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Adaptive responses of vertebrate neurons to anoxia—Matching supply to demand☆

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Abstract

Oxygen depleted environments are relatively common on earth and represent both a challenge and an opportunity to organisms that survive there. A commonly observed survival strategy to this kind of stress is a lowering of metabolic rate or metabolic depression. Whether metabolic rate is at a normal or a depressed level the supply of ATP (glycolysis and oxidative phosphorylation) must match the cellular demand for ATP (protein synthesis and ion pumping), a condition that must of course be met for long-term survival in hypoxic and anoxic environments. Underlying a decrease in metabolic rate is a corresponding decrease in both ATP supply and ATP demand pathways setting a new lower level for ATP turnover. Both sides of this equation can be actively regulated by second messenger pathways but it is less clear if they are regulated differentially or even sequentially with the onset of anoxia. The vertebrate brain is extremely sensitive to low oxygen levels yet some species can survive in oxygen depleted environments for extended periods and offer a working model of brain survival without oxygen. Hypoxia tolerant vertebrate brain will be the primary focus of this review; however, we will draw upon research involving hypoxia/ischemia tolerance mechanisms in liver and heart to offer clues to how brain can tolerate anoxia. The issue of regulating ATP supply or demand pathways will also be addressed with a focus on ion channel arrest being a significant mechanism to reduce ATP demand and therefore metabolic rate. Furthermore, mitochondria are ideally situated to serve as cellular oxygen sensors and mediator of protective mechanisms such as ion channel arrest. Therefore, we will also describe a mitochondria based mechanism of ion channel arrest involving ATP-sensitive mitochondrial K^+ channels, cytosolic calcium and reaction oxygen species concentrations.

Keywords: Hypoxia tolerance; Ion channel arrest; Neuron; Mitochondria; Calcium; ATP

1. Introduction

Vertebrate respiratory structures such as gills and lungs function as sites of gas exchange, providing an entry point for oxygen required by cellular respiration and an exit point for carbon dioxide produced by cellular respiration. Cellular respiration produces almost

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all of the adenosine triphosphate (ATP) required by a cell to carry out its various functions. ATP is the energy currency of a cell; it is produced in one location and shuttled to more distal sites where it is utilized for amongst other things ion pumping, muscle contraction, and protein synthesis. Atmospheric oxygen concentration is about 21%, and for both terrestrial and aquatic organisms (air saturated water) cellular respiration and ATP supply and demand are balanced, tightly coupled and not compromised. However, ambient oxygen levels can vary dramatically and oxygen limited environments are not uncommon on earth. For example, tide pools have a wide diurnal variation in oxygen availability, ranging from hyperoxia during daylight hours to severely hypoxic at night. Flood plains, such as the Amazon River basin, experience dramatic seasonal variations; high water and organic matter combine to greatly reduce dissolved oxygen levels. A similar phenomenon occurs in coastal marine waters and in open-ocean. Perhaps familiar to some is the severely hypoxic water created by winter ice in northern freshwater lakes. Low temperatures, reduced light penetration and impaired atmospheric gas exchange caused by ice cover kill off aquatic plant life while animal life consumes the remaining O_2. During periods of environmental hypoxia or anoxia gas exchange by gills and lungs becomes is greatly reduced and cellular respiration can become severely compromised, resulting in an uncoupling of ATP supply from demand. It is the matching of ATP supply to demand that must be achieved to survive long-term anoxic conditions. To match supply with demand an organism has two options, increase supply using anaerobic pathways or reduce demand (Hochachka, 1986). Increasing ATP supply is wasteful, since it results in the rapid depletion of glycogen reserves and only serves to shorten survival time, reducing demand is the only viable long-term strategy for a vertebrate to survive without oxygen. A reduction in demand inevitably leads to an overall reduction in ATP turnover and thus metabolic rate or as termed by Hochachka (1986)—metabolic arrest. The most significant ATP "demanding" process in most cells and particularly neurons is ion pumping to maintain or re-establish transmembrane ion gradients. Therefore, in this review we will investigate the adaptive responses of vertebrate neurons to anoxia and make a case for ion channel arrest as a fundamental adaptation for vertebrate long-term anoxic survival.

Neurons are generally regarded as the most anoxia-sensitive cells and are therefore the natural place to look for cellular adaptations permitting anoxic or hypoxic-survival in animals inhabiting regions of the earth described above. Indeed, hypoxia-tolerant neurons are found in every order of vertebrates but most of the research has focused on just a few species within these orders (reptiles, fishes and amphibians). Probably the best characterized anoxia-tolerant neurons are those from the cerebrocortex of the freshwater turtle genus *Chrysemys* (painted turtles). The western painted turtle *Chrysemys picta* survives 5 months of anoxia at 1–3 °C during winter dormancy and at warmer temperature tolerates anoxia 100–1000 times longer than mammals (Jackson, 2002). Interestingly, many turtle species, notably the southern soft-shelled turtle, are not particularly anoxia-tolerant (Crocker et al., 1999); strongly indicating that the underlying mechanisms of anoxia-tolerance are a suite of unique adaptations.

Neurons from turtles represent an extreme; these cells virtually switch off and the turtle brain becomes just about isoelectric throughout the anoxic winter dormancy. Whereas anoxia-tolerant fishes such as the crucian carp and the goldfish differ with turtles in that they and their neurons remain active during anoxic periods (Nilsson, 2001). Other fishes, such as aestivating African lungfish, enter dormant, suspended animation states during dry periods, which are possibly associated with at least some degree of hypoxia. Adult amphibians may experience low ambient oxygen during retreats to burrows and cocoons during dry seasons (e.g. the Australian frog *Cyclorana platycephala* or the American spadefoot toad *Scaphiopus couchii*), although the degree of hypoxia they encounter is probably not extreme. Spadefoot toads decrease metabolism substantially during dormancy and some information is available on the regulation of their hypometabolic condition (Storey, 2002). Species that spend the winter dormant in hypoxic water have also been of interest. The protective effect of metabolic rate depression during cold hypoxic submergence has been demonstrated in adult hibernating *Rana temporaria* (Donohoe et al., 2000). *Rana* brain maintains ATP levels for 2 days at 3 °C in anoxia before declining (Donohoe and Boutilier, unpublished data). Throughout all stages of prolonged hypoxia (water P_{O_2} 30–60 mmHg), for up to 16 weeks at 3 °C, frog brain ATP levels were maintained, although briefer anoxia at 25 °C is associated

with ATP loss (Knickerbocker and Lutz, 2001). The ability to depress metabolic rate such that ATP demands can be met by oxidative phosphorylation in an oxygen-limited environment is probably the key to the frogs' overwintering survival.

