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Brain energy metabolism and neurotransmission at near-freezing temperatures: *in vivo* ^{1}H MRS study of a hibernating mammal

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Abstract

The brain of a hibernating mammal withstands physiological extremes that would result in cerebral damage and death in a non-hibernating species such as humans. To examine the possibility that this neuroprotection results from alterations in cerebral metabolism, we used *in vivo* ^{1}H NMR spectroscopy at high field (9.4 T) to measure the concentration of 18 metabolites (neurochemical profile) in the brain of 13-lined ground squirrels (*Spermophilus tridecemlineatus*) before, during, and after hibernation. Resolved *in vivo* ^{1}H NMR spectra were obtained even at low temperature in torpid hibernators (~7°C). The phosphocreatine-to-creatine ratio was increased during torpor (+143%) indicating energy storage, and remained increased to a lesser extent during interbout arousal (IBA) (+83%). The total γ-aminobutyric acid concentration was increased during torpor (+135%) and quickly returned to baseline during IBA. Glutamine (Gln) was decreased (−54%) during torpor but quickly returned to normal levels during IBA and after terminal arousal in the spring. Glutamate (Glu) was also decreased during torpor (−17%), but remained decreased during IBA (−20% compared with fall), and returned to normal level in the spring. Our observation that Glu and Gln levels are depressed in the brain of hibernators suggests that the balance between anaplerosis and loss of Glu and Gln (because of glutamatergic neurotransmission or other mechanisms) is altered in hibernation.

Keywords: anaplerosis, brain, glutamate–glutamine cycle, ground squirrel, hibernation, magnetic resonance spectroscopy.

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The adult human brain is responsible for 20% of the body's oxygen consumption despite constituting only 2% of body weight. In contrast to the high energy demand of the human brain, torpid hibernators show a reduction in O_2 consumption by as much as 50-fold and a 10-fold reduction in cerebral blood flow, when compared with a non-hibernating animal (Frerichs *et al.* 1995). This considerable decrease in energy consumption is accompanied by specialized physiological adaptations including body temperature reduction approaching 0°C and a decrease in heart rate from 300 beats/min to 5–10 beats/min [reviewed in (Boyer and Barnes 1999; Frerichs 1999; Drew *et al.* 2001; Zhou *et al.* 2001; Carey *et al.* 2003)]. Hibernation is a regulated process involving metabolic depression (Hochachka 1986) that is controlled, in part, by the differential expression of genes common to all

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Abbreviations used: Asc, ascorbate; βHB, β-hydroxybutyrate; Cr, creatine; GABA, γ-aminobutyric acid; Glc, glucose; Gln, glutamine; Glu, glutamate; GPC, glycerophosphocholine; HLSVD, Hankel–Lanczos singular value decomposition; IBA, interbout arousal; myo-Ins, myo-inositol; NAA, *N*-acetylaspartate; NAAG, *N*-acetylaspartylglutamate; NS, not significant; PCho, phosphocholine; PCr, phosphocreatine; RF, radio frequency; RMS, root mean square; STEAM, stimulated echo acquisition mode; Tau, taurine; TCA cycle, Tricarboxylic acid cycle; VAPOR, variable power RF pulses with optimized relaxation delays.

mammals (Andrews *et al.* 1998; Bauer *et al.* 2001; Buck *et al.* 2002; Squire *et al.* 2003).

The brain of a hibernating mammal is naturally resistant to the consequences of low blood flow and hypoxia – conditions which often lead to stroke in humans. Ischemic stroke is the most common type of stroke seen clinically. It initiates a complex pathological cascade including altered energy metabolism, pH and ionic imbalances, excitotoxicity, inflammation and apoptosis [reviewed by (Dirnagl *et al.* 1999)]. Uncovering the neurochemical processes involved in hibernation has the potential to increase greatly our understanding of the human response to stresses such as cerebral ischemia and subsequent reperfusion injury. A deep hibernator, such as a ground squirrel, undergoes metabolic rate reduction followed by lower body temperature (Heldmaier and Ruf 1992; Frerichs *et al.* 1994). Identification of the key metabolic events leading to this metabolic rate reduction is critical to understand how brain activity is regulated. It may also lead to novel neuroprotective strategies in non-hibernating species such as humans.

Given the profound changes in energy metabolism during hibernation, we sought to characterize the neurochemical changes in the 13-lined ground squirrel (*Spermophilus tridecemlineatus*) throughout the hibernation season using proton magnetic resonance spectroscopy (^{1}H MRS). ^{1}H MRS has been used to measure concentrations of brain metabolites non-invasively in humans and a variety of animal models. We have previously shown that a neurochemical profile consisting of 18 or more metabolites can be measured reliably in the brain of rats (Pfeuffer *et al.* 1999; Tkac *et al.* 2001, 2003) and mice (Tkac *et al.* 2004) using ^{1}H MRS at high magnetic field. In the present study, we used this approach to measure the neurochemical profile of ground squirrels before the hibernation season (in the fall), during hibernation [torpor and interbout arousal (IBA)] and after the hibernation season (in the spring).

Methods

Animals

All animal procedures were performed according to the guidelines for the care and use of laboratory animals at the University of Minnesota and were approved by the Institutional Animal Care and Use Committee. Thirteen-lined ground squirrels (*S. tridecemlineatus*), were obtained from TLS Research (Bartlett, IL, USA) or live trapped in Minnesota, and housed at the designated animal facilities at the University of Minnesota. Squirrels were kept individually in plastic top-load rodent cages filled with pine shavings. Diet consisted of standard rodent chow supplemented with black oil sunflower seeds and water *ad libitum*. Food availability, room temperature, and photoperiod were varied artificially to induce hibernation in captivity (Table 1). Cages and bedding were changed weekly up until the first week of November when the final conditions were put into place and all animals hibernated. From October through arousal in March, the activity state of the squirrel was monitored using the sawdust technique (Pengelley and Fisher 1961).

