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Mammalian cerebral metabolism and amino acid neurotransmission during hibernation.

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Abbreviations.
bpm - beats per minute, CEN - cenothermia, ECF - extracellular fluid, GLN -glutamine, GLU - glutamate, HR - heart rate, IV - intravenous, TCA -tricarboxylic acid, NST- nonshivering thermogenesis, ST - shivering thermogenesis

Abstract

This report demonstrates that during the torpor phase of hibernation, hamsters utilize ^{14}C and ^{13}C glucose in torpor specific brain metabolic pathways. Microdialysis of ^{14}C glucose into the striatum rapidly induced a steady state labelling of ECF lactate and labelling of tissue GABA, glutamate (GLU), glutamine (GLN) and alanine in ipsilateral and contralateral striata. The same tissue metabolites were labelled in cortex, hypothalamus and brainstem after microdialysis of ^{14}C lactate into the lateral ventricle. Serine, aspartate, glycine, taurine, tyrosine and methionine were not synthesised from glucose or lactate during torpor. ECF levels of amino and organic acids were low and unchanging during torpor and increased late during arousal to cenothermia. Labelled intracellular ^{14}C GABA and GLU were not communicated to the striatal ECF or ventricular space during torpor. ^{13}C NMR demonstrated rapid formation of lactate and functional TCA cycles in GABAergic and glutamatergic neurons, and enrichment of GLN and alanine after IV ^{13}C glucose. Large changes in tissue levels of amino acids occur prior to or during entrance into torpor but not during torpor. It is proposed that cerebral intracellular dehydration, the enlargement of ECF and the biochemistries associated with brain water homeostasis may have a role in regulating hibernation.

Running title: Brain metabolism during hibernation
Key words: Glutamate, GABA, torpor

Introduction

The metabolic pathways of cerebral energy metabolism have been extensively studied at cenothermia (IUPS term replacing euthermia, typically 36-37°C) using ^{13}C NMR or analysis of radioactive content of metabolites after enrichment with ^{13}C or ^{14}C isotopically labelled glucose, respectively. Using these two complimentary procedures and other techniques, brain energy metabolism at cenothermia has been determined to be almost exclusively dependent on the oxidation of glucose (Sokoloff 1977), however ketones generated from partial oxidation of fatty acids in muscle and liver may be utilized during fasting (VanItallie and Nufert 2003). Although the proportionality of the use of glucose by neurons and astrocytes is at present controversial (Chih et al. 2001; Gladden 2004), it is recognised that *in vivo* glucose consumption by cerebral cells results from processes that are mediated by chemical reactions that require ATP. These include processes that are associated with neuron/astrocyte glutamate/glutamine cycle (Hertz and Zielke 2004; Hyder et al. 2006), in addition to biochemical house keeping reactions such as protein, amino acid and membrane synthesis which also require ATP and contribute to the basal ATP consumption. As a generalization, in a homeostatic state, cellular ATP consumption is balanced by ATP production.

Mammalian hibernation is a naturally induced, reversible depression of global metabolic rate and body temperature employed to minimize energy consumption during periods of cold when nutrient resources are limiting (Carey et al. 2003). Within the hibernating season, a single bout of hibernation for hamsters is a physiologically dynamic interval composed of four phases. A 10h entrance phase characterised by inhibition of metabolic rate and decrease of body temperature (Geiser 2004). A 100h torpor phase characterised by maintenance of metabolic rate at about 2% of basal resting metabolic rate at cenothermia and body temperature 1-2 °C above ambient temperature (4-5°C in these experiments) (Osborne and Hashimoto 2003). A 4h arousal phase characterised by engagement of non-shivering and then shivering thermogenesis (Osborne et al. 2005). A 12h inter-bout cenothermic phase characterised by recovery and preparation for the next bout of hibernation (see Figure S1 supplementary material). The brain of the torpid hamster is remarkable for its other worldliness, displaying a virtual lack of DNA, protein, mRNA synthesis (Osborne et al. 2004; Revel et al. 2007) which contribute to decreased ATP consumption. Cortical blood flow is enormously reduced (Osborne and Hashimoto 2003), the EEG is essentially isoelectric (Chatfield et al. 1951; Chatfield and Lyman 1954; Gabriel et al. 1998) while intrahippocampal synaptic connectivity is greatly reduced (Magarinos et al. 2006). In addition the brain contains a plethora of substrates and compounds at non-cenothermic levels (Lust et al. 1989; Osborne and Hashimoto 2007), yet the torpid hamster retains the ability to respond to tactile stimulation (Osborne et al. 2005), hypercapnic gas (Lyman 1951; Osborne et al. 2005) and can appropriately monitor the internal milieu of the body and coordinate spontaneous arousal, the stimulus for which remains a mystery.