Numerous mammals are hypoxia tolerant but the naked Kenyan mole rat *Hetercephalus glaber*, which lives in a burrow environment of approximately 8% oxygen, is the reigning mammalian champion. Very little is known about the hypoxia or anoxia tolerance of these mammals although there cannot be any doubt that mole rat neurons are hypoxia tolerant. Cortical and hippocampal neurons from oxygen-sensitive species such as *Rattus norvegicus* are hypoxia-tolerant during the embryonic and neonatal periods (Bickler and Hansen, 1998; Haddad and Jiang, 1993). This is not surprising because oxygen tension in fetal brain is less than half the normal value for adults (Parer, 1993). In some species the birth process or the nesting environment may be associated with hypoxia. Mammalian hibernation is associated with tolerance of hypoxia or ischemia, although hibernation is neither ischemic nor hypoxic, since blood flow and metabolism are decreased in parallel and lactic acid does not accumulate (Hochachka and Guppy, 1987). Neurons from hibernating ground squirrels tolerate hypoxia-glucose deprivation even when studied at 37 °C, suggesting that hypothermia is but one factor in the tolerance of this tissue (Frerichs, 1999).

Little is known mechanistically concerning the adaptation of marine mammals to the brain hypoxia that accompanies deeper, long-duration dives, even though their tolerance is well defined (Lutz and Nilsson, 1997). Hypometabolism occurs during diving in seals (Hurley and Costa, 2001) and it would be fascinating to determine if portions of the brain participate in energy savings by undergoing a reduction in activity during long dives.

2. Is metabolic depression regulated by ATP supply or demand?

The phenomenon of metabolic arrest or depression is extremely well documented and examples have been found in virtually all major animal phyla. A very thorough accounting of the magnitude of the metabolic depression observed in all these species can be found in articles by Guppy and Withers (1999) and Storey and Storey (1990). Perhaps surprising is the magnitude of the metabolic depression observed in small mammals and birds during hibernation and torpor which can be as extreme as 1% of resting metabolic rate in a mouse (Storey and Storey, 1990). These periods are commonly accompanied by a decrease in body temperature and are likely a strategy to conserve endogenous fuel reserves in such a small animal during its subjective night. Hypothermia raises its own host of challenges for endothermic animals, chief of which is cell swelling. Body temperature reductions differentially impact the activities of ion channels and pumps, such as the Na^+/K^+ pump. Ion channels are relatively insensitive to temperature changes and exhibit relatively small Q_{10} effects of about 2, while ion motive pumps such as the Na^+/K^+ pump can experience much higher Q_{10} effects. The disparity between ion pumping and ion channel leak leads to a redistribution of ions, cell swelling and depolarization (for review see Boutilier, 2001). However, these problems are avoided by mammals and birds that seasonally hibernate or undergo diurnal torpor and there are likely interesting neuronal adaptations to be discovered in extremists within this group, such as the arctic ground squirrel (Buck and Barnes, 2000).

Although there is little doubt that metabolic arrest occurs in nature the mechanism by which it does so remains uncertain. Furthermore, there is some discussion as to whether metabolic arrest is initiated by regulation of pathways on the ATP supply (glycolysis, oxidative phosphorylation) or on the ATP demand (protein synthesis, ion pumping) side of the equation (Fig. 1). Although metabolic arrest refers to lowered energy or ATP turnover in the hypometabolic state it is also loosely used to refer to the ATP supply pathways of glycolysis and oxidative phosphorylation as indicated in Fig. 1. Ion pumping by Na^+/K^+ ATPase and protein synthesis utilize a similar 20–30% fraction of the total ATP coupled oxygen consumption. The remainder is associated with mitochondrial proton leak (20%), Ca^{2+} ATPase (8%), actinomyosin (10%), gluconeogenesis (10%) and ureagenesis (3%; Rolf and Brown, 1997). Protein synthesis is a large ATP consumer but is rapidly reduced in hypoxic or anoxic tissues of the turtle and goldfish but not in hypoxia intolerant species (Hochachka et al., 1996; Krumschnabel et al., 2000). A sensitive detector of cellular energy status and regulator of protein synthesis—AMP kinase (AMPK) may

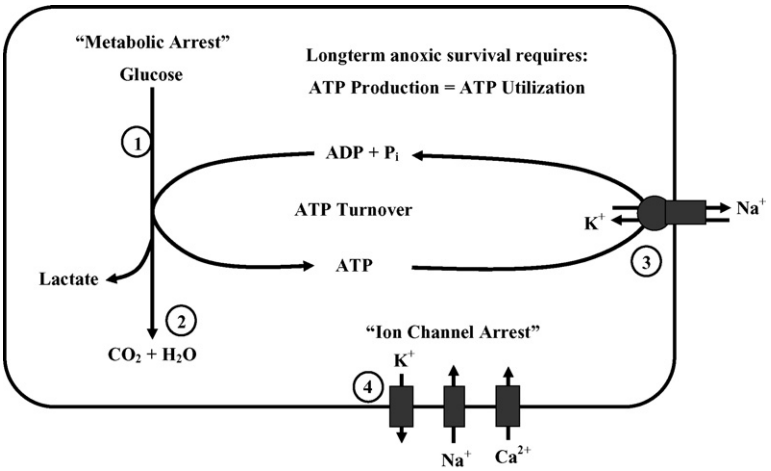

Fig. 1. An illustration showing the major components of metabolic and ion channel arrest. There are many examples of metabolic arrest in nature but how is ATP demand decreased in a coordinated fashion with ATP supply via glycolysis (1) and oxidative phosphorylation (2)? Maintenance of ion gradients by ion motive ATPases (3) is the most energy expensive cellular process. Therefore, a reduction in plasma membrane permeability to ions would result in a large ATP savings (4).

regulate the hypoxia mediated decreases in protein synthesis (Lindsey and Rutter, 2004). AMPK is activated by increases in cellular [AMP] and inhibited by high [ATP] and is regulated by differential phosphorylation. Although cellular adenylate levels do not change appreciably in anoxia-tolerant organisms, regional heterogeneity may occur and the key may again lie in phosphorylation of the enzyme. Regardless this enzyme has not been investigated in anoxia-tolerant species.