Table 1 Maintenance conditions for squirrels throughout the year

Months of the year	Ambient temperature (°C)	Photoperiod (hours light : hours dark)	Food available
Mid-March through September	23	12 : 12	Yes
October	11	12 : 12	Yes
November through mid-March	5	0 : 24	No[a]

[a]Water was available *ad libitum*.

Table 2 Experimental groups

Group (n = 7 for each group)	Month measured	Weight (g) (mean ± SD)	Body temperature (°C) (mean ± SD)
Active (fall)	September	180 ± 21	37.0 ± 1.0
Torpid hibernators[a]	December	153 ± 22	7.3 ± 1.1
Interbout arousal	February	140 ± 19	35.1 ± 1.1
Active (spring)[b]	March	133 ± 18	37.0 ± 1.0

[a]Torpid hibernators were measured ~1 month after first entering torpor. [b]Spring animals were measured ~1 week after the end of torpor when they received food and were exposed to a warmer ambient temperature.

Animals were measured by high-field ^{1}H MRS in four different conditions: active in the fall (n = 7), during torpor in the winter (n = 7), during IBA in the winter (n = 7), and active in the spring (n = 7). A total of 10 animals were measured (three females and seven males), four of which were measured in all four conditions, and six of which were measured in one, two, or three of the four conditions. Relevant animal data are summarized in Table 2. For measurements in torpid hibernators, we ensured that animals were in deep torpor during the night preceding the study using the sawdust technique. We also observed torpid animals prior to anesthesia to check for signs of IBA (increased breathing, shivers). Finally we measured the temperature of animals right after anesthesia, to make sure that their body temperature was in the range 4–8°C, similar to the temperature of animals that remained in the hibernation room. These steps allowed us to ensure that measurements were done in torpid hibernators during deep torpor.

For NMR measurements, animals (including torpid hibernators) were anesthetized using 5% isoflurane (induction) and were placed in a chamber with a circulating 30% O_2/70% N_2O mixture and 1.8% isoflurane for the remainder of the experiment. A heating/cooling tube was used to maintain body temperature at 37°C (active animals and aroused hibernators) or 6–8°C (torpid hibernators). The heating/cooling tube was connected to a circulating fluid bath (water at 70°C for experiments performed at 37°C and a 1 : 1 mix of water and ethylene glycol at −6°C for experiments performed at low temperature) controlled by feedback from a rectal temperature probe. The circulating fluid flowed via flexible plastic tubing (10 mm diameter) which ran into the magnet bore and was wrapped

below and above the animal chamber. The cooling system was supplemented with BlueIce® (RubberMaid, Atlanta, GA, USA) placed around the animal chamber for measurements in hibernators. IBA was induced by placing the animal at room temperature (~22°C) and waiting for the body temperature to approach 37°C before induction of anesthesia (typically 1\,h). After spectroscopic measurements, all animals were monitored until complete arousal (for torpid hibernators) and recovery from anesthesia.

NMR spectroscopy

Experiments were performed using a 9.4 T/31 cm bore magnet interfaced to a Varian INOVA console. Gradients (11 cm bore) reached 300 mT/m in 500 μs. The spectrometer was equipped with a strong custom-designed second order shims set (Magnex Scientific, Yarnton, Oxfordshire, UK) (Tkac *et al.* 2004). The proton transmit/receive surface coil consisted of two loops in quadrature (14 mm diameter each). Multislice rapid acquisition with relaxation enhancement images allowed positioning of a $4 \times 1.5 \times 3$ mm^3 voxel in the brain cortex for spectroscopy. Localized shimming was performed using FASTMAP with echo-planar readout (Gruetter and Tkac 2000). Proton spectra were measured using a stimulated echo acquisition mode sequence (echo time = 2 ms, repetition time = 5 s) with variable power radio frequency pulses with optimized relaxation delays water suppression and outer volume suppression as previously described (Tkac *et al.* 1999). A total of 160 scans were acquired for each voxel. Individual free induction decays were averaged into 10 blocks of 16 scans, then frequency drift was corrected before these 10 blocks were added together to obtain a single spectrum. Unsuppressed water signal was used to eliminate residual eddy currents and served as an internal concentration reference for quantification.

Quantification of NMR spectra

Spectra were analyzed using the LCModel software v2.3 (Stephen Provencher Inc., ON, Canada). LCModel fits the *in vivo* spectrum as a linear combination of model spectra from individual metabolites (Provencher 1993). Because temperature affects chemical-shift values and therefore the appearance of ^{1}H NMR spectra, two different basis sets were used for this study: one measured at 37°C and the other one measured at 5°C. The 37°C basis set has been used in many studies in our laboratory (Pfeuffer *et al.* 1999; Tkac *et al.* 1999, 2001, 2003, 2004) and was extended to include β-hydroxybutyrate (βHB) and ascorbate (Asc), which has recently been shown to be detectable by ^{1}H NMR (Terpstra *et al.* 2006).

