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REVIEW

No oxygen? No problem! Intrinsic brain tolerance to hypoxia in vertebrates

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ABSTRACT

Many vertebrates are challenged by either chronic or acute episodes of low oxygen availability in their natural environments. Brain function is especially vulnerable to the effects of hypoxia and can be irreversibly impaired by even brief periods of low oxygen supply. This review describes recent research on physiological mechanisms that have evolved in certain vertebrate species to cope with brain hypoxia. Four model systems are considered: freshwater turtles that can survive for months trapped in frozen-over lakes, arctic ground squirrels that respire at extremely low rates during winter hibernation, seals and whales that undertake breath-hold dives lasting minutes to hours, and naked mole-rats that live in crowded burrows completely underground for their entire lives. These species exhibit remarkable specializations of brain physiology that adapt them for acute or chronic episodes of hypoxia. These specializations may be reactive in nature, involving modifications to the catastrophic sequelae of oxygen deprivation that occur in non-tolerant species, or preparatory in nature, preventing the activation of those sequelae altogether. Better understanding of the mechanisms used by these hypoxia-tolerant vertebrates will increase appreciation of how nervous systems are adapted for life in specific ecological niches as well as inform advances in therapy for neurological conditions such as stroke and epilepsy.

KEY WORDS: Arctic ground squirrel, Cetacean, Hypoxia, Naked mole-rat, Seal, Turtle

Introduction

Environmental conditions vary enormously for vertebrates, both with respect to the extreme conditions tolerated by a given species at different times and with respect to average living conditions tolerated by different species. Temperature is perhaps the most obvious example: from the poles to the equator, average ambient temperatures vary widely and have been accompanied by physiological adaptations appropriate to resident species; seasonal variations in temperature and resource variability can induce dramatic changes in physiological and/or behavioral patterns including migration and hibernation. Oxygen levels also vary widely, with animals adapted to sea level, high-altitude, underground and aquatic habitats. Oxygen levels can also change dramatically on a shorter-term basis, as can occur in tidal pools or in breath-hold divers.

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Approximately 20% of the oxygen consumed by the human body is used by the brain. The greater part of this oxygen is used to produce the ATP required to maintain the membrane potentials necessary for electrical signaling with synaptic and action potentials (Harris et al., 2012). In many vertebrates, including adult humans, interruption of the oxygen supply to the brain for more than a few minutes leads to irreversible neurological damage, including neuronal death. Without oxidative phosphorylation, ATP-dependent neuronal processes including ion transport and neurotransmitter re-uptake decline sharply. Without pumping, ion gradients fail and neurons depolarize, releasing excessive levels of excitotoxic neurotransmitters such as glutamate and dopamine. The overstimulation of glutamate [*N*-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)] receptors increases intracellular calcium levels and triggers multiple internal cascades that result in cell damage and death, including activation of lipases, endonucleases and proteases, and mitochondrial-dependent apoptosis (Lipton, 1999). Not all vertebrates, however, are equally susceptible to brain damage resulting from periods of low oxygen. Specializations of brain physiology that have evolved in certain vertebrate species to cope with oxygen deprivation (hypoxia) were the subject of a symposium held during the 10th International Congress of Neuroethology in August 2012. Much has been learned about the catastrophic sequelae of hypoxia that lead to neuronal death; the study of hypoxia-tolerant species has played a major role in advancing this understanding, although significant gaps still remain.

This review focuses on specialized mechanisms in certain vertebrate brains to tolerate either chronic or acute hypoxic challenges imposed directly by extreme environmental conditions or indirectly by physiological/behavioral responses to those conditions. The brains of the particular species that are discussed here are all robustly and intrinsically tolerant to hypoxic challenge but differ markedly in the circumstances in which hypoxia occurs as a normal consequence of habitat and lifestyle. It may be present chronically, during the animal's entire life (naked mole-rats), on a seasonal basis (fresh-water turtles, hibernating ground squirrels) or during execution of particular behaviors necessary for survival (diving seals). It is hoped that consideration of the similarities and differences in how the brains of these different vertebrates cope with hypoxia will increase our appreciation of how nervous systems are adapted for life in specific ecological niches as well as inform advances in therapy for neurological conditions such as stroke and epilepsy.

Down for the count: hypoxia tolerance in the freshwater turtle brain

Among the most robust of hypoxia-tolerant vertebrates is the freshwater turtle *Trachemys scripta*, which can withstand complete anoxia for days at room temperature to weeks in winter hibernation (Jackson and Ultsch, 2010); even at room temperature, 24 h of anoxia and re-oxygenation results in no evident loss of neurons

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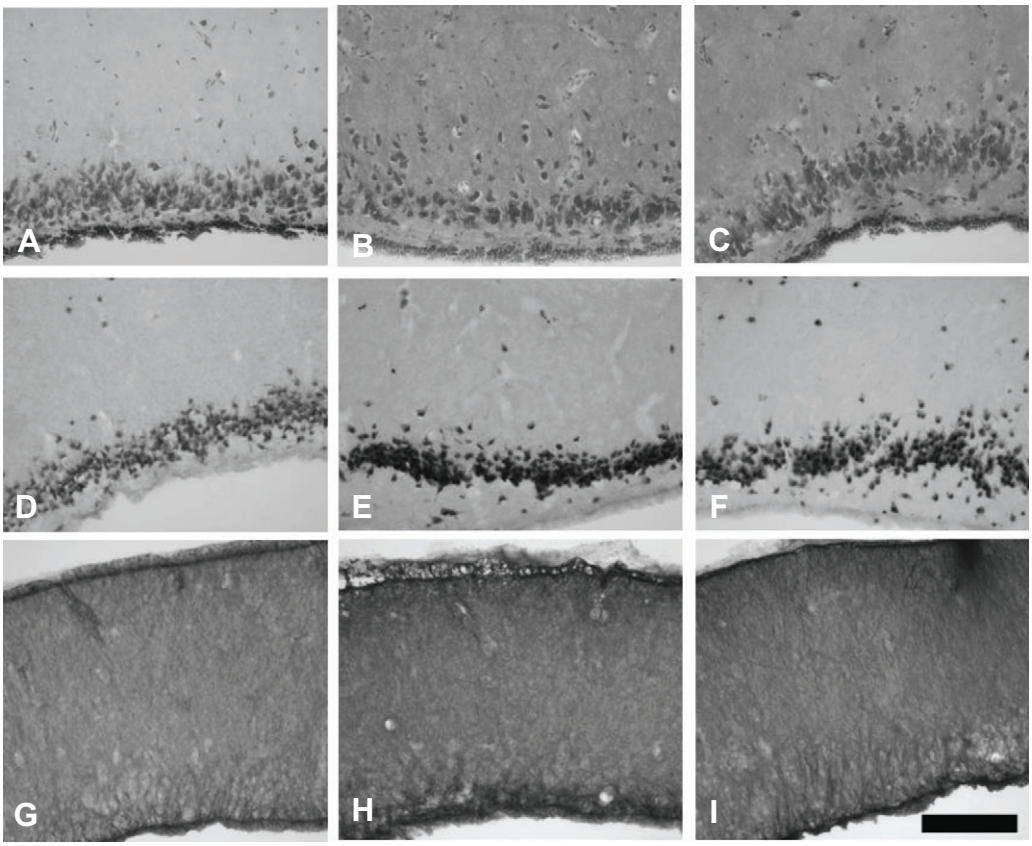

Fig. 1. Analysis of the cortex of the turtle for neuronal damage. (A–C) Cresyl Violet staining. (D–I) Immunolabeling for the neuronal marker NeuN (D–F), and the glial marker GFAP (G–I). Sections of tissue were examined from animals treated as follows: (A,D,G) Control; (B,E,H) anoxia; (C,F,I) anoxia followed by 3 days of survival. Note preservation of neuronal cell band at all time points in Cresyl Violet stained and NeuN-immunolabeled samples. GFAP signals were not increased at 3 days. Scale bar: 200 µm for all. [Figure reprinted from Kesaraju et al. (Kesaraju et al., 2009), with permission.]

(Fig. 1). Hypoxia tolerance in turtle species is not a matter of ectothermy per se, but is due to specific adaptations related to habitat: aquatic turtles with far northern ranges are more anoxia tolerant than southern animals even within a single species (probably because of the need to survive potentially long periods under ice or in hypoxic mud) (Ultsch, 2006). Interestingly, although hatchlings of many turtle species survive in the nest in their first winter through freeze tolerance (Storey, 2006) or super-cooling (Packard and Packard, 2003), in general hatchlings are far less able to tolerate anoxic submergence than adults (Reese et al., 2004). It has been suggested that this is because of their incomplete shell development, as the shell is important for lactate buffering (Ultsch, 2006). However, the development of anoxia tolerance is unknown, as only the hatchling and adult stages have been studied.

As with some of the other models discussed in this review, one mechanism to extend anoxic survival is entrance into a state of deep reversible hypo-metabolism; energy demand is reduced to meet the energy supplied by anaerobic glycolysis. Energy demanding processes are greatly suppressed; in the turtle brain these include decreases in excitatory neurotransmitter release (Milton and Lutz, 1998; Milton et al., 2002; Thompson et al., 2007), and increased neural inhibition (Lutz and Manuel, 1999; Nilsson and Lutz, 1991; Nilsson and Lutz, 1992). Decreased ion permeability (channel arrest), and the suppression of action potentials (spike arrest) also contribute to significant energy savings. Together, the reductions in ion flow and neurotransmitter release result in a reversible ‘coma’ of very reduced brain electrical activity (Fernandes et al., 1997). Protein synthesis is inhibited (Fraser et al., 2001), perhaps through epigenetic mechanisms (Biggar and Storey, 2012; Krivoruchko and Storey, 2010a), the phosphorylation–dephosphorylation of regulatory proteins (Rider et al., 2009) or through cell cycle arrest (Zhang et al., 2013).

Ion channels and neurotransmitters

Recent work has shown that many of these adaptations are intertwined, especially the interactions between neurotransmitter balance and various ion channels, with multiple and apparently redundant effects. For example, gamma-aminobutyric acid (GABA) induces anoxia-like decreases in excitatory post-synaptic potential (EPSP) activity in the normoxic turtle brain, apparently by the pre-synaptic inhibition of glutamate release. GABA also decreases ion current through glutamatergic NMDA and AMPA receptors (Pamenter et al., 2012), such that the stimulus required to generate an action potential increases more than 20-fold (Fig. 2). However, NMDA-receptor (NMDAR)-dependent excitotoxicity is also suppressed by δ-opioid receptors (Pamenter and Buck, 2008) which exist at surprisingly high density in the turtle brain (Xia and Haddad, 2001), and aid resistance to glutamate and hypoxic stress in mammals (Zhang et al., 2000). AMPA receptor currents, meanwhile, are also reduced by activation of mitochondrial ATP-dependent K^+ channels (Zivkovic and Buck, 2010), which in turn also reduce glutamate and dopamine release in early anoxia (Milton and Lutz, 2005; Milton et al., 2002). In longer anoxic exposures, glutamate release is suppressed by adenosine and GABA (Thompson et al., 2007). Adenosine in turn affects channel arrest (Pék and Lutz, 1997; Pérez-Pinzón et al., 1993), dopamine release (Milton and Lutz, 2005; Milton et al., 2002), NMDAR currents (Buck and Bickler, 1998) and cerebral blood flow (Hylland et al., 1994).