2.1. Regulation by ATP supply

Studies of the kinetic properties of the important glycolytic control enzymes phosphofructokinase and pyruvate kinase consistently show phosphorylation and decreased activities of this pair during hibernation, estivation, and overwintering in squirrel, snail and turtle tissues (Storey, 1998). There are also coordinate changes in the enzymes likely responsible for phosphorylation—protein kinase C and A (Storey, 1998). In fact, the activity of PKA and PKC and the concentration of cAMP increases within the first hour of anoxia in turtle liver and decreases thereafter, pointing to a glycolytic regulatory role during the transition to anoxia. Furthermore, protein phosphatase 1 activity decreases, consistent with a general increase in cellular protein phosphorylation levels.

The concept of regulation from the supply side garners some support from experiments on mitochondrial proton leak. Proton leak is estimated to account for 15–30% of standard metabolic rate in both endo and ectothermic species and could therefore result in significant energy inefficiencies during hypometabolic periods (Hulbert et al., 2002; Bishop et al., 2002). The hypothesis that proton leak is actively regulated in estivating and hibernating species in a channel arrest fashion and leads to increased energy efficiency proved to be incorrect. In these experiments proton leak was not actively regulated, however it did decrease passively as a result of a decrease in mitochondrial membrane potential (Bishop et al., 2002). By dividing energy metabolism into three components: (1) substrate oxidation (glycolysis, Kreb's cycle, and electron transport), (2) proton leak (across the mitochondrial inner membrane), and ATP turnover (ATP synthase and ATP consumers such as ion pumps) the contribution of each component to metabolic rate was determined in control and aestivating snail populations. Using hepatopancreas cells from these populations and the mitochondrial ATP synthase inhibitor ouabain to block proton movement into the matrix, proton leak was estimated. The response of the substrate oxidation component was also tested by titration with FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), a pro-

ton ionophore that dissipates the proton gradient. This methodology demonstrated that mitochondria from estivating snails had a reduced ability to increase respiration in response to the same concentration of FCCP as compared to control animals. There were less dramatic changes when comparisons were made between ATP consuming reactions (or ATP turnover) in aestivation and control animals. It was concluded that about 75% of the total response of mitochondrial respiration to aestivation was caused by changes in substrate oxidation and only 25% attributed to ATP turnover of the ATP demand side of the equation (Bishop et al., 2002).

Similar conclusions have been drawn from proton leak experiments on hibernating arctic ground squirrels and frogs (Barger et al., 2003; Boutilier and St. Pierre, 2002). One of the difficulties in interpreting these data is that the respiratory data are normalized to milligram cell protein or cell number. Both of these measurements can overlook decreases in the actual concentrations of the enzymes of substrate oxidation as has been pointed out in studies investigating muscle mitochondrial enzyme content and fiber-type differences (Leary et al., 2003; Dalziel et al., 2005). Furthermore, the proton leak studies are conducted on animals following several weeks of hibernation or aestivation and likely represent a period when metabolism has reached a new hypometabolic steady state, making it difficult to draw mechanistic inferences. Indeed, hepatocytes and hepatopancreas cells retain their lower metabolic rates even after being isolated from hypometabolic animals, making them excellent model systems but the lower rates are likely due to lower titers of oxidative enzymes. For example, levels of citrate synthase and cytochrome oxidase decrease by about 20 and 40%, respectively, in aestivating snails compared to control snails (Boutilier and St. Pierre, 2002). Furthermore, the situation is not as simple as it appears; the response is tissue and species specific. Aestivating in vitro frog skeletal muscle exhibits lowered respiration rates but intestine, liver, skin and fat do not, while arctic ground squirrel hepatocytes and snail hepatopancreas cells do show a respiratory depression (Boutilier and St. Pierre, 2002). We argue that reduced substrate oxidation rates in cells from hibernating and aestivating animals is the result of metabolic arrest and not the cause. To determine what the ultimate mechanisms and regulators of metabolic depression are, studies performed during the transition to a depressed metabolic state are required. A reduction in ATP demand, either ahead of or in concert with reductions in ATP supply, still remains the most logical order of events for entry into a hypometabolic state. As we will outline next, ion channel arrest would seem to be the most important first step to reducing ATP demand.

2.2. *Regulation by ATP demand: neuronal ion channel arrest and adenosine*

Regulating the demand for ATP during the transition to a hypometabolic state would seem to be the logical first step and is supported by studies showing reduced protein synthesis, Na^+/K^+ ATPase and NMDA receptor activity with the onset of anoxia (Buck, 2004). The ion channel arrest hypothesis, as proposed by Hochachka (1986), is likely a key part of the underlying mechanism to reduce ATP demand. The Channel Arrest hypothesis makes two predictions: (1) that anoxia-tolerant organisms have an inherently low permeability plasma membrane (either low channel densities or low channel activities) and (2) that they sustain a further suppression of membrane permeability when exposed to low oxygen conditions (further "channel arrest", by either suppression of channel densities or channel activities). The first prediction appears to be true. Comparisons of membrane permeability to Na^+ and K^+ in a mammal and reptile of similar size and body temperature indicate that reptile membranes are about five-fold less "leaky" than mammalian membranes (Else and Hulbert, 1987). The measurement of channel arrest has proven to be more difficult than that of metabolic arrest but there are data supporting the hypothesis. The oxygen sensing mechanisms and second messenger pathways that must be part of such a cell-based mechanism are even more elusive and have been recently reviewed (Bickler and Donohoe, 2002).

Indirect measures of ion channel arrest include the maintenance of membrane potential while Na^+/K^+ ATPase activity decreases by 75% in anoxic turtle hepatocytes (Buck and Hochachka, 1993); an anoxia mediated 42% decrease in voltage gated Na^+ channel density in turtle cerebellum (Perez-Pinzon et al., 1992); and a hypoxia mediated 50% decrease in Na^+/K^+ ATPase activity coupled with decreased Na^+ permeability and maintenance of membrane potential in frog skeletal

muscle (Donohoe et al., 2000). The only direct measures of ion channel arrest come from our lab and focus on anoxia mediated reductions in NMDA receptor open probability and whole-cell currents in turtle neurons (Bickler and Buck, 2002; Shin and Buck, 2003) and more recently goldfish (Buck et al., unpublished observations). In turtle brain cerebral cortical sheets a 62% decrease in NMDA receptor single channel open time (P_{open}) occurs within 15 min of the onset of anoxia (Bickler et al., 2000; Buck and Bickler, 1995, 1998). This decrease could be reproduced by adenosine or during normoxia with the A_1 receptor agonist cyclopentyladenosine (CPA). Thus far the ability to acutely regulate NMDA receptor activity in response to anoxia has only been described in the freshwater turtle (*C. picta*) but may generally be a neuronal adaptation of anoxia-tolerant species.