The 5°C basis set was constructed by measuring ^{1}H spectra at 5°C from individual solutions of the following 20 metabolites: βHB, Asc, scyllo-inositol, alanine, aspartate, glycerophosphocholine (GPC), phosphocholine (PCho), creatine (Cr), phosphocreatine (PCr), γ-aminobutyric acid (GABA), glucose (Glc), glutamine (Gln), glutamate (Glu), glutathione, myo-inositol (myo-Ins), lactate, *N*-acetylaspartate (NAA), *N*-acetylaspartylglutamate (NAAG), phosphoethanolamine, and taurine (Tau). Solutions were prepared at a concentration of 50 mmol/L for each metabolite in phosphate buffer (pH = 7.1) with 2 mmol/L 2,2-dimethyl-2-silapentane-5-sulfonate as a chemical-shift reference. ^{1}H MR spectra of individual metabolites were measured at 5°C under the exact same conditions as *in vivo*. Temperature was controlled using a circulating bath of water/polyethylene glycol (1 : 1) at −6°C and verified using the chemical shift of water on ^{1}H spectra (at 5°C, water resonates at 5 ppm, compared with 4.65 ppm at 37°C). Consistent scaling between the 37°C and the 5°C basis sets was verified by LCModel analysis of fully-relaxed spectra acquired at 37°C and at 5°C from the same phantom. A spectrum of macromolecules was measured *in vivo* at 5°C using inversion–recovery (repetition time 1.3 s, inversion time 0.36 s) to null the signal from metabolites (Pfeuffer *et al.* 1999) and was incorporated in the basis set.

Convergence of the LCModel fit was verified by visual inspection of residuals. Concentration values with Cramer-Rao lower bounds higher than 50% were considered unreliable and were not taken into account in the statistical analysis. A few metabolites (βHB, alanine, and scyllo) showed Cramer-Rao bounds that were higher than 50% in most animals, indicating that they were not reliably quantified under our experimental conditions. Therefore these three metabolites are not reported in our results. Finally, only the sums NAA + NAAG and GPC + PCho (total choline-containing compounds) are reported due to the high cross-correlation between NAA and NAAG on one hand and GPC and PCho on the other.

Statistical analysis

Results were analyzed for statistical significance using SPSS software (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to detect significant differences between groups. *Post hoc* tests were corrected for multiple comparisons using the correction of Bonferroni. The threshold for statistical significance was chosen at $p = 0.002$. All values are reported as mean ± SD.

Results

In vivo measurements of small molecules in the brain of active and hibernating ground squirrels present the challenge of determining metabolite concentrations over a wide range of temperatures. In this study well-resolved ^{1}H MR spectra were obtained in the squirrel brain at temperatures close to freezing (Fig. 1), thereby demonstrating the feasibility of measuring highly resolved spectra in the brain *in vivo* at low temperatures. Although spectra measured at 7°C were similar in appearance to spectra obtained at 37°C, several differences could be readily observed. A decrease in Glu signal was clearly noticeable at 2.34 ppm during hibernation. Creatine and PCr methylene resonances (~3.92 ppm) were partially resolved at 9.4 T with a peak separation between Cr and PCr of ~7 Hz at 37°C and ~10 Hz at 5°C (Fig. 1, right panel). Comparison of spectra obtained in the cortex of active animals and hibernating animals showed a clear change in the PCr/Cr ratio at ~3.92 ppm (Fig. 1, right panel). Relative peak intensities of Cr and PCr were similar in spectra from active animals (fall and spring), whereas the PCr resonance was distinctly higher than the Cr resonance in spectra from hibernators. Changes for other metabolites such as Gln and GABA were not immediately as apparent in the ^{1}H spectra because of their lower concentration and spectral overlap, but could be determined after deconvolution of the spectra using LCModel.

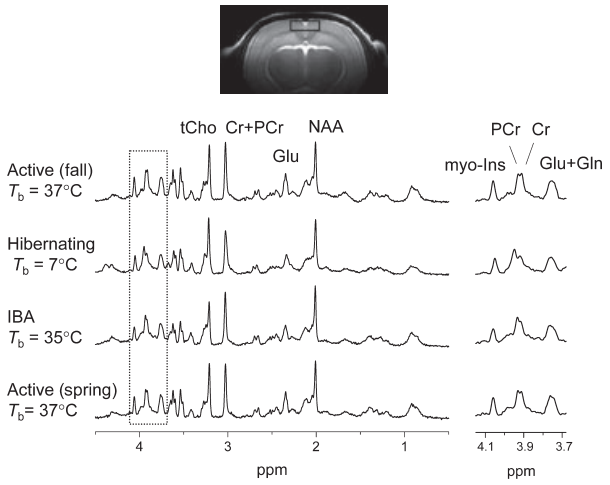

Fig. 1 *In vivo* spectra measured in the cortex of ground squirrels in the following states: active in the fall, hibernating, interbout arousal (IBA), and active in the spring. Spectra were obtained from a 18 μL volume ($4 \times 1.5 \times 3$ mm^3) and 160 repetitions with repetition time = 5 s. The image (top inset) shows the cortical brain region used for spectroscopic measurements. Each spectrum represents the sum of seven animals and is shown with 4 Hz Lorentzian to Gaussian resolution enhancement. A decrease in glutamate signal at 2.34 ppm was clearly observed during hibernation and IBA. In addition, an increase in phosphocreatine (PCr)/creatine (Cr) ratio during hibernation was visible around 3.92 ppm as shown in the expanded panel (4.1–3.7 ppm) on the right. Note that the peak height of the Cr + PCr methyl resonance at 3.03 ppm appeared noticeably smaller in spectra at 7°C compared with spectra at 37°C. Rather than a change in concentration, this reflected slight changes in chemical shifts of Cr and PCr chemical-shifts with temperature, which resulted in an 'apparent' linewidth increase for the combined Cr + PCr resonance at 7°C compared with 37°C, and a corresponding decrease in peak height, without change in total signal area. T_b, body temperature at the time of measurement.

