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## *Review Article*

# NEUROPROTECTIVE ADAPTATIONS IN HIBERNATION: THERAPEUTIC IMPLICATIONS FOR ISCHEMIA-REPERFUSION, TRAUMATIC BRAIN INJURY AND NEURODEGENERATIVE DISEASES

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**Abstract**—Brains of hibernating mammals are protected against a variety of insults that are detrimental to humans and other nonhibernating species. Such protection is associated with a number of physiological adaptations including hypothermia, increased antioxidant defense, metabolic arrest, leukocytopenia, immunosuppression, and hypocoagulation. It is intriguing that similar manipulations provide considerable protection as experimental treatments for central nervous system injury. This review focuses on neuroprotective mechanisms employed during hibernation that may offer novel approaches in the treatment of stroke, traumatic brain injury, and neurodegenerative diseases in humans. © 2001 Elsevier Science Inc.

**Keywords**—Alzheimer disease, Arctic ground squirrel, Oxidative stress, Stroke, Antioxidants, Metabolic suppression, Free radicals

## INTRODUCTION

Hibernation, a unique physiological state that evolved to survive periods of food shortage [1], is characterized by profound decreases in oxidative metabolism and body temperature during bouts of prolonged torpor, interrupted by brief periods of euthermic (37°C) body temperature [2–5]. In light of the ability of hibernating animals to survive frequent and dramatic fluctuations in blood flow without neurological damage [6,7] and, experimentally, to survive a variety of neurological insults [6–10], we hypothesized that some of the unique aspects of hibernation physiology include several neuroprotective adaptations. Here, we review a number of these neuroprotective adaptations, which might have therapeutic potential for human disease states, including ischemia-reperfusion, traumatic CNS injury, and neurodegenerative diseases.

## NEUROPROTECTIVE MECHANISMS ASSOCIATED WITH HIBERNATION

### *Hypothermia*

True hibernation is defined by prolonged bouts of torpor characterized by decreased body temperature and metabolic rate. Core body temperature in hibernating ground squirrels parallels ambient temperature down to near 0°C, where it remains for several days to weeks [2,4]. Prolonged bouts of torpor are interrupted by brief, periodic arousals where animals spontaneously rewarm for reasons that remain a matter of debate (Fig. 1) [11–15]. Such profound hypothermia, not well tolerated by nonhibernating species, likely contributes to neuroprotection.

Hypothermia is indeed one of the oldest treatments for limiting cellular injury in CNS tissues. Even a small decrease in temperature reduces the extent of ischemia and traumatic brain injury (TBI)-induced neuronal injury, glutamate release, free radical production and, consequently, may improve neuronal survival [16–19]. However, mild hypothermia by itself provides only limited neuronal protection and recently failed to improve outcome after acute brain injury in a large clinical trial

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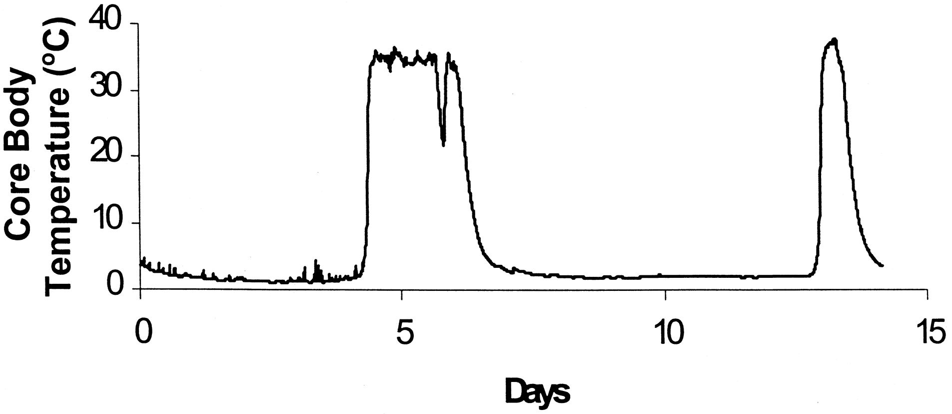

Fig. 1. Core body temperature of a hibernating arctic ground squirrel (AGS) monitored by a temperature-sensitive radio-transmitter. Over the course of the hibernation season, hibernating mammals periodically rewarm to 37°C (and briefly resume normal blood flow). These short periods of warm body temperature ($T_b$) last 24–48 h, after which $T_b$ and blood flow again plummet during entry into prolonged torpor. Handling prior to the first rewarming period shown aroused this animal. Occasionally entry into torpor is preceded by partial dips in body temperature before complete torpor is achieved.