The NMDA receptor is a high-flux ligand-gated cation channel that is highly permeable to Ca^{2+} and is required for fast excitatory neuro-transmission in the CNS. It has been identified as the major site of a pathophysiological lesion leading to irreversible anoxic injury in the mammalian central nervous system. That is, the excessive rise in intracellular Ca^{2+} characteristic of lethal cell injury has been shown to result from hyperstimulation of NMDA receptors following excessive presynaptic glutamate release during ischemic or anoxic periods (for review see Buck, 2004). The intracellular domain of the NMDA receptor is known to contain multiple phosphorylation sites. In general phosphorylation of NMDA receptors tends to increase Ca^{2+} currents and dephosphorylation decreases Ca^{2+} currents. Using both single channel patch-clamp and whole cell patch-clamp recording methods the channel has been shown to be phosphorylated by a number of protein kinases, such as: protein kinase A, protein kinase C, CaM kinase II, and tyrosine kinase. The channel has also been shown to be dephosphorylated by the associated protein phosphatases, Ca^{2+}/calmodulin-dependent protein phosphatase 2B, protein-tyrosine phosphatase and protein phosphatase 1 and 2A (see Buck, 2004; Shin et al., 2005).

The role that receptor phosphorylation status plays in the transition to anoxia in an anoxia-tolerant organism is unknown but it is reasonable to assume it does play a role. It is also reasonable to speculate that adenosine may be an important signaling molecule in brain that influences receptor phosphorylation status, since both A_1R and A_2R have been shown to have a depressant effect on neural activity (Lin and Phillis, 1991). A notable exception is in rat carotid body type II cells where adenosine, acting via A_{2A} receptors (A_2R subtype), appears to inhibit outwards currents, thereby depolarizing cells and increasing excitability (Vandier et al., 1999). Additionally, adenosine has been shown to have a tonic regulatory role in the normoxic functioning of neurons because uptake pathway inhibitors and adenosine deaminase inhibitors sharply increase its concentration in microdialysis perfusates. Moreover, [adenosine] increases in response to NMDA application in rat cerebrocortex, and can be blocked with tetrodotoxin, pointing to an activity-dependent component of adenosine release (Buck, 2004). Indeed, adenosine is well established as a potent pre- and postsynaptic neuromodulator and is likely to be one of the key metabolites involved in any neuronal anoxia-tolerance mechanism. The NMDA receptor is an important target for an effective anoxia-tolerance strategy in the turtle brain since activating the receptor in anoxic brain sheets results in a high degree of cell death, similar to that observed in anoxic mammalian brain (Bickler et al., 2000). Anoxic regulation of this receptor-ion channel is likely even more critical because ionized calcium levels in cerebral spinal fluid increase by about 6.5-fold during prolonged anoxia (Jackson, 2002).

3. Neuronal whole-cell conductance and background K^+ channels

Not only has a decrease in NMDA receptor single channel P_{open} and whole-cell currents been demonstrated to occur in anoxic turtle neurons but also a decrease in whole cell conductance (G_w). Using patch electrodes G_w was shown to decrease 27% within 20 min of the onset of anoxic perfusion (from 3.4 ± 0.3 to 2.5 ± 0.3 nS (Ghai and Buck, 1999)). Normoxic adenosine application resulted in a 32% reduction in G_w and the A_1 receptor agonist cyclopentyladenosine (CPA) could reproduce this effect. The potent A_1 receptor blocker 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) could prevent both the anoxia and adenosine mediated decrease in G_w. Moreover, the potent A_1 receptor antagonist DPCPX blocked the adenosine mediated decrease in G_w and the A_1 specific recep-

tor agonist CPA decreased G_w in a dose dependent manner, indicating that the effect likely involves a receptor based mechanism. The antagonist also prevented the anoxia induced reductions in G_w suggesting that endogenous adenosine released from cortical sheets during anoxia results in the observed decrease in G_w.

The concentrations of Ca^{2+} and Mg^{2+} increase and pH decreases in turtle blood throughout an anoxic dive (Jackson, 2002); the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) also increases in anoxic brain tissue (Bickler, 1998). When normoxic and anoxic perfusions were performed with solutions that mimic the ionic composition of turtle blood following a 1 month dive, G_w decreased significantly, and increased $[Ca^{2+}]_e$ was determined to be the cause of this decrease (Ghai and Buck, 1999). Taken together with the failure of adenosine perfusion to elicit a reduction in G_w in the presence of EGTA these data indicate that adenosine affects G_w through a change in $[Ca^{2+}]_i$. The A_1 receptor is a G-protein linked receptor able to modulate several intracellular cascades including phospholipase C (PLC)/inositol triphosphate (IP_3) and adenylate cyclase pathways (for review see Buck, 2004). Therefore, it is more probable that intracellular Ca^{2+} levels increase from A_1 receptor activation via PLC and IP_3 mediated Ca^{2+} release. The anoxia-induced increase in intracellular calcium may function as a molecular switch as described for the neuronal Ca^{2+} binding protein calmodulin, which regulates the action of various protein kinases and phosphatases. These in turn alter the phosphorylation state and thus permeability of ion channels and receptors resulting in an altered G_w. How the intracellular second messenger traffic is regulated in a way that selectively regulates the activity of the appropriate protein kinases and phosphatases is uncertain. The simultaneous measurement of $[Ca^{2+}]_i$, G_w and NMDA receptor activity during an anoxic transition would be very informative, especially as influenced by adenosine and second messenger pathway modulators.

