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Neuroprotection: Lessons from hibernators

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Abstract

Mammals that hibernate experience extreme metabolic states and body temperatures as they transition between euthermia, a state resembling typical warm blooded mammals, and prolonged torpor, a state of suspended animation where the brain receives as low as 10% of normal cerebral blood flow. Transitions into and out of torpor are more physiologically challenging than the extreme metabolic suppression and cold body temperatures of torpor *per se*. Mammals that hibernate show unprecedented capacities to tolerate cerebral ischemia, a decrease in blood flow to the brain caused by stroke, cardiac arrest or brain trauma. While cerebral ischemia often leads to death or disability in humans and most other mammals, hibernating mammals suffer no ill effects when blood flow to the brain is dramatically decreased during torpor or experimentally induced during euthermia. These animals, as adults, also display rapid and pronounced synaptic flexibility where synapses retract during torpor and rapidly re-emerge upon arousal. A variety of coordinated adaptations contribute to tolerance of cerebral ischemia in these animals. In this review we discuss adaptations in heterothermic mammals that may suggest novel therapeutic targets and strategies to protect the human brain against cerebral ischemic damage and neurodegenerative disease.

Keywords

Cerebral ischemia; Hibernation; Neurodegeneration; Stroke; Torpor

1. Introduction

Hibernation is identified by a prolonged state of energy conservation, termed torpor, that allows heterothermic mammals to tolerate the limited resource availability encountered in extreme environments (Drew et al., 2007). During the hibernation season, hibernating

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animals experience multiple bouts of torpor that are interrupted by brief periods of euthermia. Torpor can be divided into three phases, onset, maintenance and arousal, which are followed by a period of interbout euthermia. During onset and maintenance of torpor, animals exhibit overall metabolic suppression, decreased body temperature, reduction in heart rate, reduction in cerebral blood flow, decreased oxygen consumption, lowered respiratory rates and suppressed immune responses (Barger et al., 2003; Barnes, 1989; Buck and Barnes, 2000a; Drew et al., 2009; Drew et al., 2004; Drew et al., 2002; Toien et al., 2001). During arousal from torpor, rapid blood reperfusion is accompanied by enormous oxygen consumption and elevation in body temperature. Inflammatory and immune responses resume, and the capacity to scavenge free radicals is increased (Barnes, 1989; Bouma et al., 2011; Prendergast et al., 2002; Toien et al., 2001). Interbout euthermia is characterized by metabolism, blood flow and body temperature typical of a homeothermic mammal of similar size (Drew et al., 2004).

In the hibernating phase, an animal's oxygen demand is decreased to as low as 2% of euthermic oxygen demand and cerebral blood flow is decreased to as low as 10% of the euthermic phase (Drew et al., 2009; Drew et al., 2002). Even when not hibernating, arctic ground squirrels (AGS) (*Urocitellus parryii)* can tolerate at least 10 min of global ischemia without detectable neuronal injury (Figures 1 and 2) (Dave et al., 2009; Dave et al., 2006). Understanding the mechanism by which hibernators tolerate such a drastic reduction in cerebral blood flow and achieve this profound metabolic suppression has the potential to facilitate the design of novel therapies for diseases or conditions where interruptions in blood flow to the brain are inherent. In this review, we discuss the major mechanisms by which cerebral ischemic injury may be avoided during hibernation. We also present the current understanding of how the brain of hibernating species are protected from cerebral ischemia when euthermic.

We also present data that support our hypothesis (Figure 1) that hibernators have evolved physiological adaptations as a result of selective pressures associated with transitions into and out of torpor. These adaptations may have materialized as tolerance to cerebral ischemia during euthermia and an increased capacity for adult synaptic plasticity.

2. Torpor

Decreased body temperature and metabolic suppression are the two main characteristics of torpor (Drew et al., 2007; Drew et al., 2009). In very small hibernators, metabolic suppression may be regulated by temperature-dependent passive processes. In contrast, in relatively large hibernators metabolic suppression clearly precedes the temperature drop, as detected by greatly diminished oxygen consumption, suggesting a large contribution of temperature-independent processes (Drew et al., 2007; Drew et al., 2009; Geiser, 2004; Toien et al., 2011). Understanding the mechanism of metabolic suppression during torpor is important because combining features of metabolic suppression with therapeutic hypothermia may provide better protection against cellular damage following cerebral ischemia and brain trauma in humans.

The physiological mechanisms that initiate torpor in hibernating mammals are poorly understood. These mechanisms are believed to involve interactions between neural systems regulating endogenous timing, metabolism, and homeostasis. In a recent study it was observed that activation of A_1AR within the CNS is necessary and sufficient to induce torpor in arctic ground squirrels in a seasonally dependent manner (Jinka et al., 2011). Delivery of the adenosine A_1 receptor (A_1AR) antagonist cyclopentyltheophylline into the lateral ventricle of AGS reversed spontaneous entrance into torpor (Jinka et al., 2011). Moreover, delivery of the A_1AR agonist N(6)-cyclohexyladenosine (CHA) induced torpor in

all AGS tested during the mid-hibernation season but did not induce torpor in any of the AGSs tested during the summer "off-season". Torpor within the hibernation season was specific to A_1AR activation as the A_3AR agonist failed to induce torpor while the A_{2A}AR antagonist failed to reverse the spontaneous onset of torpor. Still missing, however, is an understanding of how the brain coordinates a decrease in whole animal metabolic rate. We hypothesize that central A_1AR activation suppresses thermogenesis and that the gain in this pathway is increased during the hibernation season to lower the hypothalamic temperature that will stimulate thermogenesis (i.e., decreases the lower critical temperature). It then follows that torpid metabolic rate represents the basal metabolic rate in the torpid state.