Neuroprotection at the molecular level

Despite the many pathways aimed at metabolic suppression, recent work has shown that a variety of protective mechanisms are instead activated at the molecular level in anoxic turtle brain. These include increases in heat shock proteins, anti-apoptotic factors, the MAP kinases, antioxidants and modulation of the p53 pathway.

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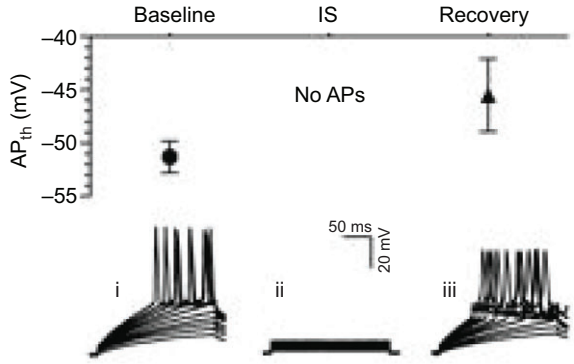

Fig. 2. Cortical neuronal membrane potential (mV) treated with an ischemic mimicking solution depolarizes to E_{GABA}. Top panel: summary of action potential (AP) threshold (AP_{th}) from stimulated neurons treated as indicated. APs could not be elicited during IS (ischemic solution) treatment. Bottom panel: sample recordings of evoked APs recorded during baseline control (i), IS perfusion (ii) and normoxic reperfusion (iii). [Adapted from Pamenter et al. (Pamenter et al., 2012); reprinted with permission from Macmillan Publishers Ltd.]

Interestingly, many of these factors may not only protect against damage under anoxic conditions, but also ameliorate oxidative stress when oxygen is restored.

Induction of the heat shock proteins (HSPs) is one of the first lines of defense against physiological stress, shifting cellular equilibrium away from apoptosis and towards survival (Lanneau et al., 2008; Obrenovitch, 2008). Although their specific roles in anoxia tolerance in the turtle are yet unknown, HSPs increase in a number of organs. Krivoruchko and Storey reported twofold to threefold elevations in several HSPs in skeletal muscle along with increases in both heat shock transcription factor 1 (HSF1) and the nuclear translocation of HSF1 in the heart and muscle (Krivoruchko and Storey, 2010b). In the brain, increases in both Hsp72 and Hsc73 were first reported by Prentice et al. (Prentice et al., 2004). The increase in the heat shock cognate Hsc73 was a novel finding, as this is one of the most abundant intracellular proteins in mammals but is considered unresponsive to stress (Snoeckx et al., 2001). Also surprising was the readily detectable normoxic levels of Hsp72, because in mammals it is essentially undetectable under control conditions (Snoeckx et al., 2001). Additional work has shown high basal levels of numerous HSPs in the brain (Kesaraju et al., 2009) and other organs (Stecyk et al., 2012) and their rapid upregulation in anoxia. Some HSPs continue to increase over 24 h anoxia in the brain; in mammals these are associated primarily with glia, leading to the speculation that in the turtle brain astrocytes may play a significant role throughout anoxia even if neurons shut down much of their function (Kesaraju et al., 2009).

The high normoxic levels of HSPs and rapid increase in response to low oxygen led to the suggestion that turtles essentially show ‘constitutive preconditioning’ in the face of anoxia, with high basal protein levels able to respond immediately to cellular stress (Prentice et al., 2004). Interestingly, Stecyk et al. reported HSP levels are also enhanced by cold temperatures, hypothesizing that turtles will experience winter temperatures before their ponds freeze over, and thus cold may further prepare them for anoxia before oxygen levels are fully depleted (Stecyk et al., 2012).

Hsp72 cytoprotection may occur through inhibition of apoptotic and necrotic cell death pathways (Giffard et al., 2008), with cell fate decided by the equilibrium between stress proteins and the apoptotic pathway (Beere, 2001), especially Bcl-2 and Bax levels. In the turtle brain, the Bcl-2:Bax ratio is maintained (Kesaraju et al., 2009) or slightly elevated (Nayak et al., 2011), in contrast to mammalian hypoxia and ischemia where increases in Bax and decreases in Bcl-2 tip the cell towards apoptosis (Feldenberg et al., 1999). In the turtle brain the balance of pro-death and survival pathways may also be affected by such factors as p53 and the MAP kinases.

The p53 transcription factor regulates the cell cycle, energy metabolism, DNA damage repair and apoptosis (Zhang et al., 2010), and is responsive to cellular stress. p53 is activated by metabolic stress, in part through AMP-activated protein kinase (AMPK) (Vousden and Ryan, 2009; Zhang et al., 2010), which increases in anoxic turtle white muscle (Rider et al., 2009). In addition, a recent study of the p53 target Tp53-induced glycolysis and apoptosis regulator (TIGAR) showed that activation reduced the generation of reactive oxygen species (ROS) and elevated levels of reduced glutathione (Wanka et al., 2012). Because the downregulation of energy pathways and protection against cell death and oxidative stress are hallmarks of anoxia tolerance, it is not surprising to find evidence of p53 activation in the turtle (Zhang et al., 2013). Interestingly, there is also cross-talk between p53 and the phosphoinositide 3-kinase–protein kinase B (PI3K/AKT) pathway (Ladelfa et al., 2011), which is upregulated in the anoxic turtle brain (Milton et al., 2008; Nayak et al., 2011).

Activated PI3K/AKT and extracellular regulated kinase (ERK1/2), generally considered to be cytoprotective, increase in turtle neurons *in vivo* (Milton et al., 2008) and *in vitro* (Nayak et al., 2011), as does Bcl-2. AKT is thought to work in part through interactions with the Bcl-2 family of proteins (Wang et al., 2007), and as with other components of anoxic survival, these pathways are linked to increases in adenosine. Blockade of the adenosine A1 receptor (A1R) prevents their upregulation, and increases levels of the pro-apoptotic factors JNK, p38MAPK and Bax (Nayak et al., 2011).

Anoxic survival mechanisms also reduce ROS damage

Unlike the mammalian brain, which shows an overproduction of reactive oxygen species (ROS) following hypoxia or ischemia/reperfusion (Hashimoto et al., 2003), the turtle brain appears to suppress ROS production upon re-oxygenation (Milton et al., 2007; Pamenter et al., 2007). As with other protective mechanisms, adenosine also impacts the production of ROS upon re-oxygenation (Fig. 3). Blockade of A1 adenosine receptors increases ROS release and cell death (Milton et al., 2007) despite high levels of antioxidants (Pérez-Pinzón and Rice, 1995; Rice et al., 1995; Willmore and Storey, 1997; Willmore and Storey, 2007). Adenosine effects on ROS may occur in part through Bcl-2, as overexpression decreases cell death during oxidative stress by enhancing antioxidant levels and suppressing free radicals (Lee et al., 2001). Recent work in the Milton laboratory has also shown that Hsp72 is involved in the reduction of ROS production (unpublished data). By affecting parts of the apoptotic pathway and possibly mitochondrial stability, then, the increases in Bcl-2, ERK1/2, AKT and certain HSPs are also likely to decrease oxidative stress during the recovery period, when ROS production might otherwise overwhelm even the high antioxidant levels of the turtle brain.

One recently discovered potential antioxidant in the turtle is neuroglobin (Burmester et al., 2000), which has also been studied in other models of hypoxia tolerance (Avivi et al., 2010; Mitz et al., 2009; Roesner et al., 2008; Schneuer et al., 2012). Neuroglobin is strongly upregulated in both hypoxia and upon re-oxygenation in the turtle (Milton et al., 2006; Nayak et al., 2009), and decreasing neuroglobin expression with turtle-specific siRNA doubles ROS

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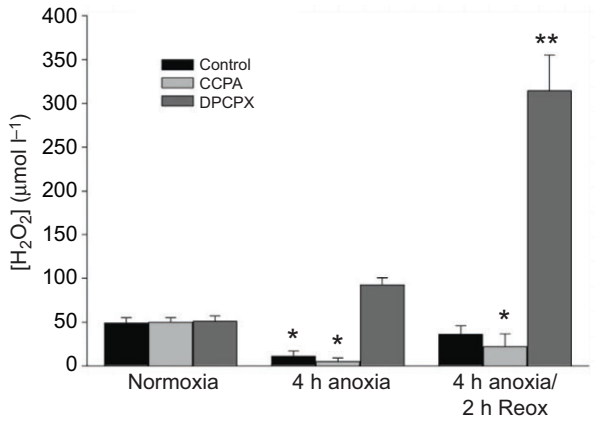

Fig. 3. H_2O_2 concentration in the medium of neuronally enriched primary cell cultures treated with either the adenosine agonist 2-chloro-N6-cyclopentyladenosine (CCPA) or antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). Anoxia for 4 h significantly decreased ROS production except in DPCPX-treated cells. Re-oxygenation increased ROS production only to normoxic levels in controls, whereas CCPA reduced and DPCPX increased ROS production. Asterisks indicate a signficant difference from normoxic cells, **P*<0.05, ***P*<0.01. Data are means ± s.e.m., *N*=3 independent experiments/group. [Figure adapted from Milton et al. (Milton et al., 2007), with permission.]

release upon re-oxygenation. However, this does not increase cell death, so it appears that turtle neurons are sufficiently protected by other mechanisms to withstand a twofold increase in ROS (Nayak et al., 2009); additional roles for neuroglobin in the turtle brain have not been investigated.

And finally, despite their remarkable tolerance to anoxia, histological examination of *T. scripta* brains that had not been exposed to anoxia in the lab showed some evidence of brain lesions, suggestive of prior damage probably occurring during long periods of anoxia during winter hibernation (S.L.M., unpublished observation). This led us to investigate the possibility of neuronal regeneration as a long-term mechanism of anoxia tolerance. When heavily damaged by global ischemia, *T. scripta* showed evidence of neuronal reproduction within 3 weeks (Kesaraju and Milton, 2009), adding yet another strategy to the toolbox of anoxic survival in these remarkable animals.