The anoxia-induced reduction in G_w may involve the regulation of non-selective, voltage-independent two pore K^+ channels, or "leakage K^+ channels" that are known to be regulated by differential phosphorylation and second messenger pathways (Talley et al., 2003). These channels are thought to contribute to basal leakage currents and may be involved in establishing resting neuronal membrane potential. Several types of leakage K^+ channels have been identified in mammalian CNS, including the two pore domain weak inward rectifier K^+ channel (TWIK-1, Lesage et al., 1997) and the outward rectifier- TREK-1 (Fink et al., 1996). Also, an acid sensitive two pore domain K^+ channel (TASK) has been demonstrated to be noninactivating at all membrane potentials tested, a manner which is characteristic of a background or leakage channel (Leonoudakis et al., 1998). Buckler (1997) has shown that anoxia induces a significant reduction in the resting membrane conductance of rat carotid body type I cells through the inhibition of a background K^+ conductance. This phenomenon has recently been extended to the central nervous system. Inhibition of TASK channels with low pH or anandamide prevents hypoxic depolarization of cultured rat cerebellar granule neurons, indicating that TASK channel underlie this response (Plant et al., 2002). Thus, it is possible that during the transition to anoxia K^+ leakage channel permeability is decreased by an adenosine mediated second messenger pathway resulting in decreased G_w. The ability to acutely regulate background K^+ channel conductance would significantly reduce the energetic cost of ion pumping and be a major neuronal adaptation to anoxic survival.

There are other findings that are consistent with changes in K^+ leakage channel permeability. The maintenance of transcellular K^+ gradients in turtle brain during anoxia is characteristic of anoxia-tolerance in this organism. When Na^+/K^+ ATPase is inhibited with ouabain during anoxia, effectively blocking the K^+ re-uptake pathway, an estimate of background K^+ leakage can be made. Under these conditions the extracellular leakage of K^+ in ouabain inhibited anoxic turtle brain occurred at 70% the rate of normoxic brain, indicating a reduced K^+ conductance (Pek and Lutz, 1997). Superfusing the brain with the adenosine receptor blockers theophylline or 8-cyclopentylthephylline (a more specific A_1 receptor blocker) significantly reduced the time to anoxic depolarization, suggesting that the reduced K^+ conductance is regulated by an adenosine receptor based second messenger cascade. Furthermore, the change in K^+ conductance is not mediated by changes in plasmalemma K_{ATP} channel permeability since inhibitors of this channel did not significantly reduce K^+ efflux following 2 h of anoxia (Pek-Scott and Lutz, 1998).

4. ATP-sensitive potassium channels

The neuroprotective effects of adenosine have been linked to K_{ATP} channels in the maintenance of extracellular glutamate in the anoxic turtle brain. During anoxia glutamate release decreases by 44% and this effect is abolished by the coordinated blockade of both adenosine receptors and K_{ATP} channels but not when blockers are applied individually (Milton et al., 2002). Similarly, Milton and Lutz (2005) found that both adenosine receptors and K_{ATP} channels regulate the maintenance of extracellular dopamine at low levels during early anoxia in the turtle brain: although no attempt was made to differentiate between the respective roles of the plasma membrane and mitochondrial K_{ATP} channel sub-populations in these studies. In mammalian ischemic preconditioning models, adenosine and mitochondrial K_{ATP} channels are involved coordinately in neuroprotection (Heurteaux et al., 1995; Yoshida et al., 2004). These studies support the hypothesis that adenosine mediation of NMDARs and mK_{ATP} mediation of ischemic preconditioning may be related mechanisms. More recently we have focused on measuring whole-cell NMDAR currents during normoxic to anoxic transitions since these measurements represent the average response of many NMDARs, not just the extra synaptic receptors studied using single-channel methods (Shin and Buck, 2003; Shin et al., 2005). Our results show that adenosine, via the A_1 receptor, attenuates NMDAR activity during normoxia in a manner similar to that seen during anoxia, and that it is reproducible with specific mitochondrial K_{ATP} channel activation. However, during anoxia adenosine did not affect NMDAR activity since A_1 receptor blockade failed to abolish the anoxia-mediated decrease in NMDAR. In contrast, specific mK_{ATP} channel inhibition prevented the anoxia-mediated decrease in NMDAR activity and blockade of mitochondrial K_{ATP} channels also abolished the normoxic effects of adenosine and A_1 receptor regulation of NMDAR currents (Pamenter and Buck, unpublished results). These results indicate that mitochondrial K_{ATP} channels play an important role in the anoxic regulation of NMDARs.

4.1. A mitochondrial link to ion channel arrest

Mitochondria play a pivotal role in cellular energy homeostasis and in the processes of apoptosis and necrosis and have been recently linked to ischemic preconditioning in mammal heart and brain. Mitochondria are also known to be a source of reactive oxygen species but there is no information on the role mitochondria play in channel arrest. Preconditioning is a well described phenomenon in which a brief sub-lethal hypoxic/ischemic bout can confer protection to a subsequent more severe and previously injurious hypoxic/ischemic bout. In the following section we will outline a putative mechanism by which mitochondria confer hypoxic protection through mitochondrial K_{ATP} channels and how this may apply to ion channel and metabolic arrest in anoxia-tolerant species.

The mitochondrial electrochemical gradient is the result of two forms of ionic interaction. The first is the concentration gradient that results from the active pumping of protons across the inner mitochondrial membrane. The second is a voltage gradient across the inner membrane, with the matrix being −180 mV with regard to the cytosol. The membrane is a barrier to protons, and thus the gradient is maintained. This allows the energy of protons traveling back along their concentration gradient to be harnessed to move ions across the membrane against their own concentration gradients. The mitochondrial membrane potential is generated primarily by the pumping of K^+ ions out of the matrix via an H^+/K^+ cotransporter, which is powered by the proton concentration gradient. The combination of the concentration and electrical gradients results in a proton-motive force, which energizes the phosphorylation of ADP to ATP via the ATP synthetase (Fig. 2).

