a Sirt1-dependent manner and in ischemic penumbra-like conditions. In this chapter, the significance of these findings is discussed in the context of other relevant, published works.

4.2 Windows of Preconditioning-Induced Ischemic Tolerance

Preconditioning-induced ischemic protection is characterized as having two distinct windows, an early one that lasts a few hours and a more robust late window that can last
several days following the preconditioning stimulus\textsuperscript{58,62}. Our observation that a single application of RPC can protect against ischemic stroke in mice for up to fourteen days demonstrates a new, extended window of protection\textsuperscript{140}. To our knowledge, this is the first evidence that a single pharmacological preconditioning treatment can induce a state of long-lasting ischemic tolerance. However, evidence from previous work hinted that an extended window might exist. In organotypic slices, we showed that one application of IPC affords protection that lasts at least four days\textsuperscript{69}. Remarkably, a paradigm of repetitive hypoxia preconditioning (varying O\textsubscript{2} exposure over a two-week period) conferred protection from focal ischemia for up to two months after treatments\textsuperscript{132}. Here we show that a pharmacological compound (resveratrol) can elicit a long-lasting state of ischemic tolerance. Elucidating the long-lasting mechanisms of pharmacological agents that can mimic preconditioning will provide a framework for the development of novel clinical applications.

Resveratrol either directly or indirectly activates and/or upregulates Sirt1\textsuperscript{145,88}, a deacetylase with the ability to induce lasting genomic and epigenomic changes\textsuperscript{137}. Increased Sirt1 expression or activity appears to be a common mechanism of IPC and RPC-induced protection\textsuperscript{69,70}. The proposed acute mechanisms for Sirt1-mediated ischemic tolerance include modulating the neuroinflammatory response via NF-κB\textsuperscript{146}, reducing/inhibiting pro-apoptotic factors such as p53\textsuperscript{147} as well as enhancing mitochondrial function via PGC1α\textsuperscript{122} and UCP2\textsuperscript{70}. Here, we found Sirt1 to be upregulated at 14 days following a single RPC treatment. A chronic upregulation of Sirt1 could promote changes in gene expression that are maintained overtime that give rise to this extended window. In line with this notion, 14 days following RPC we found enhanced Sirt1 binding to UCP2
and BDNF promoter regions. It remains to be tested whether Sirt1 is required for RPC-induction of the extended window of protection. An important distinction to make is whether Sirt1 might be involved in the induction of protection, or the maintenance of protection, or both. Future experiments, where deletion of neuronal Sirt1 (using Sirt1\textsuperscript{neu-/-} mice) is induced prior to RPC or after RPC, will identify the temporal distribution of Sirt1 activity in the extended window.

As described above, Sirt1 is known to negatively regulate UCP2 by binding directly to its promoter\textsuperscript{148}. The present study is consistent with this regulation, as we observed a decrease in synaptic UCP2 protein levels. Previous work demonstrates a reduction in UCP2 two days after RPC in rats, where protection from cardiac arrest is evident\textsuperscript{70}. This neuroprotection and decrease in UCP2 were also linked to enhanced mitochondrial efficiency\textsuperscript{70}. In another line of evidence, UCP2 knockout mice were found to be resistant to permanent focal ischemia\textsuperscript{149}. In these mice, protection was attributed to increased antioxidant defenses and a reduction in oxidative damage. Other groups have shown that overexpression of UCP2 is also protective against cerebral ischemia\textsuperscript{150} and that UCP2 knockout mice were actually more susceptible to a model of transient focal ischemia\textsuperscript{151}. Thus, the role of UCP2 as a mediator of cerebral ischemic injury is still controversial. Discrepancies could be due to variation across species, genetic compensation in knockout mouse models, the type of ischemia model employed or choice of preconditioning agent. Further studies are needed to clarify the role of UPC proteins in mitochondrial function and brain ischemia.

Sirt1 is also known to upregulate BDNF expression; the two proposed mechanisms are indirect and include Sirt1 deacetylation of MeCP2, which regulates expression of
BDNF by binding at its promoter\textsuperscript{152}, and Sirt1 inhibition of miR-134 expression, a micro-
RNA that downregulates expression of BDNF\textsuperscript{116}. Additionally, binding of Sirt1 to the
BDNF promoter has been identified in the mouse cerebellum, suggesting that a direct
interaction might occur\textsuperscript{153}. Moreover, BDNF was decreased in the brains of Sirt1 null mice
\textsuperscript{119}. BDNF is an important growth factor that promotes survival and growth of existing
neurons as well as the differentiation of new ones. It also impacts synaptic plasticity and
learning and memory\textsuperscript{154}. Our previous work shows enhanced BDNF as a mechanism for
ischemia tolerance following IPC or protein kinase C epsilon (PKC\textsubscript{e})-induced
preconditioning\textsuperscript{138}, in which increased BDNF was associated with a delay in AD. Another
group showed that intravenous BDNF administration following ischemia improves
recovery possibly through a mechanism centered on neurogenesis\textsuperscript{155}. Sirt1-mediated
elevation of BDNF might be a robust mechanism of the long-lasting ischemic tolerance
observed with RPC. Our future experiments will utilize Sirt1\textsuperscript{neu-/-} mice to further determine
the Sirt1 dependence of RPC-induced UCP2 and BDNF responses in neurons.

