

● PERSPECTIVE

Comparative insights into mitochondrial adaptations to anoxia in brain

Numerous diseases and pathologies impair the delivery of oxygen to brain, with rapid and deleterious consequences. For example, diseases related to systemic hypoxemia (e.g., chronic pulmonary disorders, cystic fibrosis), decreased oxygen carrying capacity of blood (e.g., anemia), or decreased transport (e.g., heart attack, stroke) can all reduce or entirely prevent the delivery of oxygen to brain cells, resulting in the initiation of programmed cell death pathways, necrosis, or excitotoxic cell death in brain (Pamenter, 2014). However, oxygen-limited environments are common on earth and many organisms naturally experience periods of intermittent or prolonged hypoxia or anoxia in their daily and/or annual life cycles (Bickler and Buck, 2007). This exposure exerts a strong selective pressure that has driven the evolution of a wide range of adaptations that are neuroprotective against low oxygen stress. The brains of such hypoxia-tolerant species therefore offer a working model of neuronal survival in the absence of oxygen and their study may inform the development of novel therapeutics to protect mammalian brain against diseases and pathologies related to hypoxia.

Of particular interest are naturally evolved adaptations that modify mitochondrial function to 1) provide metabolic plasticity related to the control of cellular energetics at varying oxygen tensions, and that 2) proactively initiate neuroprotective mechanisms. Studies of species that are highly tolerant to hypoxia and anoxia provide prime examples of these central roles for mitochondria in signaling oxygen variability and subsequently coordinating cellular responses to hypoxia. Indeed, mitochondria are the lynchpin of aerobic metabolism, consuming > 90% of the body's oxygen to facilitate oxidative phosphorylation, which is the process *via* which ATP is formed through the transfer of electrons along the complexes of the electron transport chain (Pamenter, 2014). This central role in aerobic energy production makes mitochondria an excellent sensor of changes in cellular oxygen tension. Furthermore, mitochondria also coordinate neuroprotective pathways that respond to low oxygen challenges in brain of hypoxia-tolerant species. These pathways mediate diverse responses including changes in transcriptional activity, organelle and synaptic function, and intercellular communication (Pamenter, 2014). For example, carefully regulated reactive oxygen species and Ca^{2+} signaling beneficially modulate neuronal proteins to limit ion flux in anoxia-tolerant freshwater Western painted turtle brain [*Chrysemys picta bellii*], preventing excitotoxic cell death during prolonged anoxic or ischemic exposures (Pamenter, 2014; Hogg et al., 2015). Conversely, in brain of hypoxia-intolerant mammals, unregulated reactive oxygen species generation and/or deleterious mitochondrial Ca^{2+} accumulation initiate cell death pathways during hypoxic or ischemic challenges (Kalogeris et al., 2014). Therefore, mitochondria are at the center of neuronal energy production in normoxia but also determine the cellular fate choice between initiating neuroprotective responses *vs.* activating cell death cascades when oxygen is limited.

Despite the central role for mitochondria in regulating cellular responses to hypoxia in general, and neuroprotective pathways in the brains of species adapted to life in hypoxia in particular, few studies have examined mitochondrial signaling in hypoxia-tolerant brain. Moreover, no previous study has directly examined electron transport chain function and metabolic plasticity in mitochondria isolated from the brain of a hypoxia-tolerant organism. In a recent study, we addressed this knowledge gap by employing high-resolution respirometry and molecular biology approaches to examine electron transport chain function and mitochondrial metabolic plasticity in the brain of anoxia-tolerant freshwater red-eared slider turtles [*Trachemys scripta*] that had been exposed to two weeks of either normoxia or anoxia (Pamenter et al., 2016). We found that

brain mitochondria from anoxia-acclimated turtles exhibited a unique phenotype of remodeling relative to normoxic controls, including: 1) decreased citrate synthase and F_1F_0 -ATPase activity, 2) markedly reduced aerobic capacity, and 3) mild uncoupling of the mitochondrial proton gradient. Furthermore, 4) brain mitochondria from normoxic and anoxic turtles were equally tolerant of an acute anoxia/reperfusion challenge.