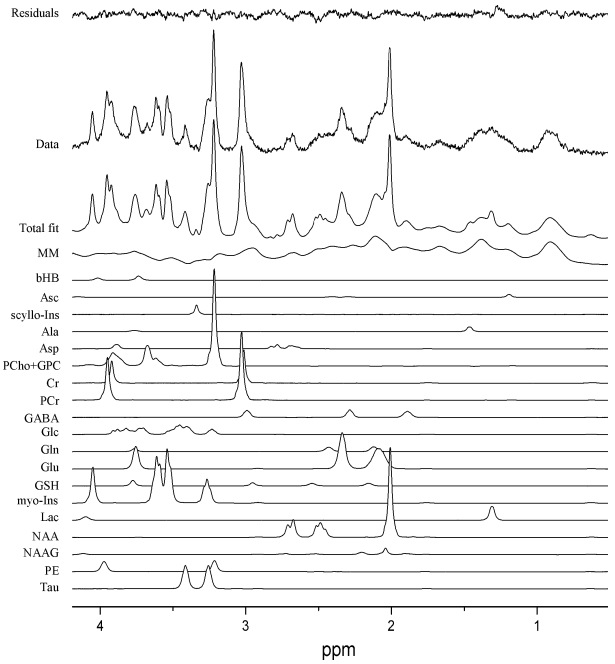

Fig. 2 LCModel fit of a proton spectrum from the brain of a hibernating squirrel measured at 7°C. From the top: residuals, *in vivo* data, total fit, macromolecules, and contribution of each metabolite to the fit. Spectra were processed with 1 Hz Lorentzian line broadening for display.

Short echo time *in vivo* spectra in torpid hibernators at ~7°C exhibited a wealth of spectral signatures as shown in Fig. 2. This illustrates that the information content of spectra obtained at 7°C was very similar to that obtained at 37°C in our previous studies. Metabolite concentrations from active ground squirrel brains measured in the fall (Fig. 3) were remarkably similar to those seen at 37°C in rats (Pfeuffer *et al.* 1999; Tkac *et al.* 2003). The only exceptions were myo-Ins, which was approximately twofold higher in active ground squirrels than rats, and Tau, which was half the concentration.

Measurements of Glu, Gln, and GABA concentrations in all four conditions – active in the fall, during torpor, during IBA, and active in the spring are shown in Fig. 3a. GABA concentration more than doubled during torpor, increasing from 0.9 ± 0.3 to 2.1 ± 0.3 μmol/g (+135%, $p < 0.0001$) and returned to basal levels during IBA and in the spring. Glutamine concentration decreased during torpor from 3.7 ± 0.7 to 1.7 ± 0.6 μmol/g during hibernation (−54%, $p < 0.0001$) and Glu showed a smaller relative decrease (−17%, $p < 0.002$), although in absolute concentration the decrease from 12.6 ± 1.0 to 10.5 ± 1.0 μmol/g was comparable with that of Gln. Total Glu + Gln concentration was decreased by 26% in torpid hibernators compared with active (fall) animals ($p < 0.0001$). Interestingly, Gln concentration quickly returned to pre-torpor levels during IBA [4.1 ± 0.5 μmol/g, not significant (NS) compared with fall] whereas Glu remained at a lower level during IBA (−20% compared with fall, $p < 0.0001$). Both Glu and Gln levels returned to their fall levels in the spring.

Measurements of Cr and PCr concentrations are shown in Fig. 3b. During hibernation, the PCr/Cr ratio more than doubled from 0.9 ± 0.3 in the fall to 2.3 ± 0.5 during torpor (+143%, $p < 0.0001$) because of a 33% increase in PCr concentration ($p < 0.0005$) and a 46% decrease in Cr concentration ($p < 0.0001$). In contrast, the *total* concentration of Cr and PCr (Cr + PCr) remained stable (Fig. 3b). Both Cr and PCr returned to their fall level in spring, yielding a PCr/Cr ratio of 1.2 ± 0.2 (NS compared with fall). The PCr/Cr ratio was somewhat lower during IBA than during torpor (1.7 ± 0.3 vs. 2.3 ± 0.5, NS) but was still elevated during IBA compared with active in the fall (+83%, $p < 0.002$).

A few additional metabolites showed significant changes during torpor (Figs 3c and d). Glucose concentration (Fig. 3c) increased from 2.4 ± 0.8 μmol/g in active animals