4.3 Neuronal vs Systemic Mechanisms of RPC and Sirt1-mediated Ischemic
Tolerance

Although it is postulated that Sirt1 mediates the neuroprotective effects of
resveratrol, it remains unclear \textit{in vivo}. Sirt1 is expressed across tissues and in different
brain cell types and thus it is crucial to identify the localization of Sirt1 that initiates and/or
terminates RPC-induced protection. In the present study, we found that neuronal Sirt1,
specifically, is essential for RPC-induced ischemic tolerance. Interestingly, cerebral blood
flow during ischemia and at the onset of reperfusion was unaffected by deletion of neuronal
Sirt1 or RPC treatment. This is important because non-neuronal and systemic processes
have been attributed to both Sirt1- and RPC-mediated ischemic tolerance. These include the sprouting and migration of endothelial cells (angiogenesis)\textsuperscript{128}, maintenance of cerebral blood flow through eNOS\textsuperscript{114}, endothelial activation of MAPK/ERK signaling\textsuperscript{129} and modulation of the inflammatory mediator NF-κB\textsuperscript{146}. Undoubtedly, these mechanisms contribute to the neuroprotective effects of RPC yet our data indicate that the activity of neuronal Sirt1 is central, perhaps interacting with endothelial or resident immune cells to exert secondary effects.

The temporal distribution of neuronal vs non-neuronal protective pathways is also a probable factor in determining the extent of their contribution. Here, resveratrol application prior to MCAo reduced infarct size when evaluated twenty-four hours following injury. The observation of neuroprotection at this early time point suggests a mechanism that targets the initial excitotoxic injury and/or the early reperfusion phase. This could be further teased apart using a model of permanent ischemia, where reperfusion of the affected tissue does not occur. In this scenario, the deleterious mechanisms of reperfusion injury do not engage the tissue and the effects of RPC solely on the initial ischemic injury or excitotoxic injury can be isolated. Then, it could be tested whether RPC-induced protection against the initial injury is maintained past the twenty-four hour time point described in the present study. These are important questions that can more specifically reveal the spatial and temporal distribution of RPC-induced protection.

Resveratrol also promotes recovery when administered post-injury\textsuperscript{35}. In this window, the acute effects of resveratrol may be more prominent, as reperfusion will induce derangements in cerebral blood flow and neuroinflammation. At this later stage, resveratrol- and Sirt1-elicited angiogenic, antioxidant and anti-inflammatory effects may
be more effective in blunting ischemic injury\textsuperscript{156}. This hierarchy of protective mechanisms could also be due to the relative expression levels of Sirt1 in different brain cell types, which \textit{in vivo} includes neurons, NSCs, NPCs and endothelial cells\textsuperscript{13}. Deletion of neuronal Sirt1 resulted in a \textasciitilde 60-80\% reduction in total brain Sirt1, demonstrating that the bulk of Sirt1 in the brain is neuronal. The role of Sirt1 in the proliferation and differentiation of NSCs/NPCs during recovery and repair in the weeks to months following ischemic stroke is also warranted.

Another interesting observation was that control and Sirt1\textsuperscript{neu-/-} mice had similar infarct sizes, indicating that neuronal Sirt1 does not inherently influence ischemic injury. This is in line with another report where mice overexpressing Sirt1 specifically in neurons were not protected from tMCAo, evidenced by similar infarct size and functional scores\textsuperscript{157}. In contrast, other studies show that Sirt1\textsuperscript{-/-} mice display larger infarct sizes than WTs\textsuperscript{112} following pMCAo and Sirt1 constitutively overexpressing mice are resistant to ischemic injury\textsuperscript{114}. This discrepancy could be reconciled by the fact that we deleted specifically the neuronal pool of Sirt1 whereas the other reports used whole-body transgenic mouse models. Additionally, our neuronal Sirt1 knockout mice are inducible while the contrasting conclusions are drawn from experiments using mice in which Sirt1 was manipulated throughout development. These findings were also obtained across three different models of ischemic injury (permanent vs transient MCAo, and global ischemia) with different outcome measures and thus the type of injury will also contribute to this variation. One final aspect that may account for discrepancies is the severity of injury. The tMCAo model in the present study produced an infarct of \textasciitilde 50\%, which could be approaching the maximal injury without significant mortality. Titration of tMCAo time to a milder injury could allow
for a more sensitive investigation into the innate role of Sirt1. Ultimately, the use of inducible whole-body Sirt1 knockout or overexpression mice across different cerebral ischemic injury models will resolve these conflicting reports.