Raising the 'dead': mechanisms of hypoxia tolerance in a hibernating species

Hibernation – prolonged periods of suspended blood flow and metabolism

Like the metabolic suppression seen in turtles, certain mammals enter periods of metabolic suppression known as hibernation. Hibernation was initially proposed as a model of resistance to ischemic brain injury because cerebral blood flow declines to levels that would produce ischemic injury in humans (Frerichs et al., 1994). It was subsequently found that brain and other organs of hibernating species resist ischemic injury better than classic rodent models even when animals are not hibernating (Dave et al., 2006; Kurtz et al., 2006) and the same is true for hypoxic injury (Bullard et al., 1960; Drew et al., 2004). Interestingly, because of metabolic suppression during hibernation, arterial partial pressure of oxygen (P_{aO_2}) is similar to other mammals. However, P_{aO_2} falls significantly during arousal from hibernation (Ma et al., 2005). Whether resistance to ischemic or hypoxic injury depends on the hibernation season, and thus seasonal expression of a hibernation phenotype, remains a matter of debate and may depend on the tissue and species studied (Christian et al., 2008; Kurtz et al., 2006). Here we provide a brief overview of hibernation and review evidence for resistance to ischemia/reperfusion (I/R) injury in hibernating species with emphasis on the arctic ground squirrel (AGS), *Urocitellus parryii*.

Hibernation is a means of systemic energy conservation that involves an orchestration of behavioral, physiological and molecular adaptations or specializations that defies the need for resources such as food and water as well as processes such as blood flow. Diverse mammalian species, including one species of primate (Dausmann et al., 2004), hibernate with similar characteristics, such as a decrease in body temperature (T_b) and metabolic rate with a minimum torpid metabolic rate that is on average 5–30% of basal metabolic rate (Geiser, 2004) and inter-bout arousals (Dausmann et al., 2004). When hibernating, animals may spend from a few days to several weeks at a time in a highly regulated and reversible state of prolonged torpor during which whole body metabolic rate, core T_b, heart rate and blood flow plummet to levels seemingly inconsistent with life support. When T_b falls below 30°C, prolonged periods of torpor are interrupted by brief intervals of euthermy during which animals spontaneously return to high, euthermic T_b of 35–37°C (Dausmann et al., 2004; Geiser and Ruf, 1995) and blood flow returns to vital organs in a heterogeneous manner (Osborne et al., 2005).

Tolerance to hypoxia, cerebral ischemia and brain injury during hibernation

During hibernation, metabolic rate drops to a minimum, which in the AGS is 1–2% of resting metabolic rate. While metabolic rate declines, cerebral blood flow decreases as much as 10-fold. Mass-weighted cerebral blood flow in hibernating animals is 7±4 ml 100 g^{-1} min^{-1} compared with 62±18 ml 100 g^{-1} min^{-1} in active, euthermic, animals (Frerichs et al., 1994). Despite prolonged ischemic-like levels in local cerebral blood flow and reperfusion-like return of cerebral blood flow upon arousal, ground squirrels show no evidence of ischemic injury (Frerichs et al., 1994; Ma et al., 2005), and some restorative processes (von der Ohe et al., 2006; Weltzin et al., 2006). In addition to hypo-metabolism, other aspects of the hibernation phenotype may contribute to ischemia tolerance in the hibernating state including cold tissue temperature, immunosuppression, anticoagulant properties of the blood and increased antioxidant defenses (Drew et al., 2001; Zhou et al., 2001).

Resistance to cerebral ischemia/reperfusion injury when euthermic

Even when not hibernating, however, AGS and other species of ground squirrels tolerate hypoxia (D'Alecy et al., 1990) and ischemic-like conditions better than ischemia-vulnerable species such as rat (Christian et al., 2008; Dave et al., 2006; Frerichs and Hallenbeck, 1998). Marked tolerance to hypoxia in fossorial species such as the naked mole rat (Larson and Park, 2009) suggests that tolerance in AGS may be the result of the fossorial nature of ground squirrels. Indeed, ground squirrels and hamsters, both semi-fossorial species, tolerate hypoxia better than other rodent species (Bullard et al., 1960; D'Alecy et al., 1990). Evidence also suggests that the AGS experiences hypoxemia regularly during emergence from hibernation and that hypoxemia and ischemia tolerance may be related to selective pressures associated with transitions into and out of torpor (Ma et al., 2005) (Fig. 4). During rewarming and in some cases, during euthermy MAPK stress pathways are activated and nitric oxide synthase (iNOS), another indicator of cellular stress, is increased (Zhu et al., 2006). Moreover, Hypoxia-inducible factor 1-alpha (HIF-1α) increases after rewarming and remains elevated

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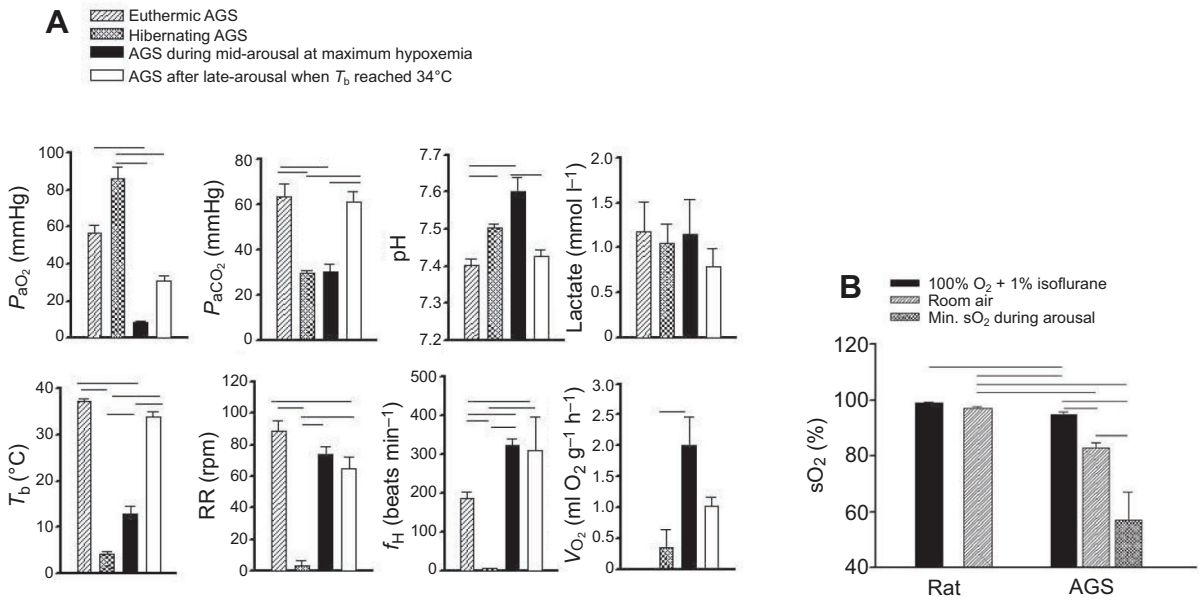

Fig. 4. P_{aO_2} and oxygen saturation during torpor and arousal in arctic ground squirrel (AGS) shows evidence of hypoxemia during arousal. Note also that in euthermic AGSs (i.e. those that are not hibernating) P_{aO_2} and oxygen saturation (sO_2) are lower than in the hibernating state or in rat. (A) Mean blood gas values, and other parameters during arousal at maximum hypoxemia (mid arousal) and during late arousal in AGS compared with euthermic (n=8) and hibernating AGS (n=7) during torpor and arousal, at the time when P_{aO_2} was minimal, and after T_b reached 34°C. (B) Hemoglobin sO_2 in euthermic AGS (n=9) and rats (n=8) under light anesthesia (100% O_2 with isoflurane, 1%) and while breathing room air. sO_2 was monitored in another group of AGS during arousal from hibernation and the minimum sO_2 recorded during arousal is shown for comparison with euthermic levels. Horizontal lines indicate significant difference between groups. T_b, core body temperature; RR, respiratory rate; f_H, heart rate; V_{O_2}, rate of O_2 consumption. [From Ma et al. (Ma et al., 2005).]

during euthermy (Ma et al., 2005). Marked synaptogenesis and activation of proliferative stress activated signaling pathways following rewarming from hibernation suggest that AGSs may benefit from restorative processes during periods of inter-bout euthermy (Drew et al., 2004; von der Ohe et al., 2006; Weltzin et al., 2006), and like turtles, restorative processes may contribute to tolerance to ischemia and anoxia (Drew et al., 2011; McGee et al., 2008; Popov et al., 2011).

Studies designed to tease out the influence of the hibernation state on protection from hypoxia and I/R in isolated brain tissue found that cold tissue temperature alone could account for enhanced protection in the hibernating state. When hippocampal slices from AGS are exposed to oxygen and glucose deprivation (OGD) at 36–37°C, resistance to injury is similar regardless of the torpid state of the animal (Christian et al., 2008; Ross et al., 2006). Tolerance to OGD *in vitro* suggested that AGSs would tolerate global cerebral ischemia *in vivo*. Dave et al. challenged summer active AGSs and rats with 8 min of asphyxia leading to cardiac arrest (Dave et al., 2006). Although asphyxia induced an immediate bradycardia and cardiac arrest in both species, only rats showed significant cell death in hippocampus, striatum and cortex 7 days after restoration of spontaneous circulation (Dave et al., 2006) (Fig. 5). In a subsequent study, summer active AGSs challenged with 10 min of asphyxia showed no significant loss of healthy neurons in the vulnerable CA1

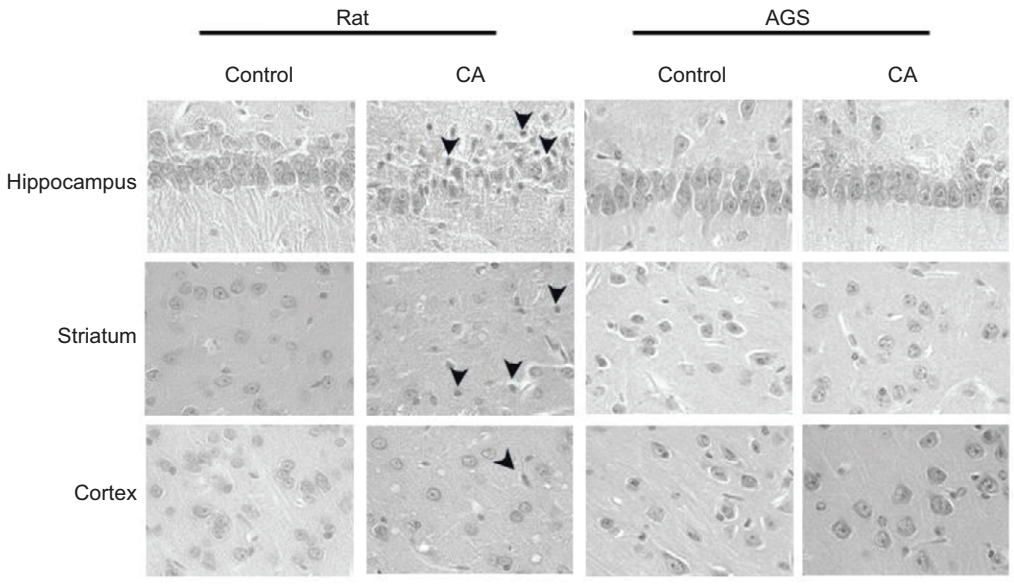

Fig. 5. Arctic ground squirrels (AGSs) resist injury in all regions studied including the hippocampus, striatum and cortex following cardiac arrest. Representative histological images of the hippocampal CA1 region of AGSs and rats subjected to sham or 8 min of asphyxia leading to cardiac arrest. Spontaneous circulation was restored within 120 s and tissues were collected for histopathology 7 days later. Sections were stained with Hematoxylin and Eosin. Arrowheads indicate ischemic neurons. All images were captured at 40× magnification. [From Dave et al. (Dave et al., 2006).]

region of the hippocampus when compared with naïve AGSs (Dave et al., 2009).