Ground squirrels are obligate hibernators and most experimental research has focused on this group of animals. On the basis of quantifications of glucose utilisation (Tashima et al. 1970), pyruvate dehydrogenase enzyme activities (Storey and Storey 2004) and gene levels (Andrews et al. 1998; Buck et al. 2002) in numerous peripheral tissues from ground squirrels, the primary metabolic paths fuelling ATP replacement during torpor were demonstrated to emphasize fatty acid beta-oxidation rather than glucose oxidation. However these analyses do not include brain tissue or tissues from other types of hibernators. Torpor in hamsters, a facultative hibernator, does not follow the pattern of autumnal fattening followed by prompt induction of torpor typical of ground squirrels but occurs only after two months exposure to a cold, dark environment and significant loss of autumnal body fat stores. An additional and

potentially significant dissimilarity in regard to cerebral glucose utilisation during torpor between obligate hibernators and hamsters is that blood glucose levels fall dramatically upon entry into torpor in obligate hibernators but are maintained at cenothermic levels during the torpor phase in hamsters (see references in (Westman and Geiser 2004)). This suggests that the metabolic fuel for torpor in hamsters is either heavily dependent upon beta oxidation and independent of plasma glucose or heavily dependent upon regulated use of plasma glucose, as it is at cenothermia.

To our knowledge *in vivo* measurements of cerebral metabolic pathways of synthesis and degradation of amino acids active during mammalian torpor have not been performed. Post-mortem analysis of brain tissue (Mandel et al. 1966; Lust et al. 1989)and *in vivo* ^{1}H NMR analysis (Henry et al. 2007) indicate significant changes in the tissue levels of free GABA, glutamate (GLU) and glutamine (GLN) during torpor that suggests an altered balance between the cerebral anaplerotic and cataplerotic pathways between torpor and inter-bout cenothermia. This provides indirect evidence that cerebral cellular metabolism during torpor is state specifically altered rather than being an energetically scaled down version of the biochemistry of cenothermia. This, coupled with the established preference of the cenothermic brain tissue for glucose as a fuel and the possibility that hamsters utilise glucose as a metabolic fuel for brain metabolism during torpor, a necessary prerequisite for an animal model of human hibernation, make an *in vivo* examination of cerebral glucose metabolism during torpor a crucial experimental objective for the understanding of the metabolic pathways required for the preservation of cerebral function and life during mammalian hibernation. This series of *in vivo* experiments are the first to use microdialysis of ^{14}C glucose or ^{14}C lactate and ^{13}C NMR to focus on the cerebral glucose utilizing metabolic pathways that remain functional during torpor in a mammalian hibernator.

Methods

Animals and housing

The following experiments conformed to the ethical guidelines of Asahikawa Medical University (ethics approval # 06004). Male hamsters *(Mesocricetus auratus)* were provided with *ad-lib* food and water and housed in 12:12 light dark cycle until attaining a body weight of 140g. Thereafter hamsters were housed individually in darkness at an ambient temperature of 4°C with *ad-lib* food and water. The surgical procedures employed in the chronic catheterization of the femoral vein, stereotaxic positioning of microdialysis guide tubes and probes in the striatum and lateral ventricles and the permanent placement of silver rings in the skin of the back under nembutal anaesthesia (65 mg/kg IP) with topical application of 4%

xylocaine have been described previously (Osborne et al. 2005) (Osborne and Hashimoto 2006). A detailed description is provided in the supplementary material.