The rate of cooling during onset of torpor is slower than during drug-induced hypothermia due to postural adjustments, decreases in respiratory frequency and profound vasoconstriction in the lower extremities (Drew et al., 2012; Milsom et al., 2001). This reduced cooling rate is consistent with decreased thermal conductance, which is reported to be lower for torpid animals than for euthermic animals (Jinka et al., 2011; Snyder and Nestler, 1990). During torpor, when body temperature can fall to as low as 0 °C without stimulating thermogenesis, a new equilibrium between heat loss and production may define the minimal torpid metabolic rate within the thermoneutral zone of a hibernating animal (Buck and Barnes, 2000b). How thermal conductance is regulated or when it is decreased is unclear. Gain in the neural pathways that coordinate heat retention may increase seasonally similar to the gain in the pathways that suppress thermogenesis.

2.1 Hypothermia

Hypothermia is one of the main features of torpor. During torpor the extent of the drop in body temperature varies from species to species. In some extreme hibernators, such as AGS, core body temperature reaches 0 °C in equilibrium with the ambient temperature. Hypothermia is shown to protect brain against ischemic injury in preclinical and clinical studies (Bernard et al., 2002; Hypothermia after Cardiac Arrest Study, 2002; Krieger and Yenari, 2004; Yenari and Hemmen, 2010). Thus, the profound hypothermia observed during torpor likely provides some degree of neuroprotection in hibernating animals. Several mechanisms are involved in hypothermia-induced neuroprotection. Cerebral blood flow is linearly decreased when core temperature decreases from 37 °C to 18 °C. Mild hypothermia prevents cerebral ischemia induced early postreperfusion hyperemia and late postreperfusion hypoperfusion, both of which are believed to contribute to cerebral ischemic damage (Yenari et al., 2008). Hypothermia prevents cerebral ischemia-induced excitotoxicity, a major mechanism of neuronal death following cerebral ischemia. Baker et al. demonstrated that intraischemic hypothermia blunts the extracellular release of glutamate in the core region following permanent focal cerebral ischemia, suggesting the role of hypothermia in reducing exitotoxicity (Baker et al., 1995). Another study observed lower glutamate and dopamine release in striatum following endothelin-1-induced focal cerebral ischemia in hypothermia treated group (Van Hemelrijck et al., 2003). The fact that the effects of hypothermia and N-methyl-D-aspartate (NMDA) antagonist MK-801 do not show additive protective effects against focal cerebral ischemia when used in combination supports the hypothesis that both of them prevent cerebral ischemic damage by reducing excitotoxicity (Frazzini et al., 1994).

In addition, several studies demonstrated that hypothermia suppresses cerebral ischemia-induced oxidative stress in different models of cerebral ischemia (Karibe et al., 1994; Katz et al., 2004). Studies have also demonstrated that hypothermia prevents oxidative stress induced cellular damage such as DNA fragmentation following cerebral ischemia (Ji et al., 2007; Nakamura et al., 1999; Phanithi et al., 2000). Phanithi et al. observed that hypothermia treatment decreases post-ischemic expression of Fas (a type-II transmembrane protein of the tumor necrosis factor family) and caspase-3 (Phanithi et al., 2000).

Hypothermia also decreases the release of mitochondrial cytochrome-c following focal cerebral ischemia (Yenari et al., 2002). Mild hypothermia (33° C) is shown to increase expression of anti-apoptotic protein Bcl-2 following global cerebral ischemia (Zhang et al., 2001). In complementary fashion, hypothermia appears to activate cell survival pathways (Yenari et al., 2008).

Hypothermia also reduces post-ischemic inflammation. Maier et al demonstrated that hypothermia-treated animals exhibit significantly less activity of the inflammatory marker myeloperoxidase in the peri-core region following focal cerebral ischemia when measured at 24 h post-ischemia (Maier et al., 1998). Post-stroke activation of transcription factor Nuclear factor-kappa B (NF-κB) leads to the expression of many inflammatory genes involved in stroke damage (Yenari and Han, 2006). Mild hypothermia treatment is able to suppress post-ischemic activation of NF-κB (Han et al., 2003; Webster et al., 2009). Post-ischemic hypothermia is able to lower interleukin-18 mRNA and protein level as well as decrease microglia activation in the developing brain (Fukui et al., 2006). This accumulation of evidence suggests that hypothermia may play a role in neuroprotection during torpor.