[20]. Although more extreme hypothermia enhances neuroprotection in hibernating ground squirrel hippocampus [9], extreme hypothermia is not well tolerated by nonhibernating species and may exacerbate tissue injury [21]. How thermoregulation and cold tolerance are accomplished in hibernating species remains a matter of investigation [4,12,22–25]. Means to improve cold tolerance in humans could be used to enhance therapeutic efficacy of hypothermia. More importantly, cytoprotection in hippocampal slices from hibernating ground squirrels is preserved at 36°C [9], indicating involvement of additional neuroprotective mechanisms.

## *Antioxidant defense*

Oxidative stress is the imbalance between free radical production and cellular antioxidant protection and is intimately associated with neuronal cell death in chronic neurodegenerative diseases, ischemia/reperfusion, and TBI [26–29]. In neurodegenerative diseases, oxidative stress plays a fundamental role not only in the initiation, but also in driving the progression of the pathogenic process [28,30]. On the molecular level, oxidative stress damages proteins, nucleic acids, and lipids [31,32], inducing specific antioxidant systems [33,34] as well as activating stress signaling pathways such as JNK and p38 [35,36]. Following ischemia or TBI, the generation of free radicals is associated with the consumption of endogenous antioxidants [37,38].

Hibernating species appear to be protected from the massive oxidative burst associated with frequent, periodic rewarming by increased concentrations of ascorbate (vitamin C) in plasma and cerebral spinal fluid (CSF) (Fig. 2) [5,39]. During arousal, when oxygen consumption peaks and the generation of reactive oxygen species is expected to be maximal, ascorbate levels progressively decrease to levels typical for euthermic animals. These data suggest that ascorbate, a known neuroprotectant [40], is poised to protect tissue from oxidative stress during arousal from hibernation. Moreover, a brief rise in plasma uric acid concentration correlates with the surge in oxygen consumption during arousal. A rise in uric acid is consistent with increased reactive oxygen species (ROS) production because ROS rapidly convert xanthine dehydrogenase to xanthine oxidase (reviewed in [5,41]),

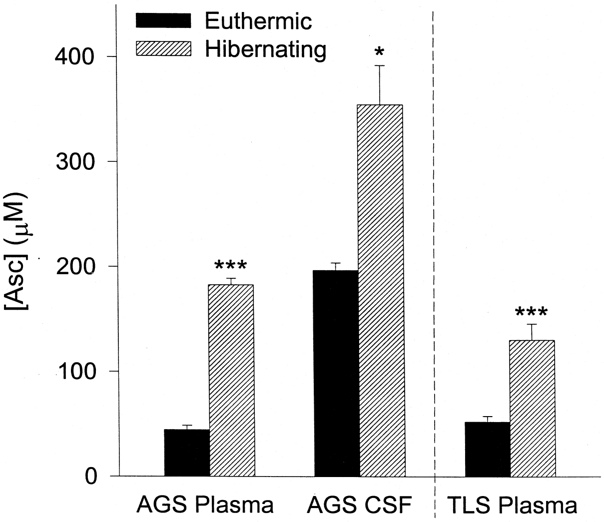

Fig. 2. Ascorbate concentration increases 3- to 4-fold in plasma during hibernation in two species of ground squirrels (arctic ground squirrel [AGS] and 13-lined ground squirrel [TLS]). Likewise, CSF concentration of ascorbate doubles during hibernation. Data shown are means ± SEM, $n = 4–8$. $*p < .05$, $***p < .001$ hibernating vs. euthermic (reprinted from [39] with permission from Elsevier Science Inc.).