Increases in the conductance of the mitochondrial inner membrane can result in a short-circuiting of these systems. The opening of ion channels that are conductive to K^+ or other ions results in an increased workload on the H^+-powered ion exchangers to return the ionic equilibrium to resting levels. The reduced proton gradient can result in increased oxidative rates (uncoupling) and may serve to reduce reactive oxygen species (ROS) production, alter Ca^{2+} homeostasis and be a protective mechanism against pathological insults as shown in Fig. 2. There is evidence that mitochondrial ion channels are also involved in ischemic tolerance, two of which are the ATP-sensitive and calcium-sensitive potassium channels (mK_{ATP} and K_{Ca}). Opening of either of these channels allows K^+ ions to flow into the mitochondria. This influx is partially countered by increasing the rate of the K^+/H^+ exchanger, which

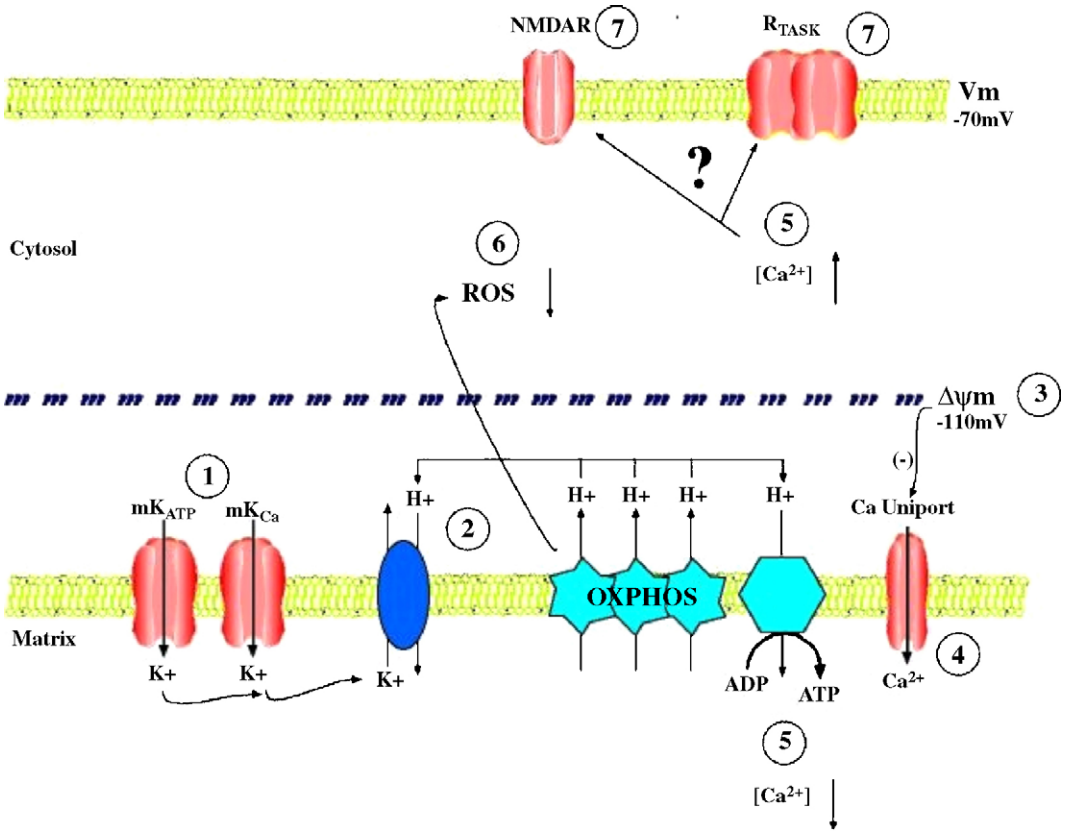

Fig. 2. Schematic showing a mitochondria-based mechanism of ion channel arrest. Opening of mitochondria K^+ channels caused by lowered matrix [ATP]: (1) causes an increased K^+ flux into the mitochondria. Elevated $[K^+]_m$ increases K^+/H^+ exchanger activity, resulting in uncoupled respiration (2) and in mitochondrial membrane potential (3). This depolarization reduces the activity of the Ca^{2+}-uniporter (4), attenuating mitochondrial Ca^{2+} uptake and subsequently elevating cytosolic $[Ca^{2+}]$ (5). Uncoupling of respiration also results in a decrease in mitochondrial ROS generation (6). Changes in $[Ca^{2+}]_c$ and [ROS] alter ion channel function (7).

pumps K^+ ions out of the matrix at the expense of the H^+ gradient. The loss of available H^+ ions to counteract the flux of K^+ ions results in a partial uncoupling of the proton gradient and a decrease in mitochondrial membrane potential ($\Delta\Psi_m$).

4.2. Mitochondrial ATP-sensitive potassium channels

Mitochondrial ATP-sensitive potassium (mK_{ATP}) channels are located on the mitochondrial inner membrane (Innoue et al., 1991). Their specific structure is unknown, but thought to be structurally similar to plasmalemmal K_{ATP} channels, which are composed of four pore-forming inward-rectifying K^+ channel subunits (K_{IR}6.1, 6.2) and four modulatory sulfonylurea receptors (SUR-1, 2). Physiologically these channels are agonized by GTP and GDP and are inhibited by ATP, ADP and long chain coenzyme A (CoA) esters. Additionally, several cellular messengers have been shown to modulate this channel, including protein kinase C (PKC), adenosine (ADO), as well as superoxide anions (Busija et al., 2004).

mK_{ATP} channels have been implicated as an integral component of mammalian ischemic preconditioning (IPC), and there are reports supporting the opening of these channels as a primary mediator of IPC protection in a variety of organisms and tissues (for a review of the role of mK_{ATP} channels in IPC see Oldenburg et al., 2003).

Heurteaux et al. (1995) showed a link between adenosine A_1 receptors and ATP-sensitive K^+ channels (K_{ATP}) in ischemic preconditioning in rat hippocampal neurons. Blockade of either A_1 receptors or K_{ATP} channels abolished preconditioning-mediated neuroprotection. Conversely, activation of adenosine receptors and K_{ATP} channels induced neuroprotection. Blockade of K_{ATP} channels also abolished the protective effects of adenosine. More recently a critical role for the mitochondrial population of K_{ATP} channels (mK_{ATP}) in ischemic preconditioning has been shown in rat cortical neurons (Kis et al., 2004; Shimizu et al., 2002).