2.2 Excitotoxicity

The brain consumes an excessive amount of oxygen for its mass because large amounts of oxygen are required to maintain ionic gradients across neuronal plasma membranes (Boveris and Chance, 1973). Ischemia-induced depletion of cellular ATP prevents neurons from maintaining membrane potential, resulting in neuronal depolarization. The loss of neuronal membrane potential results in increased intracellular calcium concentrations, leading to activation of calcium-dependent processes as well as to the massive release of neurotransmitters, including the excitatory neurotransmitter glutamate (Doyle et al., 2008; Lipton, 1999b). Increased glutamate concentration in synaptic clefts activates NMDA and α-amino-3-hydroxy-5 methyl-4-isoxazolepropionic acid (AMPA) receptors, causing excitotoxic calcium influx (Lipton, 1999b). Pharmacological inhibition of NMDA receptors during early reperfusion following cerebral ischemia is shown to reduce ischemic damage in preclinical studies (Lipton, 1999b).

Channel arrest, a term coined to describe overall down-regulation of ion channels, enhances resistance to modeled ischemia in brain tissue from torpid ground squirrels (Doll et al., 1991). Hippocampal slices harvested from torpid ground squirrels or ground squirrels during interbout euthermia are demonstrated to resist injury during modeled ischemia (Ross et al., 2006). Resistance to injury due to oxygen and nutrient deprivation, however, persists for a longer period of time in culture in slices harvested from torpid animals (Ross et al., 2006). Furthermore, the Na^+/K^+ ATPase inhibitor ouabain induced significantly higher cell death in chronic hippocampal slices from interbout euthermic AGS (ibeAGS) compared to hibernating AGS (hAGS). The resistance to ouabain-induced toxicity suggests that channel arrest is more pronounced, and the requirement for ATP to maintain ionic gradients is significantly diminished during torpor. However, some degree of ion channel arrest persists during interbout euthermia since hippocampal slices from both hAGS and ibeAGS resist the excitotoxic effects of 500 μM NMDA and 20 mM KCl (NMDA/KCl) better than do ischemia-sensitive rats. Again, resistance to this excitotoxic challenge persisted longer in hippocampal slices obtained from hAGS than ibeAGS, supporting the observation that channel arrest is more pronounced and persistent during torpor. The phenomenon of channel arrest is also observed during euthermia in arctic ground squirrels (see section 3: Euthermic phase for details). It should be noted that more direct electrophysiological studies are needed for in depth characterization of the phenomenon of channel arrest in heterothermic animals during torpor and euthermia.

Downregulation of NMDA receptors (NMDAR) in the torpid state contributes to channel arrest in hAGS. NMDAR play an important role in excitotoxicity. NMDAR are composed of NMDAR1 (NR1), a variety of NMDAR2 (NR2) subunits (NR2A-D), and NMDAR3 (NR3). NMDAR activity is regulated by either phosphorylation of NR1 and NR2 subunits or by internalization of NR1 (Forrest et al., 1994; Liu and Zhang, 2000; Nakanishi, 1992; Sakimura et al., 1995; Zhao et al., 2006b). Glutamate-induced Ca^{2+} influx is suppressed in hippocampal slices from hAGS and ibeAGS compared to rat; however, the NMDAR-mediated contribution of glutamate-induced Ca^{2+} influx is suppressed more in hippocampal slices from hAGS than in ibeAGS (Zhao et al., 2006c). Down-regulation of NMDAR in hAGS may be due, in part, to decreased phosphorylation of NR1 in hAGS compared to ibeAGS and rats. To rule out the possibility of an epiphenomenon, more direct mechanistic studies using molecular biology tools are needed. Similar observations of channel arrest are made in other ischemia / anoxia-tolerant species such as turtles (*Chrysemys picta*). Anoxia-tolerant turtles exhibit silencing / down- regulation of NMDAR activity by its dephosphorylation (Bickler et al., 2000). A previous study demonstrated that ischemia tolerance can be induced in mice by knocking out the NR2A subunit (Morikawa et al., 1998). The phenomenon of excitotoxicity during hibernation and arousal is not well understood and warrants further study. Overall, the literature suggests that hibernating animals preserve neuronal ion homeostasis and decrease excitotoxicity by decreasing NMDAR activity during hibernation.

2.3 Synaptogenesis

Another interesting feature of hibernation is maintenance and regeneration of synapses. Spines and synaptic structures retract from disuse and cold temperatures (Fu et al., 2007; Kirov et al., 2004; Roelandse and Matus, 2004); however, hibernating species may limit temperature dependent loss of synaptic profiles. During torpor, dendritic branching and synaptic profiles (e.g., spine densities) decrease in complexity, size and numbers, but rapidly re-emerge upon arousal (Magarinos et al., 2006; Popov and Bocharova, 1992; Popov et al., 1992; von der Ohe et al., 2006). Synaptogenesis may contribute to cognitive enhancement observed 24h after arousal from torpor in AGS (Weltzin et al., 2006). A linear relationship exists between body temperature and loss of dendritic branches and synaptic profiles during torpor; however, retraction is slower than in nonhibernating species such as mice (von der Ohe et al., 2006). Hippocampal slices from mice show rapid loss of actin-based motility followed by loss of the entire dendritic spine structure at later times (Kirov et al., 2004; Roelandse and Matus, 2004). By contrast, in the hibernating Golden-mantled ground squirrel (*Spermophilus lateralis)*, decreasing temperature from 17°C to 7°C does not produce further decreases in hippocampal CA3 spine density or other related parameters (von der Ohe et al., 2006). The limited temperature dependence of synaptic restructuring in these CA3 apical dendrites as well as incomplete loss of synaptic profiles at 7°C in other brain regions suggests that hibernators may limit temperature dependent loss of synaptic profiles (von der Ohe et al., 2006).