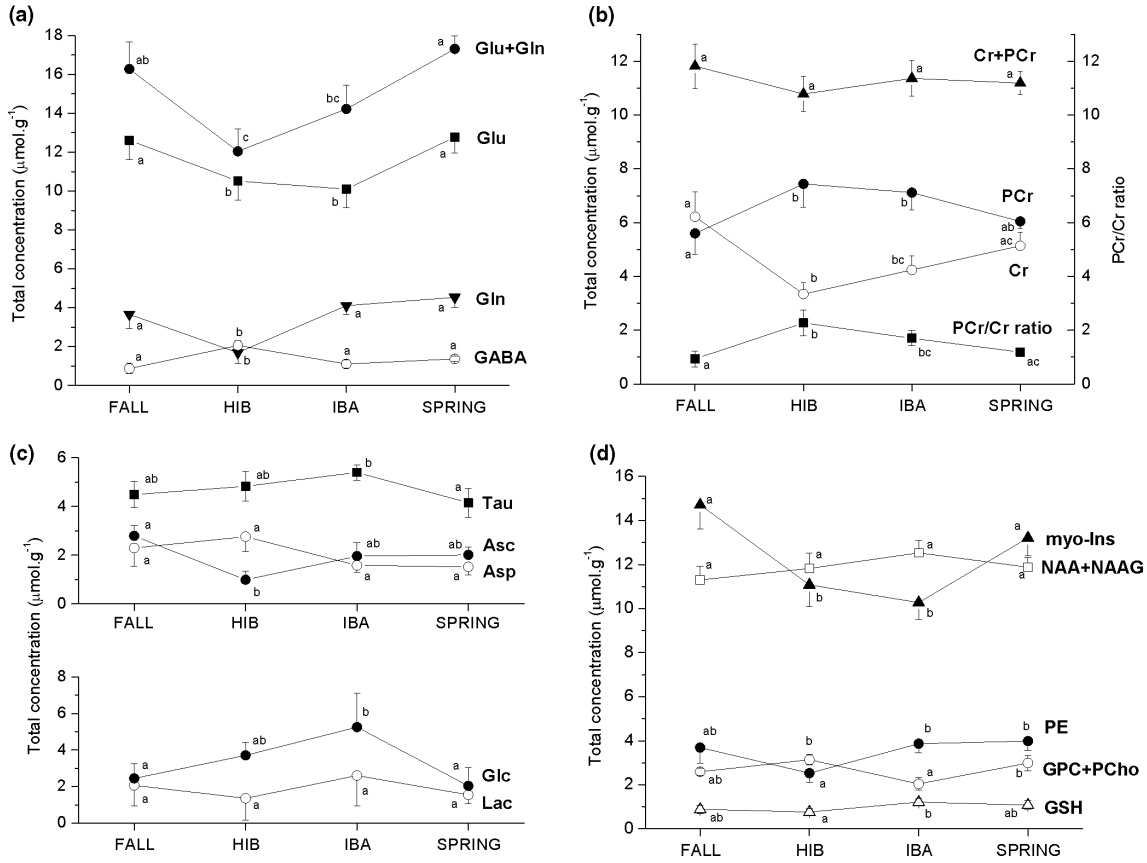

Fig. 3 Changes in neurochemical profile during hibernation and interbout arousal. (a) Changes in glutamate, glutamine, and γ-aminobutyric acid (GABA); (b) changes in creatine and phosphocreatine; (c) changes in taurine, ascorbate, aspartate, glucose, and lactate; (d) changes in myoinositol, *N*-acetylaspartate (NAA), phosphoethanolamine, choline, and glutathione. For a given metabolite, two data points are significantly different if they are not labeled with the same lower case letter. Note that all metabolites returned to their fall level in the spring.

in the fall to 3.7 ± 0.7 μmol/g in torpid animals (+51%, NS) and further increased to 5.3 ± 1.8 μmol/g during IBA (+115% compared with fall, $p < 0.0002$). Note the high standard deviation most likely reflecting a high interanimal variability in blood Glc content during IBA. Asc concentration (Fig. 3c) was reduced almost threefold from 2.8 ± 0.4 to 1.0 ± 0.4 μmol/g (−64%, $p < 0.00001$) during torpor but returned to basal levels during IBA and in the spring. Myo-Ins concentration (Fig. 3d) was decreased during torpor (−25%, $p < 0.0001$) and IBA (−30%, $p < 0.0001$) compared with fall, but returned to normal in the spring. All other measured metabolites (Tau, aspartate, lactate, NAA + NAAG, phosphoethanolamine, total choline-containing compounds, and glutathione) did not show major changes during the course of the study.

Remarkably, all neurochemical changes observed during the hibernation season were reversed after animals emerged from hibernation in spring (Fig. 3). Neurochemical profiles in fall and spring were virtually identical, with no significant difference in any of the measured metabolites.

Discussion

This is, to our knowledge, the first report of *in vivo* ^{1}H MRS in the brain of a hibernating mammal. Proton NMR spectra were obtained with high spectral resolution in the brain of 13-lined ground squirrels at low temperature during hibernation. Up to 18 metabolites were simultaneously measured in the cortex of ground squirrels before, during and after hibernation as well as during IBA. Comparison of these neurochemical profiles (Fig. 3) reveals significant changes for certain key metabolites – particularly those related to energy metabolism and neurotransmission – and provides new insights into the cellular events underlying hibernation and near-complete electrical deactivation.

Neurotransmission: glutamate–glutamine cycle and anaplerosis

Our most striking observation was that Glu and Gln levels were significantly affected during hibernation. Both Gln and Glu concentrations were decreased by 2 mmol/L during

torpor (corresponding to a relative decrease of −54% for Gln and −17% for Glu) and returned to normal in the spring. In addition, Gln returned to active levels following IBA, whereas Glu remained depressed (−20% compared with fall). These findings can be interpreted in light of the close functional relationship between neurons and astrocytes (Schousboe *et al.* 1993; Bachelard 1998; Cruz and Cerdan 1999; Gruetter 2002).