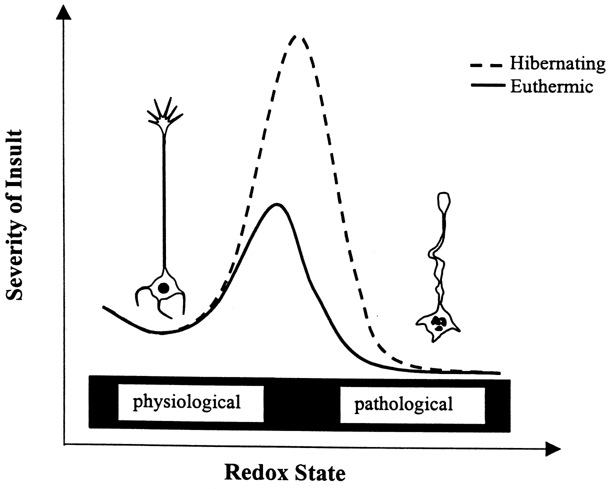

Fig. 3. Proposed relationship between redox state and severity of insult in euthermic and hibernating ground squirrels. It is hypothesized that hibernating animals require a greater insult to produce pathological consequences than euthermic animals because of enhanced antioxidant defense systems.

which catalyzes oxidation of xanthine to uric acid and superoxide. Superoxide dismutase (Cu/Zn) and glutathione peroxidase also increase during hibernation, particularly in brown adipose tissue, where metabolic activity is necessary for nonshivering thermogenesis throughout hibernation and arousal [42,43]. Taken together, these data suggest that enhanced antioxidant defense during hibernation constitutes an important neuroprotective component of hibernation physiology. It is hypothesized that a more severe insult is required to produce a pathologically equivalent response in euthermic animals compared to hibernating animals because of enhanced antioxidant defense during hibernation (Fig. 3). In this regard it is not surprising that in humans low plasma antioxidant activity is associated with greater neurological impairment following CNS insult [44]. Furthermore, a lifelong dietary pattern poor in antioxidants correlates with a higher propensity for neurological disease [45]. Consistent with these findings, antioxidants protect against the pathological consequences of brain tissue injury [46–50] and limit the progression of neurodegenerative disease [51,52,53].

### *Metabolic suppression*

Metabolic suppression, an attenuation of energy consuming processes, is yet another hallmark of hibernation, and holds promise for perhaps the most novel approach in the treatment of brain trauma and neurodegenerative disease where blood flow does not keep pace with energy demand [54]. In fact, hibernating animals exhibit an unparalleled suppression of energy demands in response to decreased energy supply and thus maintain proper energy balance [55,56]. Mechanisms of suppressed energy demand may include channel arrest [57–59], dendritic remodeling [60,61], and/or inhibition of protein synthesis [62,63].

The best evidence for channel arrest arises from studies on anoxia-tolerant turtles. Here, a significant decrease in voltage-gated sodium channel density occurs after 4 h of anoxia [64]. Similarly, NMDA receptors are “silenced” by at least three mechanisms at different times during anoxia [65,66]. To date, these findings have not been confirmed in hibernating mammals. In addition, maintenance of the cytoskeleton is thought to consume a significant proportion of cerebral energy production. Interestingly, NMDA receptor properties are intimately tied to alterations in the organization of the actin cytoskeleton. Dendritic remodeling and synapse reduction during hibernation are consistent with energy-saving, decreased maintenance of the cytoskeleton, which is also observed after surgical deafferentation. Fewer synapses would also suggest less synaptic transmission, another energy-demanding process.

Detailed mechanisms of metabolic suppression are poorly understood but entail more than temperature-dependent effects. Importantly during entry into torpor, $O_2$ consumption drops precipitously prior to a gradual decline in core body temperature [67,68]. Furthermore, $O_2$ consumption does not correlate with core body temperatures measured in arctic ground squirrels hibernating at ambient temperatures ranging from +20 to −20°C [4]. $O_2$ consumption is minimal at core body temperatures between +16 and 0°C. A 10-fold increase in $O_2$ consumption occurs, however, when ambient temperature is lowered to −20°C. This increase in $O_2$ consumption is necessary to defend a near 0°C body temperature when ambient temperature falls below 0°C [4]. Such dissociation between metabolic rate and body temperature suggests that metabolic suppression is induced and regulated by means other than hypothermia. A detailed understanding of the factors responsible for metabolic suppression during hibernation could have broad therapeutic applications.

By injecting plasma from hibernating 13-lined ground squirrels, Dawe and Spurrier [69] induced hibernation in the summer when these ground squirrels do not spontaneously hibernate. Similar experiments failed, however, in other hibernating species including Richardson’s ground squirrels [70], arctic ground squirrels, and woodchucks [71]. On the other hand, plasma from hibernating animals of some of these same species has been shown to induce hibernation in 13-lined ground squirrels [72]. Wang et al. [73] later discovered that summer hibernation in 13-lined ground squirrels was a nonspecific response that could be induced by a variety of stimuli, including saline. Nonetheless, the search continues for endogenous molecules capable of inducing hibernation [74].