Experiment 1

Amino, carboxylic acid and ammonia content of brain tissue, plasma in torpid and cenothermic hamsters.

Plasma and Tissue processing.

Brain tissues and blood samples were collected from eight hamsters with heart rate less than 10 bpm that had been torpid for 29-62 h since the onset of torpor and ten cenothermic hamsters 3h to 2 weeks after last arousing from torpor. The brain was rapidly removed and dissected at ambient 4°C into forebrain and midbrain for analysis. The forebrain region included the cortical lobes, hippocampal and striatal tissue but not olfactory bulb tissue. The midbrain region included thalamic, hypothamalic and midbrain but excluded cerebellum. Measurement of hematocrit, volume of red blood cells and acid digestion of brain tissue before HPLC analysis of amino and carboxylic acid content were performed by standard procedures.

Experiment 2.

ECF levels of amino and organic acids during torpor and arousal to cenothermia sampled by very slow perfusion microdialysis

The protocol for temperature independent sampling of the ECF during torpor and tactile stimulation induced arousal to cenothermia using very slow microdialysis has been described previously (Osborne and Hashimoto 2006) (Osborne and Hashimoto 2007). Briefly, during torpor when heart rate was less than 10 bpm and body temperature was less than 5 °C, two or three (4 µL) microdialysate samples were collected. A 24h non-perfusion interval separated the collection of each sample. One microdialysate sample was collected during the period of arousal that encompassed non-shivering thermogenesis (NST) when mouth temperature increased from 5.1 ± 0.1 to 7.9 ± 0.2 °C and HR increased from 18 to 38 ± 2 bpm. Another microdialysate sample was collected during the period of arousal that encompassed shivering thermogenesis (ST) when mouth temperature increased from 14.4 ± 0.2 °C to 36 °C, HR increased from 115 to 400 ± 8 b/min and respiratory rate increased from 60 breaths per min to peak at 140 and then decreased to about 80 breaths per min. Two consecutive microdialysate samples (CEN 1 and CEN 2) were collected upon body temperature returning to cenothermia. Microdialysates were collected from 2 consecutive bouts of torpor and arousal in 10 hamsters. The cenothermic period between these two successive bouts of hibernation was 15 ± 2 h (n = 10 recordings), a normal inter-bout cenothermic interval.

Dialysates from the first torpor and arousal bout were randomly allocated to the analysis of amino acids or organic acids. *In vitro* and *in vivo* experiments used microdialysis probes with 4-5 mm membranes (OD 250 µm, MBR 4-5, BAS, USA) perfused at 3.5 µL/h with 0.22 µm filtered art-CSF containing NaCl = 147 mM, KCl = 2.9 mM, $MgCl_2$ = 0.8 mM, $CaCl_2$ =

1.1 mM and glucose 1.2 mM. *In vitro* recoveries at 4 °C of art-CSF containing alanine, aspartate, GABA, GLN, GLU, glycine, isoleucine, lactate, leucine, lysine, methionine, phenylalanine, serine, taurine, threonine, tyrosine, tyrptophan urate and valine were greater than 90%.

Experiment 3.

Incorporation of ^{14}C label from 1-^{14}C glucose art-CSF into tissue and ECF metabolites during torpor.