Brain metabolism is compartmentalized (Van den Berg *et al.* 1969; Berl *et al.* 1970) with most Gln localized in astrocytes (Ottersen *et al.* 1992; Storm-Mathisen *et al.* 1992) and most Glu localized in neurons (Ottersen *et al.* 1992; Chapa *et al.* 2000). During neurotransmission, astrocytes take up Glu, convert it to Gln through the astrocytic enzyme Gln synthetase (Martinez-Hernandez *et al.* 1977), and send it back to neurons (Lapidot and Gopher 1994; Hertz 2004) (Fig. 4). NMR studies have demonstrated that this Glu–Gln cycle is a major metabolic pathway in the brain (Sibson *et al.* 1997; Shen *et al.* 1999; Gruetter 2002; Lebon *et al.* 2002).

In addition to their role in the uptake of extracellular Glu and its conversion to Gln, astrocytes constantly supply new carbon skeletons to neurons to compensate for losses through diffusion or Glu oxidation (Hertz *et al.* 1999). This net

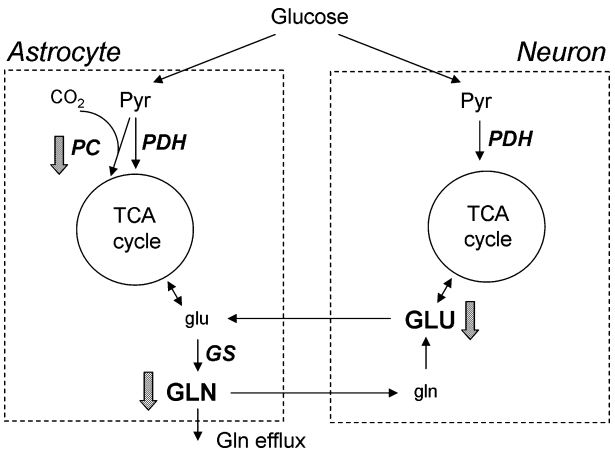

Fig. 4 Intercellular compartmentation, glutamate–glutamine cycle and anaplerosis during hibernation. Brain is compartmentalized between neurons and astrocytes. Most glutamate (GLU) is located in neurons and most glutamine (GLN) is located in astrocytes as indicated with capital letters. During glutamatergic neurotransmission, glutamate (glu) is taken up by astrocytes, converted into glutamine (GLN) through glutamine synthetase (GS) and sent back to neurons. Neuronal glutamine (gln) is then converted back to neurotransmitter glutamate (GLU). Flux of pyruvate through pyruvate dehydrogenase (PDH) does not lead to net synthesis of new carbon skeletons because each molecule entering the TCA cycle through PDH is eventually degraded into CO_2 and water. Synthesis of new carbon skeletons occurs through astrocytic enzyme pyruvate carboxylase (PC). A decrease in PC activity during hibernation would lead to decreased concentrations of glutamate and glutamine (gray arrows pointing down).

synthesis of new carbon skeletons (anaplerosis) occurs primarily by CO_2 fixation through pyruvate carboxylase, an enzyme located almost exclusively in astrocytes (Shank *et al.* 1993). It is estimated that 20–40% of all Gln synthesis corresponds to *de novo* Gln synthesis through pyruvate carboxylase (Lapidot and Gopher 1994; Merle *et al.* 1996; Aureli *et al.* 1997; Gamberino *et al.* 1997; Gruetter *et al.* 1998b, 2001; Lieth *et al.* 2001; Sibson *et al.* 2001; Oz *et al.* 2004). Recently, Oz *et al.* showed that pyruvate carboxylase flux increases with neuronal activity and hypothesized that anaplerosis is linked to neuronal activity by maintaining Glu and Gln concentrations (Oz *et al.* 2004).

In this context, our finding that Glu and Gln concentrations were decreased by about 2 mmol/L each during torpor reflects a net loss of 4 mmol/L of carbon skeleton that is being re-synthesized during IBA and in the spring. Such changes in net amino acid concentration must be regulated by anaplerotic and cataplerotic pathways. In the brain, pyruvate carboxylase is the dominant anaplerotic enzyme, and therefore changes in net anaplerosis are likely to involve pyruvate carboxylase. Oz *et al.* (2004) show that anaplerosis increases with increased neuronal activity to maintain Gln and Glu concentrations. The decrease in Gln and Glu in the present study suggests that the balance between anaplerosis and loss of Glu and Gln (e.g. because of glutamatergic neurotransmission) is altered during hibernation. In addition, because synthesis of *de novo* Gln occurs in astrocytes through pyruvate carboxylase and Gln is a precursor of Glu, changes in net anaplerosis are expected to influence Gln levels more quickly than Glu levels (Fig. 4). This is consistent with our observation that Glu changes occur less rapidly than Gln changes, as suggested by the fact that Gln quickly comes back to normal during IBA whereas Glu levels remain depressed. Our results also raise the possibility that regulation of anaplerotic activity may play a role in the dramatic reduction in metabolism that occurs during hibernation.

Neurotransmission: glutamate, GABA, and excitotoxicity

Glutamate is the main excitatory neurotransmitter and GABA is the main inhibitory neurotransmitter. The decrease in Glu and increase in GABA that we observed during torpor suggest that a coordinated decrease in excitatory Glu and increase in inhibitory GABA may play a role in protecting the brain from excitotoxic damage by decreasing energy consumption associated with synaptic neurotransmission (Nilsson and Lutz 1993). Indeed, hibernation has been proposed as a model of neuroprotection (Frerichs 1999; Drew *et al.* 2001; Zhou *et al.* 2001). Excitotoxicity caused by excess Glu in the extracellular space eventually results in neuronal death. Glutamate excitotoxicity and dysfunction in energy metabolism have been implicated in a number of diseases such as stroke and neurodegenerative disorders (Beal 1998; Doble 1999). Recently it has been shown that high extracellular levels of GABA protect cultured neurons

against damage induced by the accumulation of endogenous extracellular Glu (Velasco and Tapia 2002). The observations of GABA increases and Glu decreases during hibernation are not only consistent with our understanding of the activity of these neurotransmitter systems on arousal states such as sleep and wakefulness, but is also consistent with conclusions of 2-deoxyglucose studies in which the entrance to hibernation was proposed to be mediated by a relative activation of some brain systems and inhibition of others (Kilduff *et al.* 1990). In the current study, the cortical region which was measured corresponds most closely to the cingulate cortex, which was among the areas that underwent the greatest reduction in 2-deoxyglucose uptake in that study.