Recently, Horton et al. [75] isolated an 88 kDa opioid-like protein from hibernating woodchuck plasma and provided evidence for $\delta$-agonist activity. This peptide may play a role in neuronal survival in light of substantial evidence for protective effects of D-Ala(2),D-Leu(5)]enkephalin (DADLE) in brain and other tissues (for review see [76]). Further investigation of DADLE as a hibernation inducer in species other than 13-lined ground squirrels [77] will help confirm the role of $\delta$-opioid receptors in metabolic suppression.

In contrast to plasma-derived factors, brain extract from hibernating 13-lined ground squirrels displays greater specificity in inducing metabolic suppression. Crude extracts as well as partially purified polypeptides prepared from subcortical regions of brains taken from hibernating 13-lined ground squirrels suppressed $O_2$ consumption in rats within 30 min of iv injection [78]. This effect was independent of a decrease in body temperature, which continued to decline after $O_2$ consumption returned to normal levels. Similar results obtained with extracts from torpid nonmammalian species [79] supports the search for signaling molecules and regulatory mechanisms capable of suppressing metabolism in nonhibernating species. Although beyond the scope of this review, several studies provide evidence of protein phosphorylation and differential gene expression as regulatory mechanisms in hibernation [3,56,62,80–85].

## *CNS regulation of hibernation*

The central nervous system likely plays a key role in orchestrating metabolic suppression during hibernation. Several brain regions have received attention regarding their potential involvement in the central control of hibernation [86,87]. Studies of EEG of hibernating animals have shown that hippocampus, septum, and hypothalamus retain periodic EEG at temperatures below which EEG in other structures become isoelectric (reviewed in [88]). Changes in histamine immunoreactivity support a role for this neurotransmitter in hibernation [13,89]. Evidence further points towards a role for specific nuclei in the hypothalamus. For instance, the suprachiasmatic nucleus (SCN), an area which contains the pacemaker cells for circadian rhythms [90], may provide circadian information to the lateral septal nucleus, which then influences the medial septum [91] and subsequent arousal. Increased c-fos expression (mRNA and protein) in the SCN during arousal supports the hypothesis that this nucleus plays a role in hibernation [14,92]. Furthermore, specific morphological features in the supraoptic nucleus and paraventricular nucleus appear to be associated with the physiological changes of hibernation [93]. Finally, due to the presence of sleep promoting and temperature sensitive neurons, the preoptic/anterior hypothalamic area may play a role in hibernation [86,88].

Entrance into hibernation begins during sleep, with the early phase of entry being dominated by slow wave sleep. Thus, study of sleep promoting areas of the brain are likely relevant to CNS regulation of metabolic suppression during hibernation. Importantly, it is not yet clear if sleep is a necessary prelude to entrance into hibernation because only correlative relationships have been established. Nonetheless, because sleep induces modest declines in body temperature and metabolic rate and always precedes entry into hibernation, it has been suggested that hibernation is an extension of sleep [86, 88]. Ability to pharmacologically induce profound metabolic suppression such as is characteristic of hibernation could have significant therapeutic value in stroke and TBI.

Suppressed responsiveness during hibernation appears to represent a unique state of consciousness and is distinguished from sedation or coma in that animals maintain postural control (a tightly curled posture minimizes heat loss). Furthermore, animals may respond to touch or other sensory stimuli, albeit at an excruciatingly slow rate. Simply handling will typically induce rewarming in arctic ground squirrels except during entrance into torpor in the period just before core body temperature reaches minimal values. Even during this period, when animals are most resistant to arousal, handling may induce slow responses of the head and limbs. Finally, a whole host of coordinated physiological adaptations occur during hibernation, including suppression of basal metabolic rate, leukocytopenia, and inhibition of protein synthesis. Unlike coma, CNS suppression in hibernation thus appears to result from a highly regulated process rather than a loss of function.