In a group of seven torpid hamsters the microdialysis probe in the striatum was connected 20h after the onset of torpor and was perfused with art-CSF containing 1.4 mM 1-^{14}C glucose [50 mCi/mmol], (Moravek, Tokyo) at 1 µl/h. Dialysates were collected every 12h into a semi-sealed vessel containing 6 µl of 0.1M HCl at 4°C ambient and then frozen at -80°C until HPLC analysis of ^{14}C incorporation into amino or organic acids in ECF. Hamsters were killed after 24, 48 or 60 h of microdialysis perfusion by decapitation. Brains were rapidly removed from the cranium at 4°C ambient and frozen in liquid nitrogen. Brains were cut sagitally in an anterior to posterior direction on a freezing microtome to locate the position of the microdialysis probe in the striatum. Thirty (30) µm frozen sections were cut and dried. Normalised optical density measurements of the diffusion of the radioactive ^{14}C containing metabolites were performed after exposure to Fuji image plate (BAS III, Fuji Photo Co, Tokyo) using Image J (1.38x, NIH, Bethesda). Brain tissue (approximately 100 mg) was dissected from the ipsilateral and contralateral striatal hemispheres immediately caudal to the position of the microdialysis tract. In order to avoid formation of pyroglutamate and degradation of GLN induced by acid digestion, these brain tissues were briefly lysed in ice cold water, sonically homogenised and subject to thermal shock by refreezing at -80 °C. Protein was precipitated by dilution to 75% with ethanol and pelleted by centrifugation. The supernatant was filter (5µm ultrafree, milipore), lyphilised, diluted with water and stored at -80°C. HPLC was used to quantify amino acid contents of tissue extracts and dialysates. The HPLC elluant was collected into a flurophore (Atomlight, Packard Instrument Company, USA) and the radioactive content of amino acids (dpm) was determined by scintillation counting (LS6500 Beckman, USA).

Experiment 4.

Incorporation of ^{14}C label from 1-^{14}C lactate art-CSF into tissue and ECF metabolites during torpor.

In a group of four torpid hamsters the microdialysis probe positioned in the lateral ventricle was connected 20h after the onset of the torpor phase and was perfused with art-CSF containing 1.2 mM glucose. Dialysates were collected as per the protocol in experiment 2 and the lactate content of the CSF was measured by HPLC. In the next (2nd) bout of hibernation, the microdialysis probe was connected 20h after the onset of torpor and was perfused with art-CSF containing 1.2 mM glucose and 6.6 mM lactate at 1 µl/h for 36h. Perfusion lactate concentration was 5 mM C^{12} lactate and 1.6 mM U-^{14}C lactate [32.7 mCi/mmol], (MP Biomedicals, Tokyo). Dialysates were collected every 12h into a semi-sealed vessel containing 6 µl of water at 4°C ambient and then frozen at -80°C until HPLC analysis of ^{14}C incorporation into amino acids in ECF. Torpid hamsters were killed after 36h of microdialysis perfusion by decapitation after blood was sampled by cardiac puncture. Brains were rapidly removed from the cranium at 4°C ambient. The brain was bisected longitudinally. The brain half contra-lateral to the microdialysis probe was immediately frozen. 100mg tissue samples from cortex, hypothalamus, brainstem and cerebellum were dissected from the ipsilateral half and frozen in liquid nitrogen. Tissues and dialysates were processed and analysed for radioactive incorporation of ^{14}C into amino acids as per experiment 3. The microdialysis

probe was positioned in the ventricular cavity to facilitate diffusion of the labelled lactate into the brain.

Experiment 5.

Determination of ^{13}C labelled tissue metabolites after IV infusion of 1-^{13}C glucose into torpid hamsters and analysis by ^{13}C NMR.