Paired helical filament-like hyperphosphorylation of the microtubule-associated protein tau may be one mechanism to stabilize the cytoskeleton and synaptic structure during torpor (Arendt et al., 2003). This interpretation supports the view that while tau hyperphosphorylation is a prelude to neurofibrillary tangles a histopathological hallmark of Alzheimers disease (AD) polymerization of hyperphosphorylated tau has a positive effect on microtubule assembly and is not likely the cause of cognitive decline (Alonso et al., 2001; Alonso Adel et al., 2006). Hyperphosphorylation of tau in hibernating European ground squirrels (*Spermophilus citellus*) reverses rapidly upon arousal from torpor (Arendt et al., 2003). Suppression of cerebral metabolism leads to regional cooling in brains of patients with AD and cold-induced inhibition of serine-threonine protein phosphatase 2A may allow

hyperphosphorylated tau to accumulate in AD (Planel et al., 2004). Hibernating animals are temperature compensated in a way that avoids cold-induced inhibition of the phosphatases necessary for reversal of tau phosphorylation (Su et al., 2008). In this way, these animals may be able to reap benefits from tau phosphorylation such as stabilization of the cytoskeleton, but avoid pathological events caused by failed phosphatase activity.

Stabilization of the cytoskeleton during torpor may contribute to rapid regeneration of synapses during arousal. The rate of dendrite extension is rapid during arousal as compared to the rate in developing primate embryo or adult rat exposed to 4 months of environmental enrichment (Duffy and Rakic, 1983; Greenough et al., 1986; Magarinos et al., 2006; Markham and Greenough, 2004; von der Ohe et al., 2006). Mechanisms to minimize synaptic regression during torpor and to rapidly regenerate synapses upon arousal may contribute to successful entrance into and exit from hibernation without neuronal death.

2.4 Oxidative stress

A cerebral ischemia-induced limitation of substrates and increase in intracellular ions such as calcium leads to increased free radical release from mitochondria (Doyle et al., 2008; Lipton, 1999a; Niizuma et al., 2010). Ischemia also increases nitric oxide production by activating nitric oxide synthase (Doyle et al., 2008; Love, 1999; Samdani et al., 1997). Supra-physiological (i.e. pathological) levels of free radicals can damage proteins, lipids and nucleic acids leading to impaired cellular functions. Oxidative stress can deplete cellular NAD^+ by activating the DNA repair enzyme poly (ADP-ribose) polymerase-1 (PARP-1) (Strosznajder et al., 2010). Depletion of NAD^+ leads to impaired NAD^+-dependent processes such as glycolysis, the tricarboxylic acid cycle and mitochondrial respiration leading to suppressed ATP production and ultimately cellular energy failure and cell death (Doyle et al., 2008; Skaper, 2003). Oxidative stress can also activate matrix metalloproteases, damaging the vascular wall and resulting in increased blood-brain barrier permeability (Crack and Taylor, 2005; Doyle et al., 2008; Romanic et al., 1998). In brief, ischemia-induced oxidative stress plays a crucial role in contributing to cell death.

In AGS, oxidative stress is absent during torpor due to suppressed oxidative metabolism (Orr et al., 2009). To determine the antioxidant status of hibernating animals during hibernation and during arousal, the dynamics of the antioxidants ascorbate and glutathione in two species of hibernating ground squirrels (AGS and 13-lined ground squirrels: *Spermophilus tridecemlineatus*) were determined (Drew et al., 1999). During hibernation, plasma ascorbate levels were drastically increased (3 – 4 fold) in both hibernating species studied. Ascorbate levels were returned to pre-hibernating / euthermic levels during arousal when reactive oxygen species generation was expected to peak during maximum oxygen consumption. A similar trend was observed in ascorbate levels in AGS cerebrospinal fluid (CSF). In contrast, brain ascorbate levels were lowered by 10 – 15% in both species during hibernation. Similar high plasma ascorbate levels were reported in hibernating woodchuck (*Marmota imonax*) (Bito and Roberts, 1974). High levels of ascorbate in plasma during hibernation suggest that ascorbate is poised to redistribute to protect tissues at the time of arousal when free radical generation is expected to peak.