## *Immunomodulation*

Immune activation and inflammatory processes, which accompany stroke, TBI, and neurodegenerative diseases, likely contribute to the progressive degeneration of neurons and are a focus of therapeutic intervention [94–96]. Cerebral ischemia and TBI both produce an acute inflammatory response that is associated with an accumulation of neutrophils. In the case of ischemia, this phenomenon is most dramatic during reperfusion [97–99]. Leukocyte infiltration might exacerbate tissue damage by producing oxygen radicals, releasing proteolytic enzymes, and presenting physical obstructions to blood flow [100,101]. Neutrophil depletion or suppression of leukocyte adhesion via inhibition of the intercellular adhesion molecule (ICAM-1) adhesion site decreases

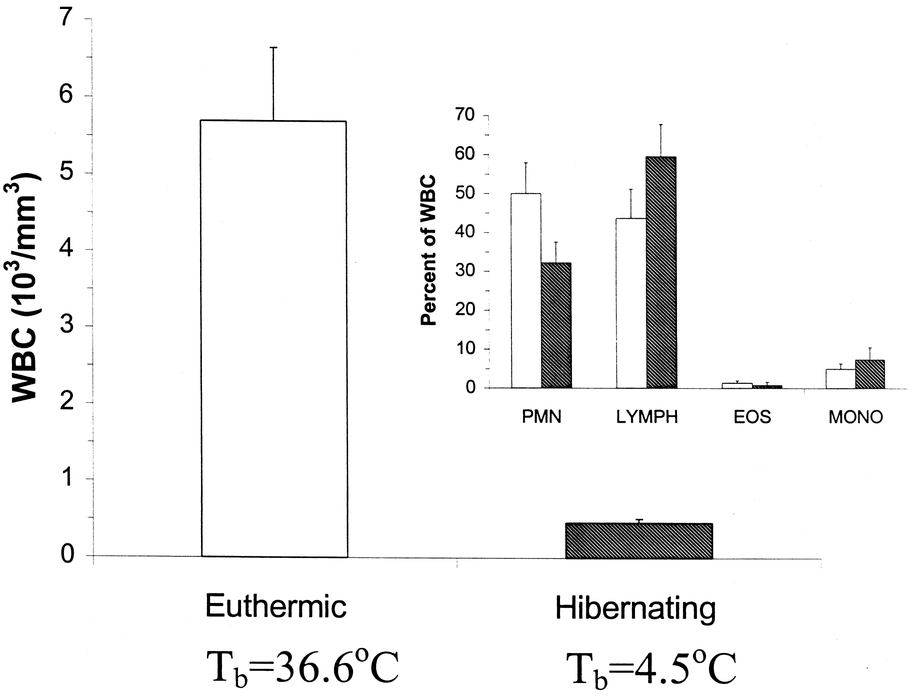

Fig. 4. Profound leukocytopenia is characteristic of hibernation. Differential white blood cell (WBC) counts typically do not change. Blood was obtained via cardiac puncture and leukocytes counted on a Coulter T-890. Data shown are means ± SEM, $n = 4-6$.

neurological damage in animal models of stroke and reperfusion injury [102–106].

Leukocytopenia, as much a hallmark of hibernation as hypothermia, [1,5,6,107–109] is thus another potentially neuroprotective adaptation. In arctic ground squirrels, leukocyte counts drop up to 100-fold during hibernation [5,10]. The decrease occurs across all cell types with no dramatic alteration in differential counts [6,10] (Fig. 4). Upon arousal, the number of circulating leukocytes rapidly returns to euthermic values [107], though not always in parallel with increased oxygen consumption or body temperature [5]. The rapid reappearance of leukocytes suggests that the cells are stored and readily available upon arousal. Szilagyi and Senturia [110] provide evidence for decreased leukopoietic activity and thus decreased production of early granulocytes, coupled with continued differentiation and maturation into late granulocytes. Accumulation of mature granulocytes may provide a pool of cells available for release upon return to normal blood flow. Curiously, Yasuma et al. [111] reported increased expression of ICAM-1 by rat microvascular endothelial cells cultured with plasma from hibernating 13-lined ground squirrels. An increase in ICAM-1 expression could account for margination of leukocytes in vascular epithelium, another proposed mechanism to account for the disappearance of white cells during hibernation. While increased ICAM-1 expression is counter-intuitive to therapeutic strategies to inhibit ICAM-1 during reperfusion [102], increased expression in hibernating mammals may remove leukocytes from the circulation prior to risk of ischemic insult upon arousal from hibernation.