Experiments were performed on hamsters (body weight 90-115 g) that had undergone two complete bouts of hibernation since femoral vein catheterization. Eighteen hamsters that had been torpid for 20h, with a heart rate of less than 10 bpm, were divided into six groups of three animals. Four groups were infused via the femoral vein catheter with 1-^{13}C glucose (200 mg/kg) in 160 µl of saline at 5-10 µl/min. The fifth group was infused as above with ^{12}C glucose (200 mg/kg). The sixth group of torpid hamsters were not manipulated. Torpid hamsters in groups 1-4 were killed by decapitation after blood sampling by cardiac puncture, 1h, 24h, 48h or 60h after infusion of 1-^{13}C glucose. Torpid hamsters in group 5 were killed by decapitation, after blood sampling by cardiac puncture, 1h after infusion of ^{12}C glucose. The sixth group of three non-infused torpid hamsters were killed by decapitation, after blood sampling by cardiac puncture, 21h after the onset of torpor. A separate group of five non-infused torpid hamsters, with heart rate of less than 10 bpm, were killed by decapitation after blood sampling by cardiac puncture, 20-88h after the onset of torpor. In all hamsters, uninterrupted torpor was confirmed by continuous measurement of heart rate of less than 10 bpm. Forebrain included cortical lobes, hippocampus and striatum but excluded olfactory bulb. The hindbrain included the thalamus, hypothalamus, cerebellum and underlying brainstem. Homogenates of frozen tissue and plasma were acid deproteinated, neutralized, treated to remove divalent cations with Chelex 100 (Sigma), filtered (0.22 µm), re-neutralised to pH 7.3 and lyophilised by standard procedures and stored at –80°C. Immediately prior to NMR measurement, the lyophilate was dissolved in 0.8 ml of D_2O containing 0.05% sodium azide, MeOH was added as an external reference (49.55 ppm) and the solution was placed in a sterile NMR tube and sealed with saran film before measurement.

NMR measurements.

The NMR spectra of extracted tissue were acquired using a JEOL FX-400HT spectrometer (T = 9.4) operating at 100.4 MHz (^{13}C) and 399.6 MHz (^{1}H). Conditions for measurement of NMR spectra are presented in supplementary material. The area of taurine CN at 48.2 ppm, inositol C4,C6, inositol C1,C3 and inositol C5 peaks at 71.3 ppm, 72.5 and 74.6 ppm respectively, did not change over the torpor phase . Inositol peak areas were used as internal references for the calculation of specific enrichments for each spectrum as per the procedure outlined in (Merle et al. 2002) that corrects for differences in nuclear Overhauser effects between spectrums should they be present. A detailed description is provided in the supplementary material.

HPLC analysis.

Amino acids.

Amino acids in standards, tissue homogenates or microdialysates were quantified using gradient reverse phase HPLC and fluorescence detection at 340/450 nm after precolumn derivatization with O-phthaldialdehyde and 2-mercaptoethanol as per a modification of a previously published method (Kehr 1998). A more detailed description is provided in the supplementary material.

Pyruvate, lactate and urate.

Pyruvate, lactate and urate in standards, acid digested tissue homogenates at pH 7.3 or microdialysates were quantified using isocratic ion exchange HPLC and absorbance detection at 225 nm. A more detailed description is provided in the supplementary material.

Glucose and ammonia.

Plasma glucose and ammonia content of tissue homogenates were analysed using a Hitachi 7170 automatic analyzer.

Statistics

All results are presented as means ± SEM. Comparisons of brain tissue contents and plasma levels of analytes between torpid and cenothermic hamsters were by unpaired t-test. Changes of ECF analyte levels during torpor and arousal to cenothermia were analysed by repeated measures ANOVA with Tukey post-hoc comparisons. Incorporation of ^{14}C into dialysate lactate was analysed by repeated measures ANOVA with Tukey post-hoc comparisons. Incorporation of ^{14}C into tissue amino acids was analysed by one way ANOVA with Tukey post-hoc comparisons. Statistical significances was set at $P < 0.05$.

Results

Experiment 1

Amino, carboxylic acid and ammonia content of brain tissue, plasma in torpid and cenothermic hamsters.