### 4.4 Sirt1 and Glycolytic Function in Brain

While the metabolic adaptations mediated by Sirt1 are well characterized in skeletal muscle, liver and adipose cells\(^9\), their manifestation in brain at the cellular level has not been identified. Given that ischemia is essentially an acute state of deficient energy substrate supply and cellular stress, Sirt1-mediated metabolic adaptations in neurons are likely to contribute to ischemic tolerance. In the present study, we found that glycolysis was the most robustly altered metabolic pathway with deletion of neuronal Sirt1. In peripheral tissues, Sirt1 inhibits glycolysis through activation of PGC-1α\(^{158}\) and repression of HIF-1α\(^{100}\) to promote the utilization of alternative energy substrates such as fatty acids for oxidative metabolism. The brain however, relies largely on continuous utilization of glucose for energy production\(^7\) in order to sustain normal function. We found that neuronal Sirt1 promotes glycolytic function as a viable adaptation in brain. Our data suggest Sirt1-mediated repression of key metabolic regulators such as GLUT1, Hk and G6PD. Characterization of the neuron-specific transcriptional program regulated by Sirt1 is necessary to understand the metabolic plasticity of the brain and has far-reaching implications in neurological disease. Dysregulation of glycolysis plays a role in the pathogenesis and/or manifestation of Huntingtin’s disease\(^{159}\), Alzheimer’s disease\(^{160}\) and Parkinson’s disease\(^{161}\), in all of which Sirt1 has a neuroprotective role\(^{108}\).

While the bulk of brain ATP is generated from the oxidation of pyruvate within mitochondria, recent studies highlight the importance of glycolysis in normal neuronal
function as well. Local production of ATP from glycolysis is crucial for fast axonal transport of vesicles, the maintenance of presynaptic function during activity and the energetic demand of action potential firing. Under energetic stress, glycolytic enzymes localize adjacent to synapses to maintain local ATP levels and in turn synaptic function. Our results demonstrate that under mitochondrial stress by anoxia, glycolysis can produce a significant amount of ATP to maintain ion gradients and delay depolarization. Strikingly, deletion of Sirt1 impaired this ability. Studies also show that the Na⁺/K⁺ ATPase, which is the main component of ion gradient maintenance in the brain, is preferentially fueled by glycolytic ATP production over mitochondria-generated ATP.

We also found that RPC increases glycolytic rate in neurons in a Sirt1-dependent manner. Sirt1 deficiency has been shown to increase glycolytic activity in Th9 cells, however other reports demonstrate that resveratrol stimulates glycolytic ATP synthesis in yeast as well as rodent liver and that resveratrol inhibits the pentose phosphate pathway. This is consistent with our findings, where in the opposite scenario, metabolite profiles of Sirt1 neu-/- mice and an increase in the rate-limiting enzyme G6PD suggest a shunting of glucose away from complex oxidation to pyruvate. More efficient utilization of glucose for energy production by promoting its full oxidation could greatly benefit neurons in the ischemic penumbra, where blood flow has been significantly restricted but some energy substrates still reach the affected tissue. RPC-induced increases in glycolytic rate were also observed under low glucose (25% of normal) and mitochondrial stress conditions, supporting a physiological relevance in penumbral-like conditions. The beneficial effects of resveratrol post-ischemia may also be mediated by Sirt1-dependent optimization of glycolytic function.
Although complete ischemia in the core region leads to necrotic cell death, the fate of cells in the penumbra depends on the extent of metabolic compromise and whether or not normal metabolic function can be recovered post-ischemia\textsuperscript{167}. In this region, accumulation of insurmountable damage will result in apoptotic cell death of neurons through many different energy-dependent apoptotic pathways\textsuperscript{168}. If cellular damage can be repaired significantly and energy metabolism can recover to an adequate rate, neurons may be spared from apoptotic mechanisms. During ischemia, oxidative metabolism (within mitochondria) and glucose utilization are decreased to less than 35\% in the core\textsuperscript{169, 170}. However, the penumbra only experiences a similar reduction in oxidative metabolism but not in glucose utilization, which may even be increased during the first two hours of ischemia\textsuperscript{171, 172}. This could be explained by the fact that glucose extraction from blood is increased in the penumbra during ischemia\textsuperscript{170} and contributes to the maintenance of glycolytic activity. In line with this notion, penumbral lactate increases\textsuperscript{173} (but not to the extent of the core), suggesting that oxygen delivery is more restricted than that of glucose. Together these data indicate that penumbral glycolysis plays an important role in the preservation of neuronal viability during ischemia. Since even larger reductions in oxidative metabolism have been observed in penumbra within one hour of reperfusion\textsuperscript{174}, glycolytic function may also be an important process in preserving neuronal viability with reperfusion of this area. Our data demonstrates that RPC enhances the energetic efficiency of neuronal glycolysis through Sirt1 and thus may represent an anti-apoptotic mechanism in ischemic penumbra. A more detailed quantification of necrotic vs apoptotic cell death as well as core vs penumbral infarct size following tMCAo may provide evidence that RPC is ultimately protecting cells in this manner. As penumbra is estimated to make up as much
as 50% of the total infarct\textsuperscript{167}, prevention of apoptotic cell death could afford significant neuroprotection.