In addition to dramatic leukocytopenia, animals during hibernation produce few or no antibodies in response to antigenic challenge; an effect that persists in vitro at 37°C [112,113]. Evidence suggests that a lipid soluble factor in brown adipose tissue from hibernating hamsters may mediate immunosuppression in this species during hibernation [114,115]. A brown adipose tissue-derived immunosuppressant is consistent with moderate seasonal variation in immune response in euthermic animals, because brown adipose tissue levels fluctuate seasonally in parallel with suppression of immune response [108]. Leukocytopenia and immunosuppression would be expected to prevent an inflammatory response during reperfusion of tissues that become metabolically active upon rewarming.

### *Hypocoagulation*

Platelet activation, fibrin formation, and leukocyte adhesion contribute to the "no-reflow" phenomenon that follows initial disruption of blood flow [100], and to progressive impairment to microvascular perfusion following ischemic brain injury [116]. Fibrin deposits and platelet accumulation thus compromise microvascular patency during reperfusion and exacerbate progressive tissue injury [95,117]. Thrombolytics have received considerable attention as potential therapeutic agents for acute ischemic stroke [118–120].

Consistent with other potentially neuroprotective adaptations, blood clotting times are increased dramatically during hibernation [121–123]. Svihla et al. [121] discovered that hibernating, but not euthermic, ground squirrels died of internal pericardial hemorrhage when 23-, rather than 26-gauge hypodermic needles were used for cardiac punctures. Subsequently, clotting times were found to be increased 5- to 6-fold in hibernating compared to euthermic animals. In hibernating ground squirrels, complete clots often never form, even after several days [121]. In addition, when clots do form, the clot quality is poor and of shorter duration in hibernating animals compared to cold-adapted euthermic animals studied at the same time of year (C. Stewart and K.L. Drew, unpublished observations). Clearly, prolongation of clotting times would alleviate risk of thrombus formation during periods of reduced blood flow during prolonged torpor. Such an adaptation might also be protective when blood flow returns to euthermic levels during frequent, periodic rewarming.

Mechanisms responsible for alterations in clotting times may vary between species. This would explain some inconsistencies reported in the literature concerning platelet numbers and plasma prothrombin concentrations [124,125]. In some cases, platelet counts decreased by as much 90% during hibernation while in other cases, platelet counts increased [125]. In arctic ground squirrels, we have seen platelet counts decrease by as much as 50% during hibernation, but hibernation related changes vary considerably and increased clotting times have been observed in animals with no apparent decrease in platelet numbers (Drew et al., unpublished observations). It is possible that cold temperatures contribute to hypocoagulation, however, even at warm temperatures clotting is significantly impaired [125]. Evidence suggests that alterations in plasma composition affecting the intrinsic clotting cascade contribute to hypocoagulation. Increases in partial thromboplastin time (intrinsic clotting pathway) in ground squirrels were associated with decreases in factors VIII and IX, two factors known to influence this test. Interestingly, plasma of hibernating squirrels did not prolong the partial thromboplastin time of active animals, arguing against the presence of an endogenous inhibitor [125].

More recently, it was reported that liver $\alpha_2$-macroglobulin (protein and mRNA) increases during hibernation in ground squirrels [80,126]. $\alpha_2$-Macroglobulin ($\alpha_2$M) is a universal protease inhibitor. The protein "baits" a variety of proteases to catalyze limited hydrolysis. This event triggers a conformational change in $\alpha_2$M that produces additional protease binding sites [127]. In this way $\alpha_2$M inhibits a number of steps in the clotting cascade. $\alpha_2$-Macroglobulin binds kallikrein and inhibits subsequent activation of factor XII in the intrinsic pathway [128]. It binds factor Xa to inhibit this activated factor at the intersection between the intrinsic and extrinsic pathways [129]; and $\alpha_2$M binds thrombin [130], the protease that cleaves fibrinogen to fibrin monomers, which polymerize to form a fibrin clot. Similarly, $\alpha_2$M binds to antigen antibody aggregates and cell membranes to regulate and often suppress immune responses [131,132]. Thus, increased $\alpha_2$M expression during hibernation may function to attenuate clot formation [126] and/or suppress immune response. Decreased coagulation would, of course, prevent clot formation when heart rate and blood flow decrease during torpor and during reperfusion upon arousal. It may not be coincidental that $\alpha_2$M polymorphisms are associated with an increased propensity to develop Alzheimer disease (AD) [133].