The amino acid content of forebrain, midbrain and plasma from torpid and cenothermic hamsters is shown in table 1. Although there are clear differences in the tissue content of amino acids between cenothermia and torpor, in general there was no difference between the effects of torpor on tissue levels of free amino acids between brain regions. Torpor was associated with global decreased in brain tissue content of aspartate, GLU, isoleucine, tyrosine and tryptophan. Tissue content of alanine, GABA, serine, threonine and lysine were globally increased during torpor. Tissue content of GLN in torpid hamsters was increased in hindbrain and tended to be higher in forebrain and cerebellum. Tissue content of histidine and taurine were globally conserved between states. Tissue content of glycine, leucine and methionine were inconsistent between regions and states. Midbrain tissue content of ammonia was unchanged between torpid (1.54 ± 0.08 µM/mg (n=8)) and cenothermic hamsters (1.69 ± 0.14 µM/mg (n=10), t=0.9, df=16, P=0.39). Ammonia content of other brain regions was not measured. There was no relationship between tissue amino acid content and duration of torpor phase. Changes in plasma levels of GLU, GLN, lysine, phenylalanine and tyrosine were consistent with the changes in tissue contents associated with torpor. Changes in plasma levels of alanine, serine, threonine, tyrosine and valine were opposite to the changes in tissue contents associated with torpor. Plasma levels of glycine, histidine and methionine were decreased during torpor. Hematocrit was significantly increased in torpor (58 ± 0.6 (n=10)) compared with hamsters that were interbout cenothermic for less than 24h (50.5 ± 0.9 (n=10)) (t=6.7, df= 18, P<0.0001) although there was no difference in the volume of red blood cells between the two states.

Experiment 2.

ECF levels of amino and organic acids during torpor and arousal to cenothermia

Figure 1 shows the temporal profile of the increase of striatal ECF levels of amino and organic acids that occurs when the hamster arouses from torpor to cenothermia. In general, basal ECF levels of amino acids at interbout cenothermia were 3-4 fold higher than during torpor. (Basal ECF levels of analytes and a summary of the incorporation of ^{13}C or ^{14}C label into cerebral tissue and ECF metabolites during torpor are presented in Supplementary Table S1 that is contained in the supplementary material). The exceptions being urate, lactate and

lysine where torpid ECF levels were 80%, 70% and 35% of cenothermic ECF levels respectively. ECF levels of analytes were not different from levels during torpor in the period of non-shivering thermogenesis despite increased brain temperature, heart rate and metabolic rate. ECF levels of all analytes were significantly increased from levels during torpor in the period of shivering thermogenesis. ECF levels of urate, but no other analyte, increased above cenothermic levels during the collection period that encompassed the maximum metabolic rate associated with shivering thermogenesis.

Experiment 3.

Incorporation of ^{14}C label from 1-^{14}C glucose art-CSF into ECF and tissue metabolites during torpor.

Diffusion of ^{14}C label from dialysis probe during torpor.

The diffusion of 1-^{14}C glucose and radioactive metabolites containing the ^{14}C label within the torpid brain is shown in figure S2 that is contained in the supplementary material.

^{14}C Labelling of ECF metabolites.

In seven torpid hamsters basal ECF levels of pyruvate 4.2 ± 0.6 µM, lactate 6.8 ± 0.4 mM and urate 79 ± 4 µM were constant for 3 consecutive 12h intervals from 20 to 56 h of torpor ($F(2,17)< 0.94$, $P>0.22$ for each analysis). The ^{14}C label from 1-^{14}C glucose was not incorporated into urate. Incorporation into pyruvate could not be determined because of co-elution of glucose in the HPLC separation. Figure 2 shows that incorporation of ^{14}C label into lactate was almost maximal by 12h and had increased slightly but significantly by the collection of the third dialysate ($F(2,17)=4.87$, $P<0.03$. In four torpid animals, amino acid analysis of 12h dialysates collected from the 48 to 60h of perfusion with 1-^{14}C glucose demonstrated low level of incorporation of the ^{14}C label into ECF GLN. No ^{14}C label was measurable in other ECF amino acids.

^{14}C Labelling of striatal tissue metabolites.