The magnitude, duration and number of neurons experiencing pathological depolarization are major determinants of cellular dysfunction, which can ultimately lead to apoptotic neuronal death in the ischemic penumbra. Here, depolarization can range from spreading depression, a slowly propagating wave of near complete depolarization characterized by collapse of ion homeostasis and neurotransmitter release that is transient and resolves, to terminal spreading depolarizations, which are also characterized by collapse of ion gradients and excitatory neurotransmitter release but cannot be recovered from since the extent of metabolic compromise is too great\textsuperscript{37}. Sustained depolarization can induce apoptotic triggering events such as overloaded intracellular Ca\textsuperscript{2+}, decreased mitochondrial membrane potential and excessive production of ROS\textsuperscript{168}. Buffering of Ca\textsuperscript{2+}, recovery of mitochondrial membrane potential and quenching of ROS are all ATP-dependent processes that can only contribute to recovery under conditions of some energetic adequacy. If homeostasis of these features is not reestablished, these hallmarks of ischemic injury will induce intrinsic apoptotic pathways that involve the activation of calpains, cleavage of caspases, formation of the MPTP, release of proapoptotic factors from mitochondria and fragmentation of DNA, among others\textsuperscript{168}. Since the extent of depolarization and recovery from depolarization-induced damaging events are both dependent on the metabolic state of the penumbral tissue, it is clear that enhanced energetic efficiency, and specifically glucose utilization, could have a large impact in preventing apoptotic cell death in neurons.
An important question remain concerning the metabolic regulation of neuronal Sirt1 in ischemia and normal brain function. Although glycolysis was the most robustly altered pathway with deletion of neuronal Sirt1, mitochondrial biogenesis is a well-characterized feature of Sirt1 activation\textsuperscript{175}. In the late window of protection induced by RPC, mitochondrial efficiency was enhanced and concomitant with Sirt1 activation and a reduction in UCP2 in rat hippocampus. Increased Sirt1 binding to the UCP2 promoter region and reduced synaptic UCP2 protein was also observed in cortex in the extended window of RPC, again suggesting regulation of mitochondrial function. Intriguingly, deletion of neuronal Sirt1 did not robustly alter abundances of metabolites associated with mitochondrial metabolic pathways such as TCA cycle or fatty acid metabolism. There were, however, alterations in several purine metabolites, potentially signifying altered mitochondrial metabolism. Many of the rate-limiting and ATP-dependent reactions within purine metabolism are localized to mitochondria and studies demonstrate that mitochondrial dysfunction disrupts purine metabolism and can lead to defective DNA repair, RNA synthesis and intracellular/extracellular signaling\textsuperscript{176, 177}. Hence, perturbations in purine metabolites in Sirt1\textsuperscript{neu\textendash/} could reflect a general state of mitochondrial stress, a possibility that deserves further attention. Glycolytic changes observed in acute slices and neuronal cultures might arise from other bioenergetics alterations and thus a more detailed analysis of the energy phenotype of neurons with RPC and/or Sirt1 deletion is warranted. This will be a focus of future experiments.