Hypocoagulation also decreases production of thrombin, a serine protease intimately involved in the clotting cascade, which may have detrimental effects unrelated to clot formation [134]. Levels of mRNA for the thrombin precursor prothrombin and Factor Xa (the protein that converts prothrombin to thrombin) are both expressed in CNS tissue [135,136]. Thrombin will directly enter brain tissue during penetrating brain injury, hemorrhagic stroke, and possibly treatment with tissue plasminogen activator (t-PA), and has been shown to produce glial scarring [137] and apoptosis [138], and is thought to exacerbate damage [139]. Thus, inhibition of the coagulation pathway during hibernation may protect tissue by decreasing production of thrombin and by suppressing clot formation.

## *Species specificity*

Physiological adaptations associated with hibernation impart profound neuroprotection in vivo [10]. Neuroprotection in tissue from hibernating animals is also observed in vitro and cannot be explained entirely by hypothermia [9]. Nonetheless, some species-specific differences exist that are independent of the state of hibernation. For example, hypothermia manifests greater tolerance to hypoxia/aglycemia in euthermic ground squirrel hippocampal slices compared to rat hippocampal slices [9]. Coincidentally, cold exposure increases activity of superoxide dismutase in euthermic ground squirrels, but not rats [43]. Finally, hibernating species display greater tolerance to hypoxia in vivo than nonhibernating species. Such tolerance is associated with hypoxia-induced metabolic suppression [8].

## CONCLUSIONS

Clinical and experimental studies show that following an acute CNS injury such as ischemic stroke or TBI, neuronal damage continues to progress for periods of several hours to days [140–142]. This time period is seen as a window of opportunity to attenuate progressive tissue damage. Although numerous therapeutic strategies have been shown to decrease ischemia-induced damage in experimental models, only t-PA has proven effective in controlled clinical trials [143–146]. While t-PA has improved prognosis for many patients, progress in the development of effective pharmacotherapies has been slow. Lee et al. [147] summarize results from a number of recent clinical trials for brain ischemia. Most were abandoned or showed no efficacy, thus emphasizing the need for novel approaches in treating stroke. Importantly, all of the results reviewed were from single agent studies. Study of hibernation physiology suggests that a variety of potentially synergistic adaptations act in concert to achieve remarkable tolerance to dramatic fluctuations in blood flow and experimentally induced brain trauma.

As with ischemia, the pathological events in AD and late onset dementia may be exacerbated and even triggered by impaired cerebral perfusion originating in the microvasculature [148,149]. Hypoperfusion disrupts energy balance and ion homeostasis and can initiate a cascade of events that ultimately leads to oxidative stress and cell death. Thus, hypoperfusion may contribute significantly to neuropathology, not only in stroke and TBI, but also in neurodegenerative diseases [150,151]. Therefore, understanding in detail the neuroprotective mechanisms underlying hibernation, a natural model of tolerance to dramatic fluctuations in cerebral blood flow and brain injury, could provide alternative therapeutic strategies. Hibernating species utilize a variety of strategies to maintain proper energy balance and minimize pathological tissue responses to trauma. Hibernation physiology emphasizes that a combination of physiological adaptations are likely necessary for the profound neuroprotection observed during hibernation. Evidence of neuroprotection associated with hibernation supports further research in combination therapies for stroke, head trauma, and neurodegenerative disease. Furthermore, the myriad of adaptations observed during hibernation may result from a single regulatory mechanism that could, one day, be duplicated in the clinic.

An important question that remains is whether a hibernation-like state induced after trauma would be protective. We find that inflammatory response around a microdialysis probe progresses as it would in a euthermic animal as soon as the animal rewarms from hibernation. Thus, the tissue response is delayed rather than attenuated. A delayed response could, however, expand the window of opportunity for transport to medical facilities. It is as yet unclear if drastic hypothermia, immunosuppression, or hypocoagulation would be advantageous to cardiovascular, renal, or intestinal tract function following trauma, as it appears to be in the slowly developing process of hibernation. Nonetheless, better understanding of regulatory mechanisms in mammalian hibernation may provide the tools to address these questions.

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## ABBREVIATIONS

AD—Alzheimer disease
$\alpha_2$M— $\alpha_2$-Macroglobulin
CSF—cerebral spinal fluid
DADLE— D-Ala(2),D-Leu(5)]enkephalin
ICAM-1— intercellular adhesion molecule
ROS— reactive oxygen species
t-PA— tissue plasminogen activator
TBI— traumatic brain injury