In addition to small molecule antioxidants, attempts have been made to determine antioxidant enzyme levels during hibernation. In another species of hibernating ground squirrel, *Citellus citellus*, the levels of superoxide dismutase (SOD) were similar in a variety of tissues in the hibernating and active states, except the levels were higher in spleen during hibernation (Petrovic et al., 1983). Similar results were also observed in the 13-lined ground squirrel. The activities and levels of the two intracellular isoforms of SOD (CuZnSOD and MnSOD) were not different between active and hibernating squirrels (Page et al., 2009). However, SOD activity was significantly higher immediately after arousal in all tissues

studied except brown adipose tissue. In a follow up study, the same group reported that CuZnSOD activity in brains of hibernating ground squirrel was higher compared with active animals (Petrovic et al., 1986). The activities of antioxidant defense system enzymes SOD, catalase, glutathione peroxidase and glutathione reductase are increased in the brains of *Citellus citellus* when animals are exposed to cold temperatures (Buzadzic et al., 1997). In hibernating Syrian hamsters (*Mesocricetus auratus*), an extracellular protein of 260-kDa exhibiting SOD-like activity was suggested to play a role in tolerance from oxidative stress during arousal from torpor (Okamoto et al., 2006).

Overall, the literature suggests that the levels of antioxidant defense molecules are increased in hibernating animals either during hibernation or at the time of arousal. It is plausible that increased antioxidant capacity during hibernation and at the time of arousal may enhance tissue protection in hibernating animals.

2.5 Inflammatory responses

Cellular as well as cytokine-mediated inflammatory responses play an important role in ischemia-induced brain damage. Cellular inflammatory responses following cerebral ischemia include activation of microglia and astrocytes and infiltration of blood cells, primarily neutrophils, into the brain (del Zoppo, 2010; Doyle et al., 2008; Taoufik and Probert, 2008). Depletion of neutrophils and suppression of leukocyte adhesion lowers ischemic damage in animal models of cerebral ischemia (Bowes et al., 1993; Dawson et al., 1996; Drew et al., 2001; Zhang et al., 1994). Tumor necrosis factor (TNF), interleukin 1 (IL-1), and interleukin 6 (IL-6) are three major cytokines that participate in ischemic damage (del Zoppo, 2010; Doyle et al., 2008). The role of TNF in exacerbating ischemic injury is controversial. Exogenous delivery of TNF into ischemic tissue is shown to aggravate injury (Barone et al., 1997; Taoufik and Probert, 2008). Antibodies to TNF and TNF binding proteins afford protection against ischemic damage, while TNF knock-out mice show reduced ischemic damage (Martin-Villalba et al., 2001; Taoufik and Probert, 2008). In contrast, mice deficient in two TNF receptors exhibited increased ischemic damage (Bruce et al., 1996; Taoufik and Probert, 2008). In general, excessive inflammatory response against ischemic tissue is responsible for increased damage.

During hibernation, the concentration of leukocytes in the circulation drops drastically. In AGS, the leukocyte count drops by more than 90% (Drew et al., 2001; Toien et al., 2001), while during arousal, the leukocyte count is restored rapidly to normal levels (Drew et al., 2001; Toien et al., 2001). Moreover, leukopoietic activity in bone marrow is drastically reduced during hibernation (Drew et al., 2001; Szilagyi and Senturia, 1972). Furthermore, there are large numbers of mature leukocytes stored in the bone marrow during hibernation (Drew et al., 2001; Szilagyi and Senturia, 1972). These data suggest that stored leukocytes rapidly enter the circulation restoring their levels upon arousal. Expression of intercellular adhesion molecule-1 (ICAM-1) on rat microvascular endothelial cells is increased when exposed to plasma from hibernating 13-lined ground squirrels (Drew et al., 2001; Yasuma et al., 1997), suggesting that increased ICAM-1 expression may be responsible for margination or extravasation of leukocytes from the circulation (Yasuma et al., 1997). In addition, antibody production from leukocytes is drastically reduced during hibernation (Drew et al., 2001; McKenna and Musacchia, 1968; Sidky and Auerbach, 1968). In hibernating hamsters and ground squirrels, fat-soluble factors released from brown adipose tissue can act as immunosuppressants during hibernation (Atanassov et al., 1995; Drew et al., 2001; Sidky et al., 1969). Low body temperature promotes storage of lymphocytes in secondary lymphoid organs, a response that depends on a temperature-dependent decline in sphingosine-1-phosphate expression (Bouma et al., 2011). Overall, these data suggest that suppressed inflammatory pathways during hibernation may be, in part, responsible for absence of brain damage in aroused hibernating animals. In particular, cerebral leukocyte invasion is a

significant contributor to deleterious neuroinflammation after stroke (Liesz et al., 2011). Further work is necessary to understand the mechanisms that cause hibernation associated lymphopenia which may have direct implications for reducing neuronal damage in stroke.

2.6 Cell death pathways

Cell death following ischemic insult occurs by necrosis, apoptosis and autophagy (Doyle et al., 2008; Taoufik and Probert, 2008). It appears that all of these processes work in parallel, because cells have been observed to switch from one type of death to another. Ionic imbalance, excitotoxicity, increased oxidative stress, DNA damage, protease activation, mitochondrial dysfunction, and the release of pro-apoptotic molecules from mitochondria are major triggers for cell death pathways (Doyle et al., 2008; Taoufik and Probert, 2008).