As Tricarboxylic acid cycle (TCA cycle) activity is required for catabolism of GABA into succinate, the increase in GABA observed in the present study may be the result of decreased TCA cycle activity (Nilsson and Lutz 1993). Interestingly a decrease in GABA has been reported in microdialysis studies of brain striatum during torpor (Osborne *et al.* 1999). In addition, extracellular concentrations of Glu measured by microdialysis do not differ between hibernating and euthermic ground squirrels (Zhou *et al.* 2002). Our findings are not necessarily contradictory with these studies as microdialysis measures extracellular concentration, whereas ^{1}H MRS measures total tissue concentration. It is important to note that there is not necessarily a direct relationship between total concentration of neurotransmitters (Glu and GABA), as measured by NMR, and neurotransmission. There is some indication, however, that total concentration of GABA, for example, may be related to increased GABAergic neurotransmission. For example, antiepileptic drugs such as vigabatrin reduce epileptic discharges, and increase GABA tissue level of GABA by inhibiting GABA transamination. Therefore, in that case, there seems to be a relationship between increased total GABA concentration and increased inhibitory neurotransmission (Petroff *et al.* 1996). Alternative methods to measure neurotransmission include microdialysis. However, because of the fact that the extracellular levels of Glu and GABA are very tightly regulated at the synaptic level, it has been suggested that microdialysis measures the 'overflow' of neurotransmitter rather than neurotransmission *per se* (Timmerman and Westerink 1997). In addition, the relevance of measuring 'synaptic' release in brain during torpor when action potentials are rare or absent is unclear. Therefore, the relationship between extracellular concentrations of the neurotransmitters GABA and Glu, as measured by microdialysis, and neurotransmission is also difficult to determine. Further progress with microdialysis probes may make it possible to address this issue in more detail (Drew *et al.* 2004a).

Energy metabolism: creatine, phosphocreatine, and glucose

Our findings show that torpor and IBA are accompanied with changes in energy storage. Most notable is the dramatic twofold increase in PCr/Cr ratio during torpor. This finding is in agreement with a previous study showing that PCr was increased by 53–82% in the brain of hibernating hamsters (Lust *et al.* 1989). Such an increase in PCr/Cr ratio is consistent with a sharply reduced ATP demand in the hibernating mammalian brain, as expected in an electrically silent animal, characterized by a flat EEG with occasional spindles (Walker *et al.* 1977). Most importantly, the high PCr/Cr ratio implies that the energy balance is preserved during torpor, e.g. to preserve ion gradients (Willis 1979; Hochachka 1986; Drew *et al.* 2004b). This preservation of energy balance has long been identified as one of the key features that allow hibernators to withstand extreme changes in physiology during what Frerichs called a 'cellular coordinated shutdown' (Frerichs 1999). Interestingly, the PCr/Cr ratio did not come back to active levels during IBA, an indication that a return to normal energetic levels may require several hours.

Glucose concentration in the brain was increased during IBA, but showed a higher variability during torpor and IBA than in active animals, as noted in a previous study (Frerichs *et al.* 1995). As brain Glc content is strongly affected by blood Glc (Gruetter *et al.* 1998a), the increase in brain Glc levels during IBA may reflect an increase in serum Glc (Brauch *et al.* 2005). Alternatively, increased brain Glc may reflect reduced brain Glc consumption (Choi *et al.* 2002).

Ascorbate

Ascorbate is a water-soluble antioxidant with well-known neuroprotective properties and is a potential neuromodulator (Rice 2000) that has recently been shown to be measurable by ^{1}H NMR (Terpstra and Gruetter 2004; Terpstra *et al.* 2006). We observed a sharp decrease in *total* Asc concentration (−64%) during torpor, with a rapid return to pre-hibernation levels during IBA and in the spring. Other studies using HPLC have reported either no decrease or a slight 15% decrease in *total* brain Asc during torpor in Arctic ground squirrels (Drew *et al.* 1999; Toien *et al.* 2001). Drew *et al.* (1999) show 15–20% less Asc in brain regions of 13-lined ground squirrels during torpor. Although the euthermic group in this study consisted of cold adapted animals that did not hibernate 15–20% lower levels of Asc in hibernating 13-lined ground squirrels is consistent with results reported in the present study. Figures 3 and 4 in Drew *et al.* (1999) are also consistent with a species difference where Asc tends to decrease during torpor in 13-lined ground squirrels, but not Arctic ground squirrels.

Alternatively, tissue used for HPLC measurements may be affected by faster post-mortem changes in warm euthermic tissue than in cold hibernating tissue. This emphasizes the advantage of using non-invasive methods such as ^{1}H MRS or microdialysis to minimize post-mortem changes.