Resistance to cerebral ischemia/reperfusion injury in the arctic ground squirrel does not require preparation for the hibernation season or the hibernation state

Resistance to brain injury following cardiac arrest in summer active (euthermic) AGSs led to doubt about how much of the tolerance to I/R noted in AGSs was due to the hibernation season and how much was intrinsic to the species. Liver and intestine isolated from the thirteen-lined ground squirrel during inter-bout arousal resist I/R injury; however, tolerance is lost or decreased when tissue is obtained from animals during the summer season (Kurtz et al., 2006; Lindell et al., 2005). By contrast, in AGS, tolerance to cerebral I/R in acute hippocampal slices is actually greater during the summer season than during winter inter-bout euthermy; where, only during inter-bout euthermy is any difference noted between active and hibernating ground squirrels (Christian et al., 2008). In other species, the state of hibernation seems to enhance tolerance relative to active (euthermic) animals and rats, although, as bath temperature is decreased, tolerance of brain slices from active animals approaches the degree of tolerance observed in slices from hibernating animals (Frerichs and Hallenbeck, 1998). In future studies better distinction between summer euthermic, winter inter-bout euthermy, and winter season animals that do not show signs of hibernation might clarify if or when the hibernating state contributes to ischemia tolerance. Importantly, cooler temperatures clearly increase tolerance and are expected to play a primary role in neuroprotection in hibernating AGS *in vivo* (Zhou et al., 2001).

Resistance to ischemia/reperfusion in the euthermic state involves events downstream of loss of ATP and NMDAR activation

Tolerance to OGD in euthermic AGSs involves aspects of enhanced ion homeostasis and events downstream of NMDAR activation. OGD leads to a loss of ATP in brain slices from AGSs as it does in brain slices from rats (Christian et al., 2008). Despite a loss of ATP, ionic homeostasis persists longer in AGSs than in rats both *in vitro* and *in vivo*, and preservation of ionic homeostasis in AGSs depends upon protein kinase C epsilon (εPKC) signaling. Dave et al. monitored ischemic depolarization (ID) in cerebral cortex during cardiac arrest *in vivo* and during OGD *in vitro* in acutely prepared hippocampal slices from AGS and rat (Dave et al., 2009). In both the *in vitro* and *in vivo* models of global cerebral ischemia, the onset of ID was significantly delayed in AGS compared with rat. During cardiac arrest, ID occurred on average at 1.9 min in rat and at 3.1 min in AGS. During OGD in hippocampal slices, ID occurred at 2.8 min in rat and at 6.6 min in AGS. The selective peptide inhibitor of εPKC (eV1-2) shortened the time to ID in brain slices from AGSs but not in rats even though εV1-2 decreased activation of εPKC in brain slices from both species. Activation of εPKC inhibits Na^+/K^+ATPase and voltage-gated sodium channels (Chen et al., 2005; Nowak et al., 2004), both of which contribute to the collapse of ion homeostasis during ischemia and may be targets of εPKC during cerebral ischemia in AGSs (Dave et al., 2009). Blocking or delaying the ID can significantly improve recovery (Anderson et al., 2005; Takeda et al., 2003).

Other mechanisms downstream of delayed ID may also contribute to cerebral ischemia tolerance in AGS. During cerebral ischemia, the loss of neuronal membrane potential that leads to ID results in the massive release of neurotransmitters, including the excitatory neurotransmitter glutamate (Lipton, 1999). Glutamate efflux into the extracellular space activates NMDA and AMPA receptors, causing excitotoxic calcium influx (Lipton, 1999).

Although ID is delayed in AGS, once it occurs glutamate efflux approximates efflux seen in rat hippocampal slices, but with minimal evidence of cell death in AGS (Drew et al., 2012). Thus, glutamate released during OGD in AGS hippocampal slices is not excitotoxic. Attenuated excitotoxicity in AGS is further supported by evidence in the semi-acute slice preparation developed by Ross et al. in which 500 μmol l^{-1} NMDA plus 20 mmol l^{-1} KCl fails to induce cell death in AGS (Ross et al., 2006). By contrast, the same concentrations of NMDA and KCl applied to slices from rat produces a significant increase in cell death (Ross et al., 2006). Protective mechanisms downstream to glutamate efflux may be related to differences in the effects of glutamate receptor activation in AGS compared with rat.

Lower levels of functional NMDA receptors located in the plasma membrane and less glutamate-induced increases in intracellular calcium are associated with ischemia tolerance in euthermic AGS. Membrane expression of NR1, an obligatory subunit of the NMDA receptor, is lower in AGS (both active and hibernating) than in rat (Zhao et al., 2006). Moreover, intracellular calcium increased by bath-applied glutamate does not exceed 400 nmol l^{-1} in hippocampal slices prepared from euthermic or hibernating AGS, whereas in rats intracellular calcium increased by bath-applied glutamate exceeds 500 nmol l^{-1} (Fig. 6) (Zhao et al., 2006). Evidence of blunted excitotoxicity and blunted calcium responses to glutamate is consistent with similar phenomena in the naked mole-rat (Peterson et al., 2012a; Peterson et al., 2012b).

AGSs do not rely on glycogen or oxidative phosphorylation for energy needs during OGD

Many organisms tolerant of low oxygen levels possess large stores of glycogen and pH buffering mechanisms that fuel and protect against pH shifts resulting from anaerobic glycolysis (Jackson, 2004; Lutz and Milton, 2004). Indeed, AGSs demonstrate enhanced pH buffering capacity because blood pH remains around 7.4 despite arterial P_{CO_2} levels of 60 mmHg (Ma et al., 2005) and arterial HCO_3^- concentrations tend to be higher in AGS than in rat. However, ischemia tolerance cannot be explained by enhanced peripheral stores of glycogen. Firstly, glucose derived from glycogen is not expected to reach ischemic tissue when blood flow is stopped during cardiac arrest. Secondly, addition of iodoacetate, an inhibitor

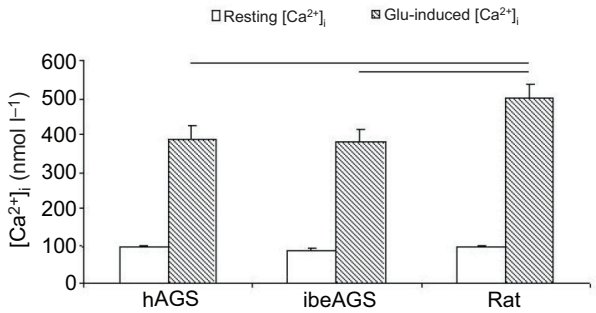

Fig. 6. Hibernating arctic ground squirrel (hAGS), AGS in the inter-bout euthermic state between bouts of torpor (ibeAGS) and rats display similar resting internal calcium concentrations $[Ca^{2+}]_i$. Glutamate induces a significant increase in $[Ca^{2+}]_i$ in all groups, however, both ibeAGS and hAGS show less glutamate-induced $[Ca^{2+}]_i$ increase compared with rat. Organotypic hippocampal slices were loaded with fura-2AM for calcium imaging under control conditions and after treatment with glutamate. Horizontal bars indicate significant difference between groups, $P<0.05$, n=12–17 slices per group. [From Zhao et al. (Zhao et al., 2006).]

of glycolysis, which would negate any benefit from glycogen, as well as the addition of NaCN, an inhibitor of cellular respiration, which would prevent use of residual oxygen in the bath, during OGD fails to increase cell death in an acute slice preparation (Christian et al., 2008).

In summary, arctic ground squirrels, similar to other hibernating rodents, experience periods of prolonged torpor when oxidative metabolism and other life-supporting processes are suspended for weeks at a time. For unknown reasons torpor is interrupted by brief, albeit regular periods of euthermy. During regular arousal to euthermy AGSs experience hypoxemia with less than 60% O_2 saturation in arterial blood (Ma et al., 2005). Despite these periods of hypoxemia, AGSs and other rodent species recover from hibernation without injury and show signs of restorative processes including synaptogenesis (von der Ohe et al., 2006) and cognitive enhancement (Weltzin et al., 2006). Similarly, when challenged by a number of experimental models of I/R, hibernating species including AGS resist injury to the brain as well as other organs. Studies show that resistance to I/R injury in the brain is independent of the hibernation season or the hibernation state (Christian et al., 2008; Dave et al., 2006; Dave et al., 2009). In hippocampal slices from AGS, oxygen and glucose deprivation produces an overflow of glutamate but dramatically less cell death than in slices from rat (Drew et al., 2012). AGS hippocampal slices also resist NMDA-induced excitotoxicity and demonstrate smaller increases in intracellular calcium following bath application of glutamate (Ross et al., 2006; Zhao et al., 2006). Taken together evidence points to species-dependent differences in NMDAR function that contributes to resistance to I/R injury in AGS brain.

When the brain goes diving: adaptations for hypoxia tolerance in diving mammals

Although in diving mammals periods of hypoxia exposure are shorter than those seen in the hibernating turtle or ground squirrel, their aquatic lifestyles, involving both breath-hold diving and exercise, may still induce severely hypoxic conditions. During diving, these animals rely on large endogenous stores of O_2 – either bound to hemoglobin in their blood or to myoglobin in their skeletal muscles – to support oxidative metabolic processes (Burns et al., 2007; Lenfant et al., 1970; Scholander, 1940). Such adaptations, along with cardiovascular and metabolic adjustments (Blix and Folkow, 1983; Folkow and Blix, 2010; Ponganis et al., 2011; Scholander, 1940), enable some seals and whales to remain submerged for a staggering 2 h (Hindell et al., 1991; Watkins et al., 1985). But towards the end of dives, arterial blood O_2 tension may still drop to only 12–20 mmHg, even during routine free diving (Meir et al., 2009; Qvist et al., 1986; Scholander, 1940), and encephalographic recordings made during simulated diving in seals have shown that cerebral integrity is maintained down to 7–10 mmHg (Elsner et al., 1970; Kerem and Elsner, 1973). These levels are lower than ‘the critical arterial O_2 tension’ of 25–40 mmHg, at which impairments from limitations in ATP production are first seen in brains of non-diving mammals (Erecińska and Silver, 2001), which begs the question: how does the brain of diving mammals cope with repeated and extreme hypoxemia during diving?