At ambient temperatures below about 30 °C torpor is interrupted by brief periods of euthermia (Dausmann et al., 2004). What induces these arousals is unknown although they represent significant energy demand. For torpid hibernators housed near their thermoneutral zone, i.e., a range of ambient termperature where torpid metabolic rate is minimal, about 70% of energy reserves required for the entire hibernation season are consumed during arousal and subsequent episodes of euthermia [reviewed in (Heldmaier et al., 2004)]. During this period of high metabolic demand, hemoglobin-O_2 saturation (or sO_2) falls from a mean of 86% to a minimum of 57% without producing neuronal histopathology or oxidative stress in AGS (Ma et al., 2005). Humans and other homeothermic mammals require sO_2 of greater than 95% to maintain healthy levels of tissue oxygenation. The partial pressure of arterial oxygen (P_ao_2) also falls in AGS from about 60 mmHg to as low as 7 mmHg. The affinity of hemoglobin for oxygen differs among species (Maginniss and Milsom, 1994) and in golden-mantled ground squirrels a higher affinity for oxygen, especially during torpor, means animals maintain high sO_2 at lower P_ao_2. However, this is not the case for AGS where a low sO_2 indicates that tissue oxygenation does not keep pace with demand during arousal.

Hypoxia inducible factor 1 α (HIF1α) is a transcription activator that is stabilized under hypoxic conditions. HIF1α activates target genes involved in ischemic preconditioning and hypoxia / ischemia adaptation (Ratan et al., 2004). Owing to robust oxygen demand during arousal, animals experience severe depletion in arterial O_2 tension (P_ao_2) and sO_2 as discussed above (Ma et al., 2005). Lower P_aO_2 and sO_2 during arousal stabilizes HIF1α (Ma et al., 2005). Activation of extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase / stress-activated protein kinase (JNK) is observed during arousal of AGS, and the levels of activated ERK and JNK are correlated with levels of HIF1α during arousal (Zhu et al., 2005). Moreover, inhibition of JNK reduced baseline survival of AGS hippocampal neurons in acute hippocampal slices, demonstrating that JNK promotes neuronal survival in this species (Christian et al., 2008). An earlier study found decreased phosphorylation of Akt, a pro-survival protein, in the brain and muscle of hibernating 13-lined ground squirrels (Cai et al., 2004). Fleck and Carey observed that expression of antiapoptotic proteins is increased during hibernation in enterocytes of 13-lined ground squirrels (Fleck and Carey, 2005). Overall, these studies demonstrate that during arousal, stabilized HIF1α and other mechanisms activate cell survival pathways. Chen and colleagues demonstrated that, in the greater horseshoe bat (*Rhinolophus ferrumequinum*), a majority of the 17 genes that are overexpressed during hibernation are responsible for regulation of apoptosis, neuronal growth, signal transduction, and neuroprotection (Chen et al., 2008). More work is required to investigate isozyme specific effects of MAPK signaling and to explore downstream consequences of chronically high expression of HIF 1α.

These studies also demonstrate that biochemically, the brains of hibernators resemble a preconditioned-like state that protects the brain from ischemia / reperfusion injury. Unlike preconditioning, AGS retain ischemia tolerance and do not require a sublethal stimulus to

induce tolerance. Ischemic preconditioning was found to induce changes in gene expression consistent with suppression of metabolic pathways and immune responses and reduction of ion channel activity (Stenzel-Poore et al., 2003), all of which are characteristic of hibernation and thought to contribute to neuroprotection in the hibernating state (Drew et al., 2001). Stenzel-Poore et al. (2007) suggest that preconditioning is associated with reprogramming of the cytotoxic cellular response. This reprogrammed response resembles the naïve response in hibernating species. Overall, activation of cell survival pathways during hibernation and /or arousal may protect brains of hibernators from ischemia / reperfusion injury.

2.7 Adenosine A1 receptors

A_1AR activation induces ischemia tolerance in homeothermic species through activation of phospholipase C, production of diacylglycerol and subsequent activation of protein kinase C, epsilon (PKCε) (Di-Capua et al., 2003; Lange-Asschenfeldt et al., 2004; Raval et al., 2003). The role of A_1AR activation was also demonstrated in an *in vitro* model of anoxic preconditioning (Perez-Pinzon et al., 1996). In other models of ischemic preconditioning A_1AR activation was shown to confer neuroprotection via opening of ATP-sensitive potassium channels (Heurteaux et al., 1995; Reshef et al., 2000). Anoxia tolerance in certain species of turtle also requires adenosine binding to A_lAR (Perez-Pinzon et al., 1993).

Although seasonal sensitization of A_1AR signaling is clearly poised to contribute to ischemia tolerance, a role for adenosine in ischemia tolerance in AGS has not been observed. Resistance to ischemic injury *in vivo* or *in vitro* is independent of the hibernation season (Christian et al., 2008; Dave et al., 2006, 2009). Moreover, the delay in anoxic depolarization is not reversed by an A_1AR antagonist (Dave et al., unpublished), nor is ischemia tolerance attenuated by an A_1AR antagonist despite an OGD-induced increase in adenosine release in brain slices harvested from AGS during the hibernation season (Drew et al., unpublished). Overall, these studies suggest that activation of A_1AR is not necessary for the robust ischemia tolerance in hibernators.