In both the contralateral and ipsilateral hemispheres from six torpid hamsters dialysed for 24, 48, or 60h the ^{14}C label from 1-^{14}C glucose was incorporated into alanine, GLU, GLN and GABA. It was not incorporated into any other amino acid. A linear relationship existed between the incorporation of ^{14}C label into metabolite (dpm/µM/mg of tissue) on the contralateral and ipsilateral hemispheres versus duration of microdialysis. The R^2 values of the relation for each amino acid were alanine (R^2=0.85), GLU (R^2=0.73), GLN (R^2=0.64) and GABA (R^2=0.81). These relations were used to normalise the radioactive labelling between contralateral and ipsilateral hemispheres. Figure 3 shows that the ^{14}C label from 1-^{14}C glucose was increasingly incorporated into GLU, GLN, alanine and GABA over the course of the dialysis with 1-^{14}C glucose art-CSF while the total tissue content of amino acids did not change over the torporous incubation period.

Experiment 4.

Incorporation of ^{14}C label from U-^{14}C lactate art-CSF into ventricular and tissue metabolites during torpor.

^{14}C Labelling of metabolites in ventricular CSF.

Lactate concentration in the ventricular CSF during torpor of the first bout of hibernation in 4 hamsters was 6.2 ± 0.5 mM. Amino acid analysis of three consecutive 12h microdialysates collected during 36h of perfusion with U-^{14}C lactate art-CSF during torpor of the second bout of hibernation showed constant levels of all measured amino acids. The ^{14}C label in ECF GLN in the 1st 12h CSF was sample was 155 ± 15 dpm. This increased in successive samples and was 497 ± 31 dpm in the 3rd 12h CSF sample ($P <0.001$) but total ECF levels of GLN (200 ± 20 µM) were unchanged between the 3 samples. No ^{14}C label was measurable in other ECF amino acids. The plasma of all torpid hamsters contained low levels of radioactive ^{14}C label (1384 ± 146 dpm/20 µL). HPLC analysis of plasma demonstrated that the radioactive fraction was not lactate or amino acid.

^{14}C Labelling of cerebral tissue metabolites.

The ^{14}C label from U-^{14}C lactate was incorporated into tissue alanine, GLU, GLN and GABA in cortical, hypothalamic and brain stem tissue in all four hamsters dialysed for 36h during torpor. Incorporation of label into metabolites in cerebellum was inconclusive and absolute levels were close to background. The absolute level of ^{14}C labelling in each amino acid was greatest in tissue closest to the microdialysis probe being hypothalamus = cortex>brain stem and lowest in cerebellum. The pattern of labelling in substrates was the same in each brain area suggesting no obvious regional differences.

Experiment 5.

Incorporation of ^{13}C label into plasma, forebrain, hindbrain tissue during torpor.

Plasma.

Figure 4 shows the time course of change in plasma glucose levels over the torpor phase in response to IV glucose infusion. Plasma glucose from five non-infused hamsters torpid for 20 to 88h was maintained at 9.7 ± 0.3 mM. One hour after IV infusion of ^{12}C glucose (200 mg/kg) plasma glucose had increased to 14.1 ± 0.6 mM (n=3). The concentration (and enrichment) of ^{13}C glucose in plasma was approximately 4.3 mM (32%) one hour after IV infusion (200 mg/kg) and had decreased to 0.8 mM (7%) by 60h after IV infusion. Mean plasma lactate level was 0.86 ± 0.03 mM (n=12) and showed no relationship to the duration of the torpor phase. The ^{13}C label from IV infused glucose was not detected in any metabolite in the plasma.

Brain.