Previous studies have also shown a three to fourfold increase in plasma Asc during hibernation and a twofold

increase in Asc in the cerebrospinal fluid (Drew *et al.* 1999). In addition, a rapid decrease in plasma Asc was observed during IBA which paralleled the increase in O_2 consumption (Toien *et al.* 2001). These findings suggest that Asc levels are tightly regulated in the brain and that there are dynamic changes in both extracellular Asc levels (increase) and total Asc levels (decrease) during hibernation.

Seasonal versus torpor-specific changes in metabolite concentrations

The present study evidenced both seasonal changes that were observed throughout the hibernation season in torpid hibernators and aroused hibernators alike (e.g. Glu concentration) and changes specific to torpor (e.g. Gln and GABA concentration). Seasonal changes would refer to changes that are present throughout the hibernation season. However, because we measured all aroused hibernators about 1 h after initiating arousal, we cannot exclude the possibility that some metabolites concentrations need more than 1 h, perhaps several hours, to return to their baseline concentration. Such a return to baseline would not have been detected in our study. For example, our data show that the PCr/Cr. ratio is more than doubled during torpor compared with active animals. During IBA, the PCr/Cr ratio is lower than during torpor, but still higher during IBA than in active (fall and spring) animals. A possible interpretation is that the PCr/Cr ratio goes back relatively slowly to its baseline value during IBA. The same could hold true for Glu. Addressing this question would require measuring another group of animal at a later time point after initiating IBA.

Our study did not include a 'summer' group. By measuring the concentration of brain metabolites in the fall (just before the start of the hibernation season) and in the spring (right after the end of the hibernation season), we ensured that we would detect changes that were specific of torpor and IBA, as opposed to changes that occur before the hibernation season. However, there may also be changes between summer and fall that we were not able to detect because we did not measure a 'summer' group. Indeed, it is conceivable that animals measured in the fall (just before the hibernation season) could already have undergone significant changes in brain metabolite concentrations compared with summer animals. For example, the high myo-Ins concentration and the low Tau concentration observed in squirrel brain compared with rat brain may reflect species-specific differences, but they may also reflect metabolic adaptations in the squirrel brain in fall compared with summer. Measurement of a 'summer' group will allow us to address this question in the future.

Anesthesia

One limitation of our study is the fact that animals were anesthetized for the duration of MR measurements. Anesthesia is necessary for awake animals to avoid movement and minimize stress to the animals. Anesthesia also relaxed muscular tone in torpid hibernators, facilitating installation of animals in the chamber and positioning of the detection coil above the head. We chose in this study to anesthetize both awake animals and torpid hibernators with the same concentration of isoflurane. We did not evaluate the depth of anesthesia, raising the possibility that the depth of anesthesia may have been different in anesthetized active animals and in anesthetized torpid hibernators because of their different breathing pattern. However, experiments performed in rats with either 1% or 2% isoflurane did not show any substantial changes in the concentration of brain metabolites. Therefore, we consider it unlikely that the concentrations changes observed in this study are due to a differential effect of anesthesia between the different groups of animals.

Perspectives of ^{1}H MRS for the study of hibernation and neuroprotection

Hibernation is characterized by a dramatic reduction in blood flow reminiscent of ischemic stroke in humans (Frerichs *et al.* 1994). For this reason, hibernation has generated tremendous interest in the search of neuroprotective strategies after a stroke. Although many neuroprotective mechanisms appear to be specific to torpor (e.g. metabolic suppression, hypothermia), and although the brain of hibernators is not hypoxic, the brain of hibernators has been shown to have intrinsic neuroprotective properties (Frerichs and Hallenbeck 1998). Moreover, hibernators appear to survive hypoxia and ischemia better than non-hibernators even when they are in the euthermic state (Drew *et al.* 2004b; Dave *et al.* 2006). It has been suggested that this tolerance to hypoxia during euthermia may help hibernators tolerate transitions in and out of torpor (Ma *et al.* 2005). In spite of differences between hypoxia–ischemia and hibernation, a better understanding of the mechanisms leading to hypoxia–ischemia tolerance in hibernators (particularly euthermic hibernators) may provide novel directions for the treatment of stroke.

Among new potential therapeutic strategies are hibernation-inducing drugs (Borlongan *et al.* 2004) and systemic cooling or selective head cooling methods (Tooley *et al.* 2003; Gluckman *et al.* 2005; Thoresen and Whitelaw 2005; O'Brien *et al.* 2006). ^{1}H MRS could be used as a tool to reveal neurotransmitter and brain metabolite alterations *in vivo* in such therapeutic strategies. Previous ^{31}P MRS studies have demonstrated the usefulness of MRS to monitor changes in PCr and ATP during hypothermia (Laptook *et al.* 1995; Taylor *et al.* 2002; Litt *et al.* 2003; O'Brien *et al.* 2006).

Conclusion

We conclude that the brain of hibernators can be studied non-invasively using *in vivo* ^{1}H MRS even at near-freezing body

temperatures. A range of changes in energy metabolism and neurotransmission in the cortex of ground squirrels during hibernation was thereby detected. The increase in PCr/Cr ratio indicates increased energy storage, and maintenance of energy balance. Although the link between total neurotransmitter concentrations and neurotransmission is tentative, the increase in GABA and decrease in Glu suggest that a coordinated decrease in excitatory neurotransmission and increase in inhibitory neurotransmission may play a role in avoiding brain damage during metabolic arrest. The dramatic decrease in Gln during hibernation and rapid recovery of Gln during IBA, as well as the smaller and slower changes in Glu, support the hypothesis that anaplerosis is depressed and does not compensate for loss of Glu and Gln during hibernation.

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