4.5 Conclusions, Significance and Future Directions

Here we have identified a novel and robust mechanism of RPC-induced ischemic tolerance \textit{in vivo} that requires metabolic regulation of neuronal Sirt1. It is important to put
this finding in the context of other preconditioning-induced paradigms to get a global view of the innate protective responses to cerebral ischemic injury. Sirtuin proteins are implicated not only in RPC\textsuperscript{70}, but in IPC\textsuperscript{69} and PKC\textsubscript{e} preconditioning\textsuperscript{124}. In addition to Sirt1, Sirt5 (the major mitochondrial de-malonyl, -glutaryl and -succinyl –ase) is also a mediator of preconditioning-induced protection and given the diverse substrates and subcellular distribution of Sirtuin proteins, it is likely that they work in concert to mediate adaptations in different cellular compartments, which culminate in a protective state of cellular metabolism. Investigation into the interplay between Sirtuins and their respective targets will enhance our understanding of how cellular metabolic regulation can combat ischemic injury. Aside from Sirtuins, many other pathways also contribute to this phenotype (discussed in Chapter 1.2). This is evidenced by omics-based studies that continue to identify the vast transcriptional and translational features of the preconditioning response. The synergy between metabolic and other cellular pathways that converge on DNA, RNA and protein targets is likely a crucial aspect of ischemic tolerance and will need to be teased apart in future studies. The combinatorial treatment of different preconditioning agents that can activate complementary pathways and act in parallel will ultimately provide the most robust neuroprotection. More preclinical studies are needed to validate this approach; however, several lines of evidence already demonstrate the efficacy of preconditioning agents regarding its translational potential.

Dirnagl and colleagues nicely described the validity of preconditioning as a therapeutic approach in clinical scenarios of cerebral ischemia\textsuperscript{178}. In addition to ischemic stroke and cardiac arrest, there are a host of conditions or surgical procedures that leave patients susceptible to bouts of ischemia. These include carotid endarterectomy, coronary
artery bypass graft, transient ischemic attack (TIA) and sub-arachnoid hemorrhage, among others\textsuperscript{178}. These patients, and those affected by or at risk for stroke, make up a population in the millions that can benefit significantly from preconditioning-based therapies. A marked advantage of preconditioning in ischemic stroke treatment is that it affords an addition therapeutic window. Ultimately, a combination of prophylactic treatment and those that facilitate recovery post-injury will provide the greatest impact. Already, the clinical efficacy of preconditioning has been demonstrated in the protection of peripheral organs through remote limb preconditioning, which refers to the ability of brief periods of ischemia in a limb to induce ischemic tolerance in organs remote from the site of ischemia\textsuperscript{179}. Similar studies in brain will be paramount in moving preconditioning therapies towards the stroke clinic.

Likewise, pharmacological preconditioning agents are of immense translational value. The efficacy of resveratrol in the treatment of cerebral ischemia is promising. It has passed phase I clinical trials for the treatment of several medical conditions\textsuperscript{180} and has been deemed safe for use in humans at and above the equivalent preconditioning doses\textsuperscript{48}. Our results and those of other labs show that desensitization to prolonged treatment does not occur\textsuperscript{74,75,77,140}, demonstrating its effectiveness as a prophylactic treatment. Clinically relevant routes of administration have also been validated, such as IV and oral applications\textsuperscript{49}.

The efficacy of RPC has been validated across labs, species and routes of administration, yet many preclinical aspects of RPC still merit evaluation. Most studies describe neuroprotective mechanisms in young rodents and have not confirmed findings in aged animals, the cohort most affected by stroke. Studies have heavily favored the use of
male animals and thus further investigation into sex-based dichotomy of RPC-mediated protection is also warranted. Future studies need to evaluate the therapeutic potential of RPC in combination with treatments that facilitate recovery when administered post-injury. In addition to RPC, administration of resveratrol post-injury can also induce neuroprotection\textsuperscript{35} and thus might prove a robust agent in both therapeutic windows. Sirt1 is also an attractive, druggable target with translational potential. Recently developed Sirt1-specific activator compounds (SRT1720 and SRT2014) allow for direct, specific and more robust activation of Sirt1\textsuperscript{50}, making them promising candidates for therapeutic purposes. In addition to resveratrol, these Sirt1 activating compounds might be promising candidates for therapeutic intervention in cerebral ischemia.
References


177 Allsop, J. & Watts, R. W. Activities of amidophosphoribosyltransferase (EC2.4.2.14) and the purine phosphoribosyltransferases (EC2.4.2.7 and 2.4.2.8), and the phosphoribosylpyrophosphate content of rat central nervous system at different stages of development--their possible relationship to the neurological dysfunction in the Lesch-Nyhan syndrome. *J. Neurol. Sci.* **46**, 221-232 (1980).