Cerebral substrate supply

Cerebral blood flow is generally well maintained during prolonged simulated diving in seals, despite the otherwise widespread vasoconstrictor response (Blix et al., 1983; Zapol et al., 1979), and the challenge encountered by the diving brain therefore is different from that of the ischemic brain. However, blood-borne glucose will be delivered in steadily declining amounts (Guppy et al., 1986), particularly during long dives, because glucose then cannot be readily replenished from the liver due to its much reduced blood supply (Blix et al., 1983; Zapol et al., 1979). However, data from freely diving seals suggest that blood glucose depletion does not limit diving capacity (Guppy et al., 1986), which in part is related to the favorably low brain-mass-to-blood-volume ratio of seals (Hochachka, 1981). Brain metabolism may also be further supported by endogenous stores of glycogen, which are larger in divers than in non-divers by a factor of two to three but still quite small compared with those of skeletal and cardiac muscles (Kerem et al., 1973). Thus, the steadily declining blood O_2 content remains the major challenge to the diving brain.

O_2 diffusion and possible roles of neuroglobin

One possible compensatory adaptation to the hypoxemic challenge would be to maintain a high cerebral capillary density, which would reduce the diffusion distance and thereby improve the flow of O_2 to neurons. Indeed, two studies suggest that the brain capillary density of seals and whales is higher than in typical non-diving species (Glezer et al., 1987; Kerem and Elsner, 1973).

Downstream, cellular O_2 flow might be enhanced by means of facilitated intracellular diffusion, i.e. as achieved by myoglobin in skeletal muscle cells (Wittenberg and Wittenberg, 2003). Diving mammals maintain high levels of both myoglobin and hemoglobin and might therefore be expected to also carry higher loads of the neurally based neuroglobin. This globin, discovered about a decade ago (Burmester et al., 2000), is thought to play a key role for maintenance of aerobic metabolism in neural tissue, possibly by facilitating O_2 diffusion (Burmester and Hankeln, 2009). However, neuroglobin may also have other functions related to hypoxia defense, such as the detoxification of reactive oxygen species (ROS) (Burmester and Hankeln, 2009) that are generated during and after diving (Zenteno-Savín et al., 2002). Interestingly, studies in the deep-diving hooded seal (*Cystophora cristata*) have revealed that their cerebral neuroglobin levels are not higher than those of rodents or man (Mitz et al., 2009). Instead, the protein has an unusual cellular distribution, with higher levels in glial cells (astrocytes) than in neurons. This distribution, which contrasts with that in terrestrial mammals (Mitz et al., 2009), was later confirmed in other seal species (Fig. 7A) (Schneuer et al., 2012). Because neuroglobin has repeatedly been shown to be closely associated with mitochondria, thereby implying a key role in oxidative metabolism (Burmester and Hankeln, 2009; Mitz et al., 2009), these findings suggest that in the seal brain, glial cells are more involved in aerobic metabolism than are neurons. This further implies that seal brain neurons depend more heavily on anaerobic metabolic pathways, whereas glial cells/astrocytes may remove and metabolize the lactate that thereby is produced, and that seal brains may have a reversed lactate shuttle system compared with non-diving mammals (Mitz et al., 2009; Schneuer et al., 2012), a hypothesis that is currently under investigation. Whales, in contrast, have recently been shown to have a typical mammalian neuroglobin distribution, with higher levels in neurons than in astrocytes (Fig. 7B), but in whales neuroglobin mRNA expression levels are 4 to 15 times higher than in seal, cow (*Bos taurus*) and ferret (*Mustela putorius furo*) brains (Fig. 7C) (Schneuer et al., 2012). This finding is consistent with a possible role for neuroglobin in facilitated diffusion and local storage of O_2 within whale neurons. It, thus, appears that neuroglobin may convey brain hypoxia tolerance in both seals and whales, but that its role is quite different in the two orders.

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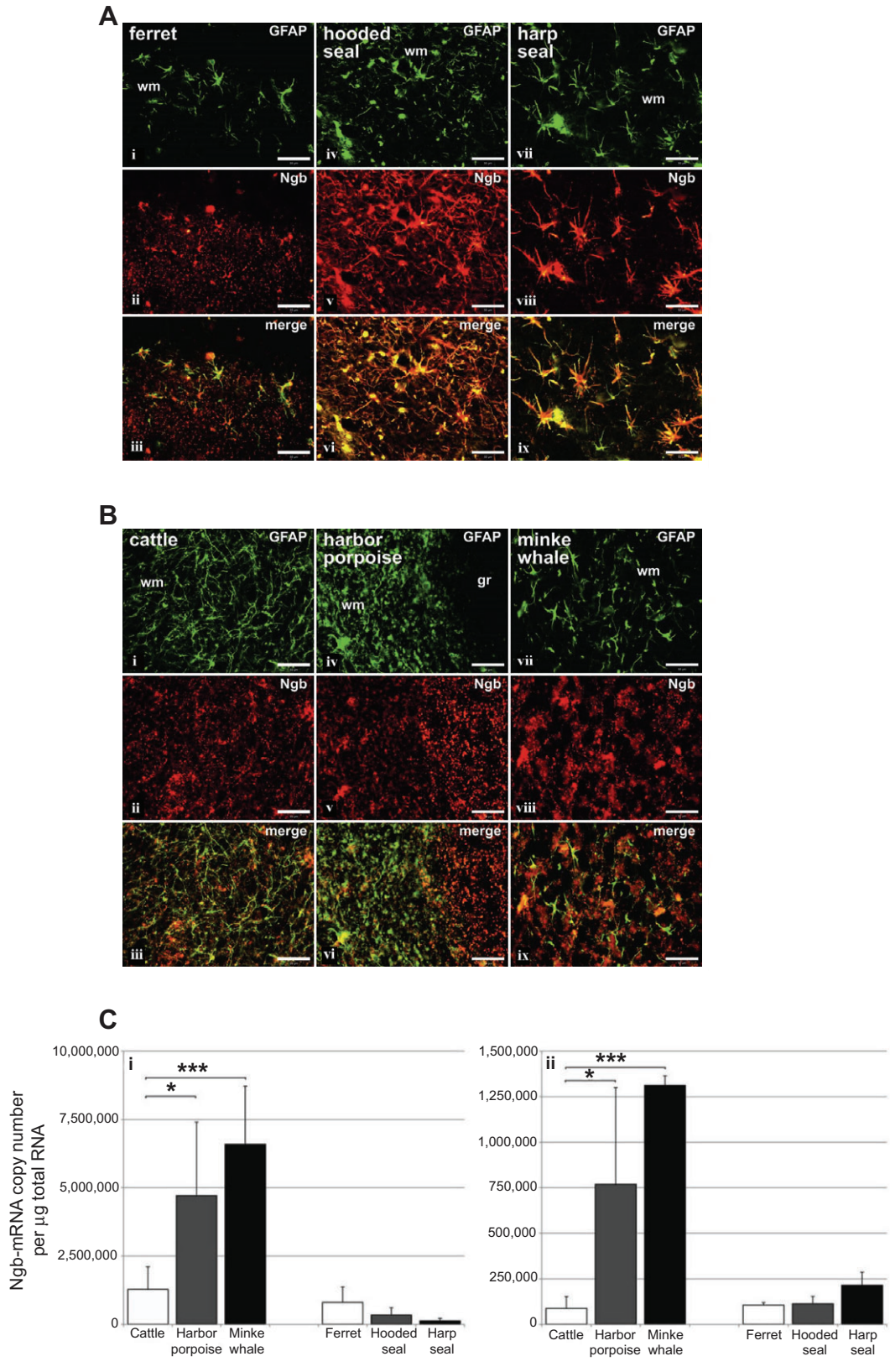

Fig. 7. Neuroglobin distribution. (A) Immunofluorescence of the glial marker glial fibrillary acidic protein (GFAP; green) and neuroglobin (Ngb; red) in the cerebrum of ferret, *Mustela putorius furo* (i–iii), hooded seal, *Cystophora cristata* (iv–vi) and harp seal, *Pagophilus groenlandicus* (vii–ix), showing colocalization of GFAP and Ngb (merge; yellow) in astrocytes in hooded and harp seals (vi,ix), but to a much lesser extent in ferrets (iii), in which Ngb immunoreactivity was observed in cortical neurons (c). (B) Immunofluorescence of GFAP (green) and Ngb (red) in the cerebrum of cattle, *Bos taurus* (i–iii), the odontocete harbor porpoise, *Phocoena phocoena* (iv–vi) and the mysticete minke whale, *Balaenoptera acutorostrata* (vii–ix), showing Ngb immunoreactivity in neurons of the cerebral cortex in all species (ii,v,viii) and only marginal colocalization of GFAP and Ngb (merge; yellow) in astrocytes (iii,vi,ix). (C) Quantification of Ngb mRNA expression in (i) the cerebral cortex and (ii) the cerebellum of whales and seals compared with cattle and ferret. In both brain regions, whales show significantly higher Ngb mRNA levels compared with cattle, ferret and seals (***P≤0.001, *P≤0.05). wm, white matter; gr, gray matter. Scale bars: 50 µm. [Reprinted and modified from Schneuer et al. (Schneuer et al., 2012), with permission from Elsevier.]

Enhanced cerebral anaerobic capacity

Cerebral tolerance towards extreme diving hypoxia may, thus, depend on a high anaerobic capacity. Earlier studies in seals subjected to simulated diving have shown that lactate levels rise in the venous effluent from their heads (which presumably is dominated by cerebral venous drainage), but only towards the end of long dives, when arterial blood O_2 tension drops below 25 mmHg and the difference in arterio-venous blood oxygen content starts to decrease (Hochachka, 1981; Kerem and Elsner, 1973). This suggests that the brain's dependence on anaerobic metabolism rises as the animal becomes

severely hypoxemic. The maintained blood glucose supply (Guppy et al., 1986) in combination with fairly large endogenous glycogen stores (Kerem et al., 1973) and a high enzymatic capacity for anaerobic glycolysis in brains of divers (Messelt and Blix, 1976; Murphy et al., 1980; Shoubridge et al., 1976) all indicate enhanced cerebral anaerobic capacity, although not likely to be sufficient to support normal resting brain function (Hochachka, 1981). However, *in vitro* recordings from spontaneously active isolated neocortical slices from hooded seals have shown that these may maintain a high spiking activity for up to 60 min in severe hypoxia (Ramirez et al., 2011), and hooded seal cerebellar slices may even maintain a high spontaneous activity for durations of 10–15 min in chemical anoxia (2 mN NaCN) (L.P.F., S. Ludvigsen and S. Geiseler, unpublished observations), as also previously demonstrated in eider ducks, *Somateria mollissima* (Ludvigsen and Folkow, 2009). The ATP required to maintain neural integrity and activity under the latter conditions must have been derived through anaerobic pathways, because CN^- effectively blocks oxidative phosphorylation.