Attenuation of NMDAR activity in the anoxic turtle brain may involve mK_{ATP} channels in a fashion similar to a mechanism proposed by Holmuhamedov et al. (1998). They reported that mK_{ATP} channel activation in heart caused K^+ influx into the mitochondria resulting in depolarization of the mitochondrial membrane. As a consequence, Ca^{2+} was released from the mitochondria, elevating $[Ca^{2+}]_i$. In turtle cerebrocortical neurons, $[Ca^{2+}]_i$ increased by 35% and inactivation of anoxic NMDAR receptors scaled in a dose dependent manner with changes in $[Ca^{2+}]_i$ (Bickler et al., 2000). The elevation of $[Ca^{2+}]_i$ may originate from mK_{ATP} channel-induced release of Ca^{2+} from the mitochondria. Studies on turtle cortical neuron NMDAR regulation in our lab have shown that activation of mK_{ATP} channels reduces NMDAR whole-cell currents by about 40%. Additionally, the anoxic reduction in NMDAR currents is abolished by the perfusion of either the mK_{ATP} specific blocker 5HD or the general K_{ATP} channel blocker glibenclamide (Pamenter and Buck, unpublished observations). It also appears that intracellular Ca^{2+} mediates mK_{ATP} attenuation of anoxic NMDAR activity since inclusion of the Ca^{2+}-chelator BAPTA in the recording electrode abolishes both the anoxia and mK_{ATP} channel induced reductions in NMDAR currents.

4.3. Mitochondrial calcium-sensitive potassium channels

The most recently identified mitochondrial inner membrane channel is the Ca^{2+}-sensitive K^+ channel (mK_{Ca}). This channel was identified first in human glioma cells and is functionally similar to plasmalemmal BK-type channels (Siemen et al., 1999). They are multi-conductance state channels whose P_{open} is both voltage and $[Ca^{2+}]$ dependent. Channel activity increases with a depolarization of the mitochondrial membrane potential and/or by elevations of $[Ca^{2+}]_m$ (Siemen et al., 1999). Like surface membrane BK-type Ca^{2+} channels, this channel is blocked by the Ca^{2+}-channel blocker charybdotoxin (ChTX).

mK_{Ca} channels form part of the background K^+ conductance of the mitochondria at resting $[Ca^{2+}]_c$ in cardiac mitoplasts (~200 nM). This conductance increases with increasing $[Ca^{2+}]_c$ and is abolished by inclusion of ChTX but not by the mK_{ATP} channel blocker 5HD. Additionally the perfusion of the mK_{Ca} channel opener NS1619 increases K^+ uptake two-fold while ChTX abolished K^+ uptake (Xu et al., 2002). Importantly, these authors also found that mK_{Ca} currents in mitoplast-attached patches increased when the $[Ca^{2+}]$ outside the pipette was increased, suggesting that the Ca^{2+} sensor of the channel is located on the matrix side of the mitochondrial membrane. Therefore, channel activity increases as matrix calcium increases in response to elevated cytosolic calcium sequestered in the mitochondria. Such an accumulation of calcium occurs during ischemia.

Xu et al. (2002) found that activation of mK_{Ca} channels in cardiac myocytes with NS1619 conferred cytoprotection during global ischemia and reperfusion experiments that was similar in magnitude to the activation of mK_{ATP} channels or ischemic preconditioning. These results were confirmed by Cao et al. (2005) who found that NS1619 reduced infarct size and lactate dehydrogenase (LDH) release during ischemia in cardiac tissue. The mK_{Ca} blocker paxaline abolished both IPC protection and the specific protection induced by mK_{Ca} activation. mK_{Ca} induced protection was not affected by mK_{ATP} blockers (Xu et al., 2002; Cao et al., 2005). Cao et al. (2005) also demonstrated that cardioprotection due to the activation of mK_{ATP} channels with diazoxide was not impaired by blockade of mK_{Ca} channels. These results suggest that the two channels function independently although their mechanism of action is probably similar. Sato et al. (2005) confirmed this independent activity by showing that diazoxide and NS1619 oxidize flavoproteins additively in guinea pig ventricular myocytes.

Together, these data independently confirm the central role of K^+ influx into the mitochondrial matrix in protection from ischemic injury. The specific mecha-

nism of protection conferred by these channels is not known, however opening of mK_{Ca} channels partially uncouples the mitochondria in a manner similar to mK_{ATP} channel opening.

4.4. Mitochondrial uncoupling and calcium-homeostasis

Mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_m$) are maintained by a balance between mitochondrial Ca^{2+} uptake and Ca^{2+} efflux. Calcium uptake into the mitochondria occurs via the activity of the Ca^{2+}-uniporter and the rapid mode (RaM) Ca^{2+} channel, both of which are powered by $\Delta\Psi_m$ (Sparagna et al., 1995). As the gradient is decreased due to ion channel opening, calcium uptake via these two mechanisms decreases and $[Ca^{2+}]_m$ is altered. The Ca^{2+} efflux systems include a Na^+/Ca^{2+} exchanger and its associated Na^+/H^+ antiporter, as well as a Ca^{2+}/H^+ exchanger. Thus in the mitochondria, both the uptake and release of Ca^{2+} are facilitated by the mitochondrial proton gradient (for a review of mitochondrial calcium cycling see Gunter et al., 2000).

Several studies have indicated that IPC and pharmacological opening of mitochondrial ion channels results in mild uncoupling of the mitochondrial membrane and attenuation of Ca^{2+} accumulation in the mitochondria. Holmuhamedov et al. (1999) found that diazoxide application decreased the rate of Ca^{2+} uptake into isolated rat heart mitochondria. Diazoxide also increased the rate of Ca^{2+} release from isolated mitochondria that had been pre-loaded with Ca^{2+}. Both of these responses to mK_{ATP} opening occurred in a dose dependent manner that was linked to a decrease in $\Delta\Psi_m$. In intact cardiomyocytes, diazoxide application decreased $\Delta\Psi_m$ and reduced $[Ca^{2+}]_m$; 5HD blocked the reduction of both. Preconditioning and mK_{ATP} opening have also been shown to limit $[Ca^{2+}]_m$ accumulation in rabbit heart (Murata et al., 2001). Finally, Wang et al. (2001) found that IPC or diazoxide treatment attenuated ischemia-induced mitochondrial Ca^{2+} accumulation and depolarized the mitochondrial membrane potential. Opening of K_{Ca} channels induces ischemic protection in the same manner. In guinea pig ventricular myocytes, opening of K_{Ca} channels depolarizes the mitochondrial membrane potential and attenuates mitochondrial calcium accumulation during ischemic insult (Sato et al., 2005).

Prevention of mitochondrial Ca^{2+} accumulation may confer protection in a variety of ways. In anoxia-sensitive organisms undergoing periods of ischemia, $[Ca^{2+}]_c$ slowly elevates and $[Ca^{2+}]_m$ increases concomitantly. When $[Ca^{2+}]_m$ becomes overloaded at 1–3 μM $[Ca^{2+}]_c$, permeability transition (PT) pores open and cellular injury results (for a review of the role of the PT pore in apoptosis/necrosis see Crompton, 2000). Uncoupling of the mitochondrial membrane and the subsequent attenuation of mitochondrial Ca^{2+} uptake could have a significant effect on $[Ca^{2+}]_c$.