The findings of our study are important because they suggest that 1) brain mitochondria from one of the most anoxia-tolerant species identified possess endogenous defense mechanisms that are chronically activated, but 2) also retain the malleability to undergo remodeling during prolonged anoxic exposure, likely in order to activate and regulate neuronal defenses against this challenge (e.g., Hogg et al., 2015). The occurrence of endogenous protective mechanisms in mitochondria is an important observation because the primary cause of mitochondrial damage during ischemia/reperfusion injury is deleterious reactive oxygen species generation upon the reintroduction of oxygen. This sudden reactive oxygen species production induces damage locally to the mitochondrial membrane and electron transport chain complexes, but also globally within the cell to DNA, membrane proteins, and other organelles (Kalogeris et al., 2014). It is important to note that low levels of reactive oxygen species are a natural by-product of oxidative phosphorylation, wherein a small portion of the oxygen consumed for aerobic cellular metabolism is converted to the superoxide anion radical (Pamenter, 2014). However, mitochondria possess an efficient antioxidant system; enzymes like superoxide dismutase and glutathione peroxidase, as well as compounds such as reduced glutathione, nicotinamide adenine dinucleotide phosphate, and ascorbate eliminate superoxide anion radicals produced in mitochondria. Conversely, after ischemia and reperfusion, a state of oxidative stress occurs and components of the respiratory chain that had been reduced during the ischemic period are rapidly refilled by molecular oxygen, producing superoxide anion radicals in excess, which overwhelm these antioxidant defenses (Kalogeris et al., 2014; Pamenter, 2014). Amazingly, excess reactive oxygen species generation is prevented in freshwater turtle brain during the onset of anoxia and following reperfusion (Pamenter et al., 2007), and this tissue survives both prolonged anoxia and also ischemic challenges that induce reactive oxygen species generation and cell death in mammalian brain cells (Pamenter, 2014).

This proactive modulation of reactive oxygen species production during anoxia- and ischemia-reperfusion in turtle brain can be traced to the mitochondrial adaptations that we describe in our re-

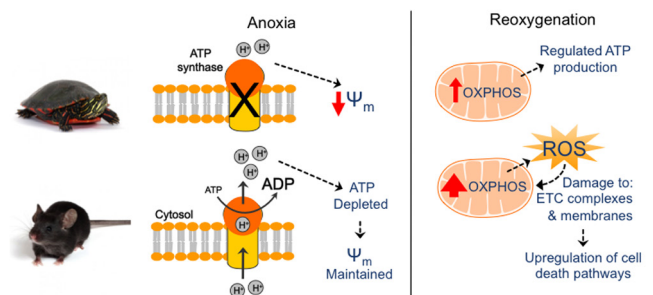


Figure 1 Turtle brain mitochondria are protected against anoxia/reperfusion injury.

Anoxia-tolerant turtle brain mitochondria decrease F_1F_0 -ATPase activity, electron transport chain (ETC) respiratory flux, and mitochondrial membrane potential (ψ_m) in anoxia (upper panels). During reoxygenation, these adaptations help to throttle the rate at which oxidative phosphorylation (OXPHOS) is reengaged, which prevents deleterious reactive oxygen species (ROS) generation and protects mitochondria and brain cells from oxidative damage. Conversely, in anoxia-intolerant mouse brain mitochondria (lower panels), the activity of the F_1F_0 -ATPase is reversed in the absence of oxygen, resulting in the hydrolysis of ATP into ADP to maintain ψ_m , which results in depleted cellular ATP stores and a buildup of ADP. During reoxygenation, accumulated ADP causes OXPHOS to accelerate rapidly, resulting in large-scale ROS generation, which damages ETC components and mitochondrial membranes, leading to the induction of cell death pathways.

cent study (Pamenter et al., 2016). Of particular importance is the apparent ability of these mitochondria to streamline metabolic flux through the electron transport chain, which likely permits turtle brain mitochondria to essentially shut down oxidative phosphorylation in a controlled manner in the absence of oxygen. This is important because a coordinated shut down of this system would help to prevent the deleterious backflow of electrons along the electron transport chain; electron backflow is characteristic of uncontrolled throttling of oxidative phosphorylation in hypoxia-intolerant brain mitochondria when molecular oxygen is not present to serve as the terminal electron acceptor (Kalogeris et al., 2014; Pamenter, 2014). In support of this, we observed that: 1) mitochondrial states II, III, and IV respiration were all decreased following two weeks of anoxia in turtle brain, 2) total electron chain capacity decreased by $\sim 1/3^{\text{rd}}$, mediated largely by a substantial decrease in complex I activity relative to total electron transport chain capacity, and finally, 3) the mitochondrial proton gradient was partially uncoupled following acclimatization to anoxia (Pamenter et al., 2016). Partial uncoupling of the proton gradient, caused by the passive leak of protons across the mitochondrial inner membrane, would reduce the driving force for reverse electron flow during anoxia and thereby reduce the generation of reactive oxygen species during reperfusion. Taken together, these adaptive changes likely dampen the flux of electrons through the electron transport chain during reoxygenation following anoxia or ischemia and limit the generation of deleterious reactive oxygen species. Thus, turtle brain appears to turn down the gain of its oxidative phosphorylation apparatus during periods of anoxia, which would allow for a slow and controlled recovery when oxygen is reintroduced following a low oxygen challenge. Unfortunately, to our knowledge, no other studies have examined mitochondrial function in the brain of a hypoxia-tolerant species. However, a recent study examined respiratory flux and reactive oxygen species production from thorax mitochondria isolated from *Drosophila melanogaster* that had been raised under chronic hypoxia (4% O₂) for > 200 generations (Ali et al., 2012). This study demonstrated that state III respiration and reactive oxygen species production were decreased by > 30 and 60%, respectively, in hypoxia-acclimated flies, relative to naïve flies. These differences agree well with our observations in anoxia-adapted turtle brain mitochondria; however, additional comparative studies in other hypoxia-tolerant or hypoxia-adapted species are clearly warranted.