Evidence of cerebral hypometabolic responses

Regardless of mode of ATP production, the diving brain would benefit if its metabolic activity was depressed during the asphyxic challenge. Some depression is likely to result from the Q_{10} effect of the up to 3–4°C drop in brain temperature that has been documented in harp seals (*Pagophilus groenlandicus*) and hooded seals during simulated diving (Fig. 8) (Blix et al., 2010; Odden et al., 1999). Such brain cooling probably also protects neurons by limiting both primary and secondary injury through its other known effects on metabolic, molecular and cellular events (Yenari and Han, 2012). Also, data from harbor seals (*Phoca vitulina*) that were subjected to simulated dives in the laboratory suggest that cerebral O_2 uptake does decrease towards the end of long dives, but a simultaneous rise in lactate release implies that this drop – at least in part – reflects insufficient oxygen supply, rather than metabolic depression (Kerem and Elsner, 1973). However, evidence for direct intrinsic hypometabolic responses to hypoxia and/or chemical anoxia have been derived from other tissues: in *in vitro* studies of both seal liver (Hochachka et al., 1988) and kidney slices (Hong et al., 1982), which seem to display both metabolic arrest (depressed ATP production rates) and channel arrest (reduced ion permeability) in response to O_2 deprivation (Hochachka et al., 1988; Murphy et al., 1980). A similar (pilot) study using seal brain slices suggested normal mammalian hypoxia sensitivity (Hochachka et al., 1988), but more recent studies show that the cerebral hypoxia tolerance of these animals is due, in part, to intrinsic neuronal properties, because intracellularly recorded cortical pyramidal neurons of acute brain slices from hooded seals maintain near-normal resting membrane potential (Fig. 9) and the ability to generate action potentials when stimulated, even when subjected to severely hypoxic conditions that cause mouse neurons to rapidly depolarize (Folkow et al., 2008). Also, as in eider ducks (Ludvigsen and Folkow, 2009), isolated cerebellar slices from seals can have differential responses to hypoxia or chemical anoxia, in that some sites maintain spontaneous activity whereas others shut down but recover even after 1 h of insult (L.P.F., S. Ludvigsen and S. Geiseler, unpublished observations). This suggests that some neurons may enter into an inactive state while others remain active, which possibly reflects a reconfiguration at the organ level that would allow some networks to continue to control vital functions while others conserve energy by entering into a hypometabolic state (Ramirez et al., 2007). Studies of cellular processes that may underlie the unusual neural hypoxia tolerance of diving mammals and which could also explain their apparent high resistance towards post-dive oxidative stress and damage (e.g. studies of Ca^{2+} influx rates and NMDA receptor functions of neuroglobin and of cerebral antioxidant capacity) are under way, but at a pace that is set by the inherent logistic difficulties involved in accessing and studying these mammals from this perspective.

Buried alive! Arrested development and hypoxia tolerance in the naked mole-rat

In contrast to the other species discussed so far, fossorial animals experience chronic environmental hypoxia, rather than seasonal or occasional episodes of oxygen deprivation. African naked mole-rats (*Heterocephalus glaber*), are unusual even among other subterranean and other mole-rat species. Most notably, they are cold-blooded (Buffenstein and Yahav, 1991), they are the longest-lived rodent known – with lifespans exceeding 30 years (Buffenstein, 2008) – and they live in a eusocial structure similar to ants and bees (Jarvis, 1981). A key aspect of the naked mole-rat's lifestyle in the context of our investigations is that they live in colonies with a great many individuals, some colonies having more than 300 members (Brett, 1991). The combination of a large number of extremely

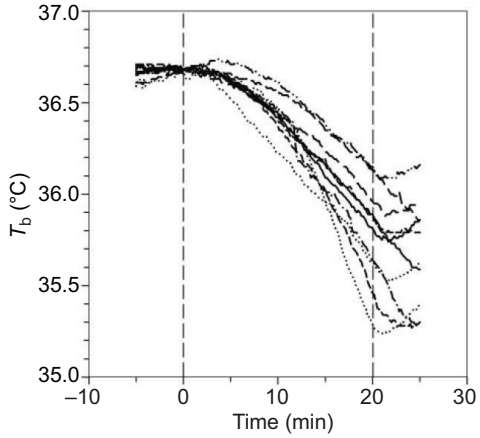

Fig. 8. Changes in brain temperature (T_{brain}; °C) of a hooded seal during 10 experimental dives lasting for 20 min (between vertical bars) and during the first 5 min of the recovery period. Lines for individual dives were normalized to fit the median brain temperature at the start of diving (t=0). [Reprinted from Blix et al. (Blix et al., 2010).]

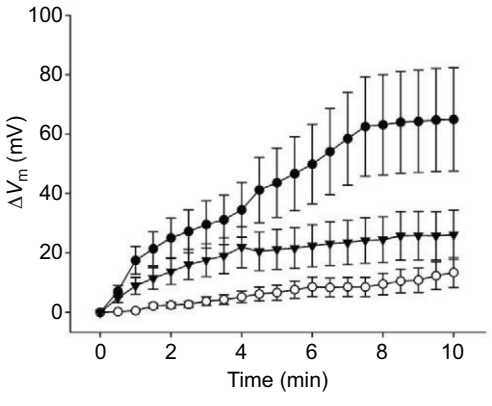

Fig. 9. Average membrane potential changes (ΔV_m) in cortical pyramidal neurons of hooded seals and mice, as evaluated every 30 s during the initial 10 min of severe hypoxia. Closed circles, adult mice (n=14); closed triangles, neonatal mice (n=10); open circles, adult hooded seals (n=7). Values are means ± s.e.m. [Reprinted from Folkow et al. (Folkow et al., 2008), with permission from Elsevier.]

social animals living in a crowded subterranean space where ventilation is poor means that naked mole-rats are exposed to chronically low levels of O_2 (and high levels of CO_2). Although gas concentrations have not been measured in naked mole-rat burrows in nature, in other fossorial species O_2 levels can be as low as 6–14% and CO_2 levels can be as high as 6–10% (Arieli, 1979; van Aardt et al., 2007). Because most of these measurements are for burrows of solitary animals or small colonies, levels in large colonies of naked mole-rats may reach even more extreme values. Consistent with living in an oxygen-deprived environment, naked mole-rats have high-O_2-affinity hemoglobin (Johansen et al., 1976), and a low resting metabolism (Buffenstein and Yahav, 1991).

In addition to the chronic environmental hypoxia of fossorial life, naked mole-rats also experience acute hypoxia during certain behaviors such as foraging and tunnel excavation. We studied the acute response of naked mole-rat brain tissue to hypoxic challenge in two ways using hippocampal slices maintained *in vitro* (Larson and Park, 2009). In the first, we measured the oxygen sensitivity of synaptic transmission in slices from naked mole-rats and mice. Slices were maintained in 'interface-style' chambers where the lower surface is exposed to the artificial cerebrospinal fluid perfusate and the upper surface is exposed to 100% O_2 (less 5% CO_2 to maintain pH with a bicarbonate buffer system). In this type of chamber, the slice draws O_2 directly from the chamber atmosphere and it is standard practice to use a 'carbogen' gas mixture containing enough CO_2 (5%) to buffer the pH of the artificial cerebrospinal fluid (ACSF) and the balance (95%) containing pure O_2. The situation regarding O_2 availability to the tissue is very different from that *in vivo*; however, it is worth noting that the timing of neuronal responses to hypoxia in interface slice chambers more closely models *in vivo* responses to ischemia than in slices submerged in artificial cerebrospinal fluid where the O_2 supply is limited to that dissolved in the medium (Croning and Haddad, 1998). After a baseline period, various portions of the oxygen in the atmosphere above the slice surfaces were replaced with N_2 for 30 min, and then returned to 100% O_2. Synaptic transmission was measured as the amplitude of field excitatory post-synaptic potentials evoked by stimulation of Schaffer-commissural fibers in field CA1. Slices from both mice and naked mole-rats tolerated replacement of half the oxygen atmosphere with nitrogen equally well. However, further reductions of the O_2 supply caused divergent effects on mouse and naked mole-rat slices. Fig. 10 shows example data from slices exposed to 15, 10 or 0% O_2 (Fig. 10A,B,E). In each case, the mouse slice showed a much more rapid and severe decline in function compared with the naked mole-rat slice. Summary data show that slices from naked mole-rats are much more resistant to hypoxia challenge (Fig. 10C), and are much more likely to recover after severe hypoxia (Fig. 10D) than slices from mice.

In a second set of experiments, we measured the time required to abolish all electrical activity in slices from mice and naked mole-rats after replacement of all O_2 in the slices' atmosphere with N_2 (nominal anoxia). In mice, removal of O_2 triggers a rapid (1–2 min) suppression of synaptic transmission, followed by a sudden loss of antidromic spikes and fiber volleys (presynaptic action potentials) and a spreading depression-like extracellular DC potential shift due to synchronized loss of membrane potentials and efflux of potassium. In mice, the anoxic depolarization occurs approximately 3.5 min after anoxia onset at 35°C. In slices from naked mole-rats, the suppression of synaptic transmission is delayed and progresses more slowly, with the anoxic depolarization occurring about 13 min after anoxia onset (Fig. 10F). All of these changes occurred more slowly at 30°C but the difference between mouse and mole-rat remained.

These results indicate that isolated brain slices from naked mole-rats are remarkably tolerant of acute hypoxic challenge. Anoxic depolarization is one step in a cascade of neuronal processes that occur during oxygen deprivation, including alterations in metabolic enzymes and ion channels, release of neurotransmitters (glutamate, adenosine) and activation of receptor-coupled signaling mechanisms (Erecińska and Silver, 2001; Lipton, 1999). A key element of the hypoxia cascade that determines whether the cellular response is reversible or leads to cell death is the accumulation of free intracellular calcium ions, which trigger cytotoxic mechanisms (Deshpande et al., 1987; Lee et al., 1991). In a study using the calcium indicator, fura-2, we found that calcium accumulation during hypoxia is very much lower in brain slices from naked mole-rats compared with age-matched mice (Peterson et al., 2012a). Fig. 11 shows change in calcium over time, before, during and after a 10 min exposure to hypoxia for neonatal and weanling (considered adult-like) mice (Fig. 11A) and naked mole-rats (Fig. 11B). The downward deflection in the curves corresponds to increasing levels of intracellular calcium. For both species there was significantly more calcium taken up by slices from older animals. However, the species differences at both ages are dramatic (Fig. 11C). In fact, the 10 min exposure had no appreciable effect on the neonatal naked mole-rats, so we had to test an additional group with a longer duration (Fig. 11D) to produce an effect.