Opening of mK_{Ca} channels by NS1619 provides protection against ischemia in isolated perfused rat hearts. Opening of the PT pore by atractyloside abolishes the protective effects of both IPC and NS1619, suggesting that cardioprotection induced by mK_{Ca} opening prevents pore formation (Cao et al., 2005). Furthermore, the same authors found that blockade of the pore decreased the infarct size and LDH release following ischemic insult. Korge et al. (2002) showed that activation of mK_{ATP} channels decreased mitochondrial accumulation of Ca^{2+} during ischemia and that this prevented PT pore formation and cytochrome c loss from the mitochondria. In the anoxia-tolerant western painted turtle, $[Ca^{2+}]_c$ increases significantly in the first few hours of anoxia and the protective decrease in NMDAR activity is regulated by changes in $[Ca^{2+}]_c$ (Bickler et al., 2000). Additionally, chelation of $[Ca^{2+}]_c$ by BAPTA blocks the protective effects of diazoxide application on NMDAR activity (Pamenter and Buck, unpublished observations). Taken together, these data suggest that the protective effect of mK_{ATP} opening and potentially mK_{Ca} activation involves downstream changes in $[Ca^{2+}]_c$. Therefore changes in mitochondrial Ca^{2+}-handling and the subsequent change in $[Ca^{2+}]_c$ may act as a signaling mechanism to down-regulate cellular processes during periods of low oxygen.

4.5. Mitochondrial uncoupling and ROS production

Mitochondria are a major source of ROS under normal physiological conditions. The production of ROS is directly linked to the rate of oxidative phosphorylation, which is regulated partially by mitochondrial $[Ca^{2+}]_m$

and partially by $\Delta\Psi_m$. As this potential is reduced due to ischemic opening of ion channels, ROS production is altered. ROS are potent cellular messengers and redox signaling has been implicated in the regulation of many cellular processes. The primary radical produced in the mitochondrion is the superoxide anion (O_2^-). This radical is produced by complexes I and III of the electron transport chain. The specific site of superoxide production within complex I is not known, however superoxide formation here occurs when electron transport is reversed due to a high proton-motive force. Experiments with the complex I inhibitor rotenone abolish the majority of superoxide formation, suggesting that superoxide production is primarily due to reverse electron transport from succinate to NAD^+ and not due to complex III activity (Liu et al., 2002). Similarly, experiments by St. Pierre et al. (2002) implicated complex I as the primary production site of H_2O_2 via reverse electron flow. The same study found that complex III produces H_2O_2 only in the presence of inhibitors and not under normal physiological conditions. This area is controversial and recent evidence points to an important role for complex III in ROS generation. Reducing the expression levels of the Rieske iron–sulfur protein component of complex III employing RNAi (interference) techniques resulted in a corresponding decrease in mitochondrial ROS production (Guzy et al., 2005). These studied also demonstrated that a fully functioning complex III is required for HIF-1α stabilization and therefore oxygen sensing. Not only are electron transport complexes capable of producing ROS but recently the Kreb's cycle enzyme α-ketoglutarate dehydrogenase was shown to be a significant source of H_2O_2 (Starkov et al., 2004).

The rate of the reverse electron flow that is responsible for the generation of free radicals in the mitochondria is regulated by the mitochondrial proton gradient. Therefore, a partial uncoupling of the mitochondrial membrane potential will alter the rate of free radical production. Opening of mitochondrial ion channels such as the mK_{ATP} and mK_{Ca} channels will result in an uncoupling of the mitochondrial proton gradient and subsequently alter the production of ROS. Superoxide anions in large quantities are highly deleterious to the cell, however a resting concentration of these anions are constantly being produced by the mitochondria and alterations in the rate of radical formation may act as a redox signaling mechanism, potentially regulating downstream messengers such as PKC (Oldenburg et al., 2003).

There is considerable evidence that redox signaling plays a role in ischemic tolerance. Vanden Hoek et al. (1998) reported an early increase in ROS production during hypoxic preconditioning. The blockade of mK_{ATP} channels with 5HD abolished both the protective effects of IPC as well as the hypoxia-induced generation of ROS. Similarly, Pain et al. (2000) found that opening mK_{ATP} channels with diazoxide reduced the infarct size in ischemic rabbit hearts and that this protection was abolished by inclusion of free radical scavengers, suggesting diazoxide triggers cardioprotection via the generation of free radicals. In addition it is believed that ROS may form a positive-enforcing feedback loop on mK_{ATP} channels. Mitochondria-derived nitric oxide (NO) can activate mK_{ATP} channels and this messenger has also been implicated in IPC (for a review see Bolli, 2001).

It is possible that an initial decrease in available oxygen such as occurs during ischemia or anoxia may decrease the rate of oxidative phosphorylation, resulting in a change in mitochondrial ROS production. This altered redox signal could then feed-forward to activate mitochondrial ion channels such as the mK_{ATP} channel. Opening of these channels would then partially uncouple the mitochondria, potentially altering cellular Ca^{2+}-dynamics and activating other second messengers to down-regulate energy-expensive ion channels, pumps and cellular processes. Indeed, ROS production during hypoxia has been shown to increase by 45–100% depending on cell type and has been linked to increases in $[Ca^{2+}]_i$ (Waypa et al., 2001, 2002).

5. Summary

Oxygen depleted environments are common on earth and represent both a challenge and an opportunity to organisms that survive within them. The vertebrate brain is the most sensitive tissue to oxygen lack and it is likely to exhibit unique adaptations to enable vertebrate life without oxygen. Depressing metabolism or metabolic arrest is a commonly observed strategy to survive under such conditions. And in a metabolically arrested state the matching of ATP supply and demand is critical. To reduce ATP demand and achieve this match ion channel arrest must also be a critical

neuronal adaptation to hypoxic and anoxic survival. Furthermore, mitochondria play a key role in determining cellular fate following ischemic or anoxic neuronal injury (apoptosis versus necrosis) and are ideally situated to serve as a cellular oxygen sensor and mediator of protective mechanisms such as ion channel arrest.

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