Another key contributor to this proactive dampening of reactive oxygen species generation during reperfusion may be the downregulation of F₁F₀-ATPase activity in anoxic turtle brain. Specifically, we reported that turtle brain mitochondria exhibited an $\sim 85\%$ reduction in F₁F₀-ATPase activity following two weeks of anoxia acclimatization. This difference was manifest in enzyme activity but not in protein content, suggesting that the change is due to post-translational modifications, which can be rapidly reversed in a controlled manner following reperfusion. This is an important adaptation because in anoxia, mitochondria transition from being the powerhouse of the cell to becoming a liability that futilely consumes limited cellular energy stores. Specifically, when deprived of oxygen to serve as the terminal electron acceptor in the electron transport chain, the mitochondrial F₁F₀-ATPase reverses, hydrolyzing ATP into ADP in order to maintain the proton-motive force, thereby robbing the cell of valuable fuel reserves (St-Pierre et al., 2000). A large buildup of ADP during anoxia provides an enormous drive to mitochondrial respiratory flux upon the return of molecular oxygen (Figure 1), which is a primary contributor to the deleterious burst of reactive oxygen species typically observed during reperfusion (Kalogeris et al., 2014). By downregulating F₁F₀-ATPase activity during prolonged anoxia, turtle brain mitochondria not only protect available fuel reserves, but also prevent a rapid acceleration of oxidative phosphorylation upon reperfusion (Figure 1), thereby enabling a slow and controlled recovery of mitochondrial metabolism following a low oxygen challenge. This mechanism has also been previously described in muscle mitochondria from anoxia-tolerant frogs (St-Pierre et al., 2000), and in heart mitochondria from anoxia-tolerant freshwater turtles (Galli et al., 2013); however, no other studies have examined this mechanism in mitochondria from the brain of any other species.

In conclusion, studies exploring cell death mechanisms induced in mammalian brain when challenged with anoxia or ischemia-reperfusion constitute a reactive approach to the problem, as they are generally focused on trying to repair damage that has already occurred. Conversely, comparative studies of naturally occurring endogenous neuroprotective mechanisms in the brains of hypoxia-tolerant species provide a window into proactive approaches to protecting brain cells that have evolved in response to natural environmental challenges. Such a proactive approach drastically reduces the need for neuronal regeneration strategies to replace cells that die as a result of ischemic or hypoxic challenges; however, it is fascinating to note recent studies demonstrating that adult turtle brains are nonetheless able to activate neurogenesis pathways when needed (Kesaraju and Milton, 2009). Research examining such strategies in anoxia-tolerant turtle brain has consistently revealed a central role for mitochondria in 1) the activation of neuroprotective cellular responses, 2) the prevention of deleterious reactive oxygen species generation and cellular Ca²⁺ overload, and 3) the activation of neurogenesis. These results agree well with the handful of other studies that have examined protective mechanisms related to anoxia-tolerance in mitochondria isolated from non-brain tissues in anoxia-tolerant species. This agreement is exciting and suggests that there may be a common suite of adaptations that have evolved within the mitochondria of multiple species to protect cells against low oxygen stress; however, many more studies are required, particularly of brain mitochondria across species, to test this hypothesis. Such studies will not only shed light on the regulation of mitochondrial function in isolation, which is an important step in understanding how the brains of hypoxia-tolerant species tolerate low oxygen challenges, but also provide great potential to inform the development of novel therapeutics against hypoxia-related pathologies in mammal brain.

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Matthew E. Pamenter*

Department of Biology, University of Ottawa, Ottawa, ON, Canada

*Correspondence to: Matthew E. Pamenter, Ph.D., mpamenter@uottawa.ca.

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