The extreme tolerance of naked mole-rat brain to hypoxia calls to mind the well-known tolerance of mammals as neonates (Bickler, 2004) and led us to suggest that hypoxia tolerance in the naked mole-rat brain might result from retention of juvenile characteristics into the adult period (Larson and Park, 2009). The results from the calcium imaging experiments are consistent with this and, incidentally, the ability to load slice neurons from naked mole-rats with fura-2 far beyond weaning age is also highly suggestive (Peterson et al., 2012a). The 'neoteny hypothesis' for naked mole-rat brain also explains two other observations that we made in our initial studies of neuronal electrophysiology in hippocampal slices: a lack of synaptic (paired-pulse) facilitation and an insensitivity to exogenous adenosine – two features only observed in hippocampus of typical lab rodents very early in postnatal development (Larson and Park, 2009).

In order to pursue this hypothesis further, we examined the expression of NMDA receptor subunits in brains of neonatal and adult mice and naked mole-rats. NMDA receptors are important mediators of hypoxia-induced excitotoxicity and show dramatic shifts in subunit composition from the neonatal period to adulthood in rats and mice: most forebrain neurons express the GluN2B subunit at high levels in the neonatal period and high levels of GluN2A in adulthood. Moreover, Bickler and colleagues have shown that the GluN2D subunit expressed at higher levels in neonatal rat brain actually prevents calcium fluxes rather than mediating them in response to hypoxic conditions (Bickler et al., 2003). We used immunoblotting to compare NMDA receptor subunit expression changes from neonatal to adult periods in mice and naked mole-rats brain (Peterson et al., 2012b). Although expression levels between species could not be directly compared since the antibody may not have the same affinity for proteins in both, we could compare the relative decrease (or increase) in expression from the neonatal to adult periods within each species. GluN2A and GluN2B did not show significant between-species differences in relative change from neonate to adult. However, adult naked mole-rat brain retains a remarkably higher proportion of GluN2D (66% of neonate) compared with adult mouse brain (13% of neonate). This is highly significant since GluN2D was implicated

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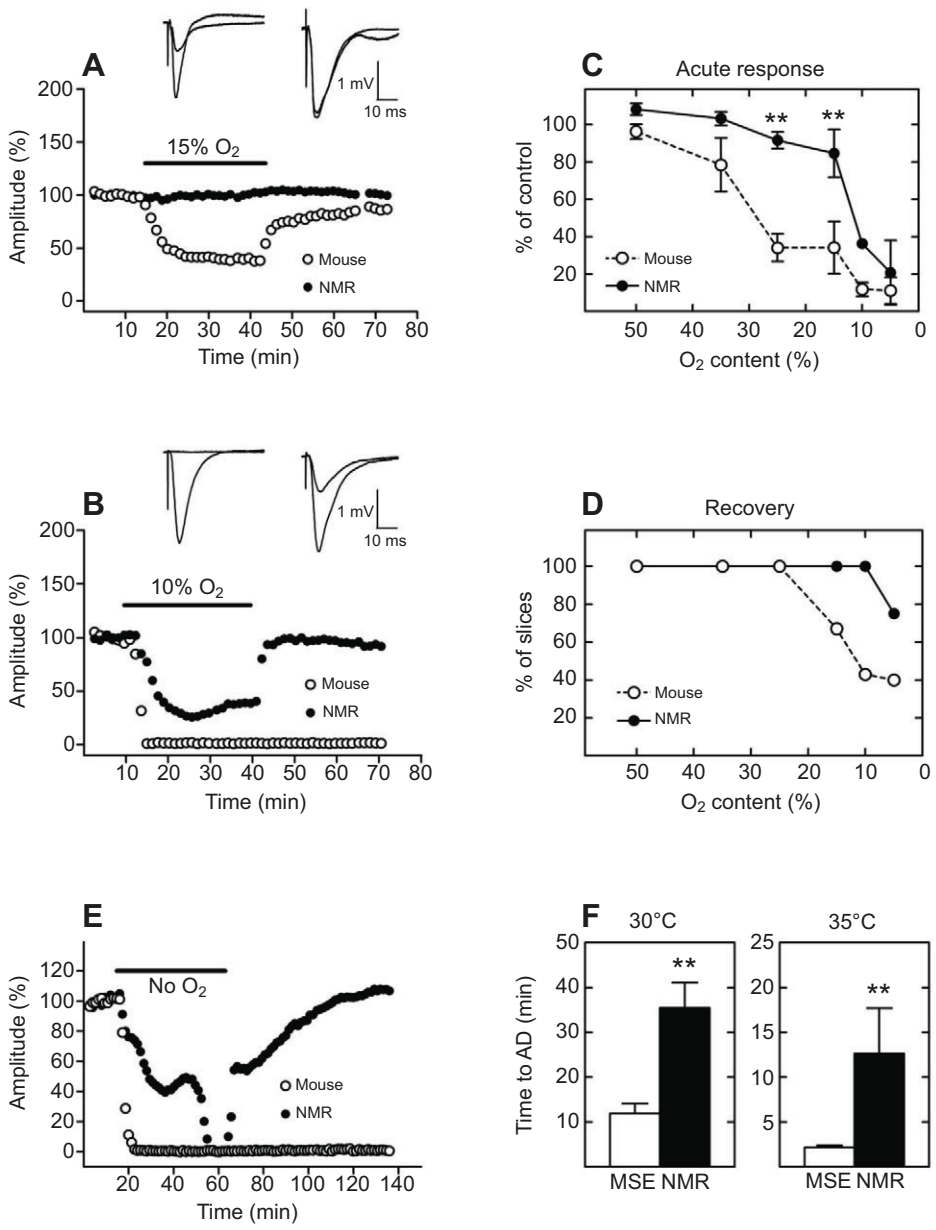

Fig. 10. Resistance of naked mole-rat (NMR) hippocampus to hypoxia. Field excitatory postsynaptic potential (EPSP) amplitude in hippocampal slices before, during (horizontal bar) and after replacement of 95% oxygen (O_2) in the slice chamber with 15% O_2/80% N_2 (A) or 10% O_2/85% N_2 (B) for 30 min. Inset traces show EPSPs recorded before and during hypoxia for mouse (left) and mole-rat (right). (C) Decrease in field EPSP amplitude in NMR (n=25) and mouse (n=48) slices at varying O_2 content (means ± s.e.m.). (D) Percentage of slices showing any functional recovery after re-oxygenation. (E) Response to removal of all O_2 from the chamber atmosphere. (F) Duration of exposure to O_2-free atmosphere needed to produce anoxic depolarization (AD) in slices from mice (MSE, n=19) and NMR (n=13) incubated at 30 or 35°C. All NMRs were at least 1 year old and mice were at least 2 months old. **P<0.01. [Adapted from Larson and Park (Larson and Park, 2009) and reprinted with permission.]

in hypoxia tolerance in neonatal rat brain (Bickler et al., 2003) and its expression in naked mole-rat brain shows retention of a neonatal feature into adulthood.

These findings provide partial support for the hypothesis that naked mole-rat brain is hypoxia tolerant because it has arrested development. Could this be a general mechanism that is used by many fossorial species or is it peculiar to mole-rats or even unique to *H. glaber*? Fig. 12 shows time to anoxic depolarization in slices from three species of African mole-rat (Bathyergidae), one species of European mole-rat (Spalacidae), two other families of terrestrial rodents (three murids and two cricetids), and three other mammalian orders (Marsupiala, Carnivora and Lagomorpha). Among the eutherians, the naked mole-rat and common mole-rat (*Cryptomys hottentotus*) stand out as hypoxia tolerant. The common mole-rat is more distantly related to the naked mole-rat than to the Damaraland mole-rat (*Cryptomys damarensis*). The latter, despite its close resemblance to naked mole-rats in habitat and social organization, did not show unusual hypoxia tolerance. It remains to be seen whether or not the common mole-rat shows other manifestations of arrested development. It would also be of interest to test fossorial species other than mole-rats under the same conditions. As noted above, hippocampal slices from arctic ground squirrels have significantly delayed electrophysiological responses to combined oxygen and glucose deprivation, compared to rats (Dave et al., 2009).

If the neoteny hypothesis for hypoxia tolerance in naked mole-rat brain holds up under further mechanistic and comparative scrutiny, it may explain some other, seemingly unrelated, unusual traits of these animals. For example, peripheral insensitivity to chemical irritants (LaVinka et al., 2009), lack of a functional substance P nociceptive pathway (Park et al., 2003), poor thermoregulation (Buffenstein and Yahav, 1991), absence of fur, and high-affinity hemoglobin (Johansen et al., 1976) may all be juvenile characteristics retained into adulthood. Slowed or arrested development might even contribute to the extraordinary lifespan (Buffenstein, 2008) of these remarkable animals. It is tempting to speculate that environmental conditions (fossorial) and patchy food resources (Bennett and Faulkes, 2000) might have driven a

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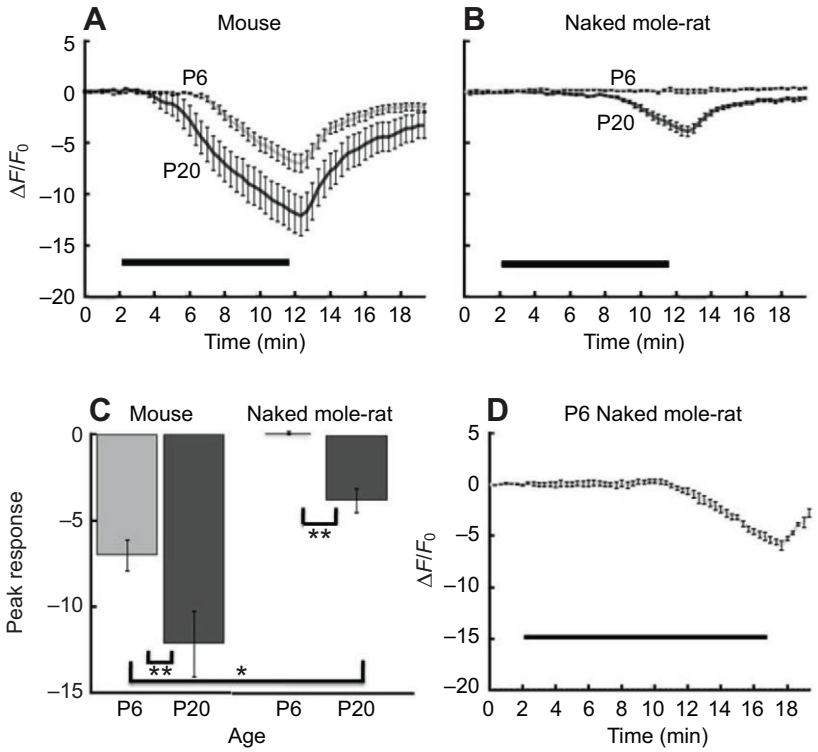

Fig. 11. Increase in internal calcium from exposure to hypoxic bath solution. (A) Data from postnatal day 6 (P6; 11 slices, 6 animals) and P20 (11 slices, 6 animals) mouse hippocampal slices. Values on the *y*-axis indicate the percentage change in calcium-mediated fluorescence within CA1 neurons in the field of interest with negative values corresponding to an increase in calcium (calcium decreases the fluorescent signal). Images were collected every 20 s over 20 min. The horizontal black bar indicates the 10 min when hypoxic bath solution was in the recording chamber. In these experiments, slices were superfused with artificial cerebrospinal fluid (ACSF) saturated with 95% O_2 and 5% CO_2 in the baseline and recovery periods; hypoxic solution was ACSF saturated with 95% N_2 and 5% CO_2. Error bars indicate ± s.e.m. (B) Data from P6 (14 slices, 5 animals) and P20 (10 slices, 3 animals) naked mole-rat slices. (C) Summary data showing the change in maximal calcium with age for mice and naked mole-rats for a 10 min exposure. **P*<0.05 and ***P*<0.01, according to the Newman–Keuls test. (D) Data from P6 (7 slices, 2 animals) naked mole-rat slices with an extended hypoxia exposure (15 min). Note that in all panels, animals in the P6 groups actually ranged in age from P5 to P7, and animals in the P20 groups actually ranged in age from P18 to P22. [Adapted from Peterson et al. (Peterson et al., 2012a) and reprinted with permission.]