2.8 Other neuroprotective mechanisms

Cerebral blood flow falls to ischemic-like levels during torpor without apparent neuronal histopathology or neurological deficits (Frerichs and Hallenbeck, 1998a). Unlike ischemia, however, torpid hibernators do not experience deficits in brain energy or O_2 supply because the decreased blood flow meets the needs of decreased metabolic demand. This suggests that hibernators are not in an ischemic state once they have entered torpor (Henry et al., 2007; Lust et al., 1989). This balance between supply and demand may wane, however, during the metabolically demanding process of arousal (Ma et al., 2005). Although energy charge is not affected by arousal in hamsters (Lust et al., 1989) low sO_2 in AGS suggests that this species experiences deficient O_2 delivery during arousal. Nonetheless, “reperfusion” during arousal from torpor is not injurious (Ma et al., 2005). In contrast, reperfusion of ischemic tissues in nonhibernating mammals can induce vasospasm, with subsequent disruption in blood flow as well as inflammatory and pro-oxidative and pro-nitrosative processes that contribute to tissue damage and poor recovery following stroke and cardiac arrest (Hossmann, 1997; Palomares and Cipolla, 2011).

Consistent with previous studies (Krilowicz, 1985), torpid AGS have higher levels of blood ketones than 24 h-aroused AGS (Christian et al., 2008). Rapid processing of ketone bodies during the first 24 h of arousal indicates that ketones are an important source of energy during arousal and may play a neuroprotective role (Andrews, 2007; Maalouf et al., 2007). Further work is needed to understand the relationship between ketone catabolism and neuroprotection in hibernation.

3. Euthermic phase

In vitro studies demonstrate resistance to modeled ischemia in hippocampus from both hAGS and ibeAGS compared to the more ischemia-sensitive rat (Ross et al., 2006). Brains of ibeAGS retain a significant capacity to tolerate energy deprivation even when not in a torpid state. Tolerance in ibeAGS appears to involve channel arrest, but of a more limited scope than the channel arrest that occurs during torpor in hAGS. Relative to rat, glutamate-induced Ca^{2+} influx is suppressed in both hAGS and ibeAGS and membrane expression of the essential NR1 subunit for NMDAR is lower in hAGS and ibeAGS than in rat (Zhao et al., 2006a).

These *in vitro* observations of tolerance were extended to model ischemia into the *in vivo* condition by using a clinically relevant model of cardiac arrest. Using this model, it was demonstrated that summer euthermic AGS resist injury from an ischemic insult even when the body temperature is maintained at 37 °C (Dave et al., 2009; Dave et al., 2006). Based on hippocampal slices harvested from hibernating and euthermic 13-lined ground squirrels, Frerichs and Hallenbeck observed that at 36°C the duration of ischemia tolerated by slices prepared from active ground squirrels did not differ from slices prepared from rats, and slices from both rats and active ground squirrels were more vulnerable to modeled I/R than slices prepared from torpid ground squirrels (Frerichs and Hallenbeck, 1998b). Later it was demonstrated that slices from euthermic AGS tolerate oxygen / glucose deprivation better than do rats, even at 37°C (Ross et al., 2006). These studies thus suggest that ischemia tolerance exists in AGS not only during torpor / hibernation but also during euthermia and that the degree of tolerance in AGS may not be matched by all hibernating species. Surprisingly, an earlier study demonstrated that euthermic AGS maintained lower P_aO_2 and elevated P_aCO_2 compared to rat (Ma et al., 2005). It is possible that owing to chronic mild degrees of anoxia and hypercapnia, ischemia-tolerant pathways are active in euthermic AGS. In support of this speculation, euthermic AGS brains displayed HIF1α protein levels that were as high as levels observed after arousal and more than double the levels observed in tissue from hibernating AGS (Ma et al., 2005). Upregulation of HIF1α and HIF1α-regulated gene expression plays an important role in hypoxia-induced ischemic tolerance (Bergeron et al., 2000; Bernaudin et al., 2002; Bruer et al., 1997; Gidday et al., 1994; Jones and Bergeron, 2001; Miller et al., 2001; Prass et al., 2003; Ruscher et al., 2002).

In addition, we have observed that *in vivo,* and in hippocampal slices, ischemic depolarization is delayed in euthermic AGS when compared to rats, supporting the idea that channel arrest is present to some extent even in euthermic AGS (Dave et al., 2009). It should be noted that we had not observed any spontaneous torpor in these summer active animals. Neuronal depolarization during ischemia represents a collapse of ion homeostasis ultimately leading to neuronal death (Kaminogo et al., 1998). Blocking or delaying ischemic depolarization can significantly improve ischemia recovery (Anderson et al., 2005; Takeda et al., 2003). Also, inhibition of PKCε during *in vitro* ischemia reversed the delay in ischemic depolarization in slices from euthermic AGS but had no effect on the timing of depolarization in slices from rats (Dave et al., 2009). PKCε activation inhibits Na^+/K^+-ATPase and voltage-gated sodium channel (VGSC) (Chen et al., 2005; Nowak et al., 2004), both key players in ion homeostasis and its collapse during ischemia. This observation indicates that PKCε activation is required for the robust ion homeostasis in euthermic AGS brain tissue during experimental ischemia. It is possible that in AGS neurons, PKCε is poised to regulate this balance by decreasing VGSC and Na^+/K^+-ATPase function. Activation of PKCε plays an essential role in induction of ischemia tolerance in many organs including brain and heart (Perez-Pinzon, 2007; Savitz and Fisher, 2007). In summary, we and others have demonstrated that, ischemia tolerance pathways are chronically active in euthermic hibernators.

4. Summary

Mammals capable of hibernation represent a robust example of tolerance to cerebral ischemia that is unmatched by any other model of ischemia tolerance. Hibernation is characterized by profound decreases in metabolic demand and body temperature as well as decreased blood flow does not produce an ischemic state. Brief, interbout arousals occur frequently throughout the hibernation season and appear to be the most physiologically challenging aspect of heterothermy where blood flow may not meet cellular demands. Moreover, synapses lost during prolonged torpor rapidly return during interbout arousal. Thus, tolerance to rapid changes in blood flow and metabolic demand as well as for the capacity to stabilize and regenerate synapses likely evolved as means for successful hibernation (Figure 1). Humans, in contrast, suffer significant consequences in response to limited blood flow where they experience significant injury in response to ischemia and reperfusion, in addition to having limited capacity for synapse regeneration. There is much to be learned about cellular, molecular and systems-wide mechanisms that protect heterothermic mammals from ischemia and promote synaptic regeneration upon arousal. Currently, little is known about these mechanisms; however, clear appreciation of the degree of ischemia tolerance these animals display opens the door for further in depth mechanistic studies. Better understanding of regulatory mechanisms will guide discovery of therapeutics designed to mimic natural means of ischemia tolerance and adult synaptic plasticity.

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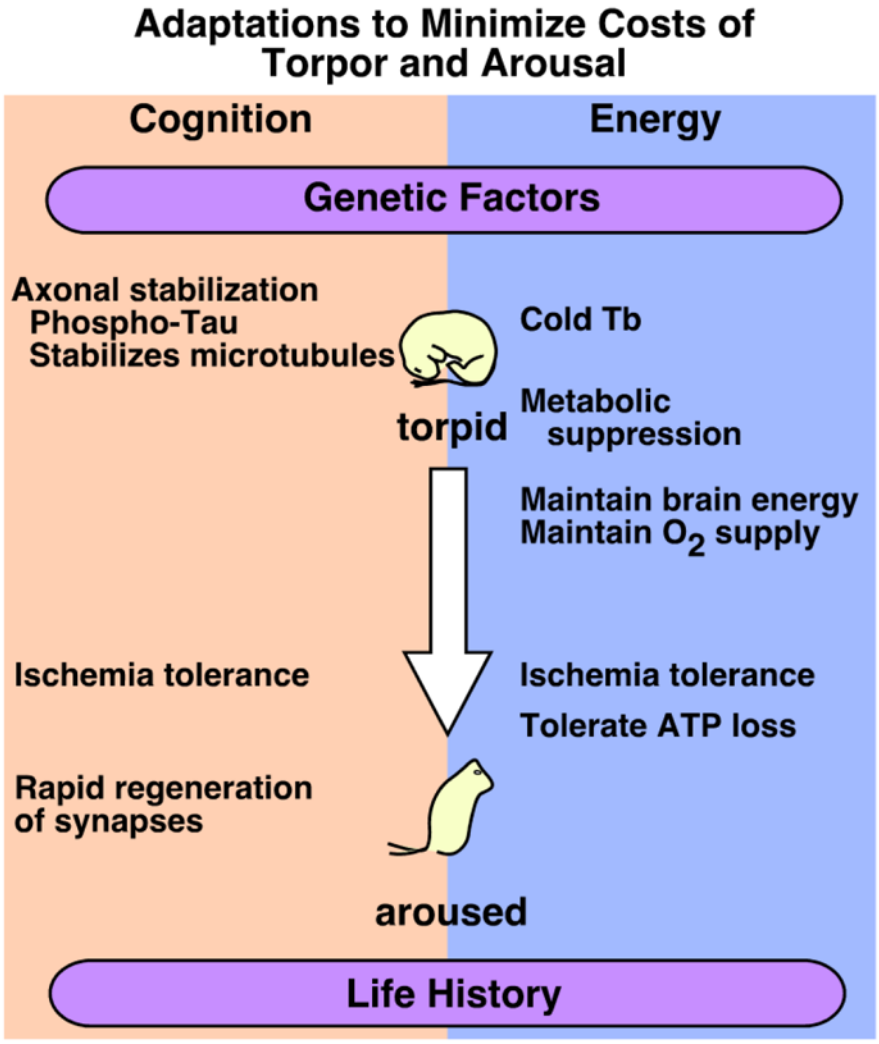

Figure 1. Schematic diagram depicting the adaptations used to minimize the cognitive and energetic costs of arousal

Genetic factors and potentially life history contribute to the ability to tolerate multiple arousals from the torpid state without cognitive deficits or cellular pathology. Rapid regeneration of synaptic profiles during arousal prevents cognitive deficits and likely contributes to improved mnemonic function 24 h after arousal. Cold body temperature (Tb) combined with metabolic suppression during torpor conserves energy. Overall maintenance of cellular homeostasis during arousal promotes neuronal cell survival and contributes to ischemia tolerance in hibernating species.