(conceptually) simple ontogenetic timing change that allowed development of intrinsic brain tolerance to hypoxia, a communal lifestyle (eusociality), and extremely long life.

Discussion

Consideration of what is known about intrinsic brain tolerance to hypoxia in these different species seems to raise more questions than have yet been answered. For purposes of discussion and synthesis, we offer two dimensions along which the different organisms and their hypoxia tolerance mechanisms can be compared and contrasted. These dimensions are not really orthogonal but may capture a large part of the variance between species.

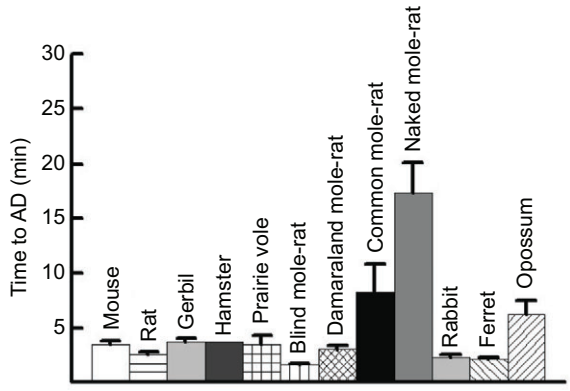

Fig. 12. Comparison of hypoxia sensitivity of hippocampal slices from diverse species of rodents and other mammalian orders. Histograms show average (means ± s.e.m.) time to induce anoxic depolarization (AD) in field CA1 after nominal anoxia (complete replacement of slice atmospheric O_2 with N_2). Brains from four common laboratory rodents were sampled (*Mus musculus*, Muridae, *n*=36; *Rattus norvegicus*, Muridae, *n*=4; *Meriones unguiculatus*, Cricetidae, *n*=2; and *Mesocricetus auratus*, Cricetidae, *n*=1), the prairie vole (*Microtus ochrogaster*, Cricetidae, *n*=5), the blind mole-rat (*Spalax sp.*, Spalacidae, *n*=3), three African mole-rats (*Cryptomys damarensis*, *n*=4; *Cryptomys hottentotus*, *n*=4; *Heterocephalus glaber*, *n*=11; all Bathyergidae), a lagomorph (*Oryctolagus cuniculus*, *n*=8), a carnivore (*Mustela putorius*, *n*=3), and a marsupial (*Monodelphis domestica*, *n*=3). (Unpublished data from J.L., S. Pawlowska and T.J.P.)

The first dimension has to do with the nature of the hypoxic threat imposed by the habitat and lifestyle of the animal. As was pointed out, a key mechanism to extend hypoxic survival is entrance into a deep but reversible hypo-metabolic state. The inducing conditions, duration and level of this hypoxia-tolerant state vary widely in the different species we have considered. It may be a lifelong adaptation to chronically low levels of ambient oxygen as in naked mole-rats; it may not be needed at all times but available for use during appetitive behavior patterns such as diving in seals and cetaceans; it may involve hibernation, a physiological state that is induced not by hypoxia per se but rather is entered as a means to conserve calories in seasons when food resources are scarce and requires flexibility in cerebral and systemic blood flow, as in arctic ground squirrels; or involve all three of these timescales and environmental and/or organismal inducers. It can be useful to consider chronic adaptations as distinct from those that are induced by a hypoxic challenge, whether the chronic adaptations are associated with chronic environmental hypoxia or merely the threat of unpredictable episodes. Thus we would distinguish chronic specializations that are preparatory and reduce the effects of hypoxia from specializations that are acute and reactive in nature.

The second dimension relates to the functional nature of the specialization. From the work presented here, we can distinguish a few categories: (1) dampening of the effect of systemic hypoxemia on the brain by improving O_2 delivery or using molecular O_2 buffers (e.g. neuroglobin); (2) reduction or restriction of energy consumption; (3) alterations to cellular metabolism (aerobic/anaerobic) in glia and neurons; (4) protection against excitotoxic damage; (5) protection against anoxic stress-induced necrosis and apoptosis; (6) prevention of oxidative stress during re-oxygenation; and (7) promotion of restorative processes.

1. Species-specific preparatory adaptations such as a particularly high capillary density in the brain have been described for diving mammals but do not seem to have been measured in the other species. This modification would bias the brain to receive a larger share of the O_2 supply when levels get low, given that brain blood flow appears to be well-maintained during diving (Blix et al., 1983; Zapol et al., 1979). Neuroglobin could serve to buffer O_2 levels as a preparatory mechanism. This seems to be the case with regard to cetaceans, although neuroglobin levels were not found to be higher in diving seals than in typical non-diving mammals, although its distribution is unusual. A reactive role for neuroglobin is suggested by its upregulation by hypoxia in turtles, possibly as a mechanism to buffer reactive oxygen species during re-oxygenation (see also below). Neuroglobin levels were found to be higher in the blind mole-rat (*Spalax*) brain under normoxic conditions, but downregulated during hypoxia (Avivi et al., 2010).

2. Low resting metabolism can be seen as a preparatory mechanism for energy conservation when hypoxic or ischemic episodes occur, at least in naked mole-rats and in hibernating arctic ground squirrels, although it does not explain intrinsic brain tolerance of hypoxia. Reactive mechanisms to reduce energy demand during hypoxic episodes are manifold and best described in the turtle: they include ‘channel arrest’, ‘spike arrest’, release of adenosine/inhibition of glutamate release, release of GABA, and inhibition of protein synthesis, among others. Such mechanisms may not be as significant in arctic ground squirrels or naked mole-rats, but may involve select populations of neurons in diving mammals.

3. A shift of cellular metabolism toward anaerobic pathways (in neurons or glia) would be a reactive adaptation to hypoxic episodes and may be important in seal brain, but not in arctic ground squirrels. A shift to glycolysis must be vital during very prolonged periods of anoxia in the turtle.

4. Protection against excitotoxic damage induced by hypoxic release of glutamate and its interaction with NMDA receptors is an important preparatory mechanism in turtles, arctic ground squirrels and naked mole-rats; it has not been assessed in diving mammals. In turtle, excitotoxicity is dampened indirectly by δ-opioid receptor-mediated suppression of NMDA receptor currents and may be subject to reactive regulation as well. In arctic ground squirrels, NMDA receptor expression is lower than in hypoxia-sensitive rats, whereas in naked mole-rats, NMDA receptor subunit expression (GluN2D) favors suppression of calcium fluxes in response to hypoxia.

5. Protection against hypoxia-induced apoptosis probably involves both protective and reactive mechanisms in turtles, through constitutive and induced changes in expression of heat shock proteins, other stress-associated proteins and apoptosis mediators. There are also some indications that protection of neurons from non-excitotoxic death may be important for hypoxia tolerance in the arctic ground squirrel as well. We know very little about the cell death pathways in naked mole-rats and diving seals.

6. The generation of reactive oxygen species during re-oxygenation (recovery) after an episode of acute hypoxia is a final aspect of the hypoxic catastrophe. The mechanisms by which ROS damage is avoided or ameliorated are beginning to be unraveled in freshwater turtles; as noted above, neuroglobin may be an important mediator, perhaps also in diving mammals. High levels of antioxidants in the brain of the arctic ground squirrel may help to alleviate ROS damage during recovery from hibernation.

7. Restorative processes including neurogenesis or synaptic remodeling may play a role in turtles and arctic ground squirrels, an aspect of the response to hypoxia that has not been studied in naked mole-rats and diving seals.

Hypothermia is a significant factor in hypoxia tolerance in most of the animals reviewed here. Freshwater turtles, being cold-blooded, are clearly subject to suppressed cellular metabolism when ponds freeze over; hibernation is associated with hypothermia in arctic ground squirrels. Naked mole-rats are the only mammals with significantly reduced body temperature (28°C) in their thermostable natural habitat; physiological thermoregulation is very poor in these animals (Buffenstein and Yahav, 1991; McNab, 1979). Aside from the effect on metabolic rate, the significance of hypothermia in naked mole-rats on brain hypoxia tolerance is less clear. Decreases in brain temperature may also be a reactive protection mechanism in diving seals.

Study of hypoxia-tolerant species has revealed many ways to mitigate the hypoxic catastrophe in neurons; however, one area that is yet to be addressed is the physiological cost. Presumably, deployment of special mechanisms for hypoxia tolerance must divert resources from other brain functions. As an example, synapses in naked mole-rat hippocampus do not show paired-pulse facilitation, a robust short-term synaptic plasticity mechanism normally present in many forebrain synapses in all other species that have been studied. Is this related to hypoxia tolerance? There has been little or no discussion of the potential behavioral or cognitive costs incurred by adaptations for hypoxia tolerance. Can they be measured? Are they significant? Meaningless? Or is there something unexpected yet to be learned from a comparative study of hypoxia tolerance in vertebrates?

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The authors declare no competing financial interests.

Author contributions
J.L. wrote the Introduction and Discussion sections, co-wrote (with T.J.P.) the section on hypoxia tolerance in the naked mole-rat, and revised the article. K.L.D. wrote the section on hypoxia tolerance in hibernating mammals, and revised the article. L.P.F. wrote the section on hypoxia tolerance in diving mammals, and revised the article. S.L.M. wrote the section on hypoxia tolerance in freshwater turtle brain, and revised the article. T.J.P. co-wrote (with J.L.) the section on hypoxia tolerance in the naked mole-rat, and revised the article.

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