Figure 5 is a stack plot of ^{1}H decoupled ^{13}C NMR spectra from approximately 1.5 gm of acid digested torpid hamster hindbrains depicting the time course of incorporation of ^{13}C label into metabolites until the periods 1h (spectra B), 24h (spectra C) 48h (spectra D) 60h (spectra E) after a 36 minute infusion of 1-^{13}C glucose. Endogenous ^{13}C levels 1h after ^{12}C glucose infusion in torpid hamsters (spectra A). Hindbrain tissue from 3 hamsters was combined to generate each spectrum. In spectra B and C, the resonances of glucose (C1 apha and C1 beta), probably of blood vessel origin, were measured. By 1h after infusion the ^{13}C label of glucose was incorporated into the C3 of lactate (spectra B). By 24h after infusion (spectra C) the ^{13}C label was incorporated into resonances for GABA (C2, >C3=C4), GLU (C4, > C3=C2), alanine (C3), lactate (C3) and pyroglutamate (C4, > C3=C2). The label from GLN has been incorporated into pyroglutamate as a consequence of acid digestion and alkalination during tissue preparation. There was no evidence of incorporation of ^{13}C label into glycogen (C1), inositols (C1, C3, C4, C5, C6), taurine (C1, C2), creatinine C2, glucose (C2a, C5a) or aspartate (C3). The ^{13}C label is incorporated in an unknown peak at 69 ppm that may be lactate C2 or dihydroxyacetone phosphate (DHAP) at 68 ppm. The pattern of incorporation of the ^{13}C label into metabolites of the forebrain was indistinguishable from the spectra of hindbrain, with the exception that specific enrichments are smaller and alanine appears to be labelled by 1h spectra (C). The pattern of labelling at 48h (spectra D) and 60h (spectra E) is essentially similar to 24h (spectra C) however the specific enrichment of each labelled compound increases with duration of torpor. The percentage enrichment of forebrain and hindbrain was averaged for each spectrum and is presented in Figure 6. The specific enrichment of GLM was corrected to include the C4 >C3=C2 labelling pattern of pyroglutamate (Willker et al., 1995).

Discussion

Plasma.

The initial finding of this study is that plasma glucose level is regulated to balance tissue uptake of glucose during torpor in hamsters. IV enrichment of plasma with 1-^{13}C glucose induced a modest hyperglycemia, with the normal 10 mM plasma level being increased to 14 mM. At this stage it is not known if uptake of glucose by tissues is an insulin dependent response. The lack of ^{14}C or ^{13}C label in plasma alanine or lactate and the constant level of unlabeled plasma lactate over the duration of torpor suggests that the products of glucose oxidation, with the exception of bicarbonate, are predominantly retained within the tissue of synthesis.

Glycolysis during torpor.

The blood brain barrier remains functional during torpor since plasma and ECF amino acid concentrations are distinct and ECF ^{14}C labelled lactate and amino acids are not communicated to the plasma. The first prominent finding in the metabolic chain of glucose oxidation is that despite the isoelectricity of the brain (Chatfield et al. 1951; Chatfield and Lyman 1954; Gabriel et al. 1998) and the very low metabolic rate during torpor (Osborne and Hashimoto 2003), 1-^{13}C glucose supplied to the blood is transported across the blood brain barrier and metabolised to lactate by brain tissue within less than 1 hr after IV infusion (NMR spectra B). A similarly rapid time scale is associated with the uptake and metabolism of ^{14}C glucose by cerebral cells and the establishment of a steady state of ^{14}C labelled lactate in the striatal ECF within 12h of the onset of microdialysis. Thus it appears that brain tissue of hamsters retains a functional pyruvate dehydrogenase complex torpor. This contrasts with peripheral tissues in hamster (unpublished data) and the ground squirrel, an obligate hibernator (Tashima et al. 1970; Andrews et al. 1998; Buck et al. 2002; Storey and Storey 2004).

In the brain of the torpid hamster the concentrations of lactate in the ECF and CSF (~6-7 mM) are considerably higher than in the plasma (0.9 mM), consistent with the trend of measurements in cenothermic humans and rats (Abi-Saab et al. 2002). At cenothermia, the higher level of lactate in the ECF has been offered as supportive evidence for the Astrocyte/Neuron Lactate Shuttle theory (Pellerin and Magistretti 1994) where active transport of lactate from astrocytes into the ECF makes lactate available for neuronal metabolism during periods of enhanced metabolic activity associated with action potential dependent release, uptake and recycling of GLU and GABA (Abi-Saab et al. 2002; Hyder et al. 2006; McKenna et al. 2006). However, in contrast to cenothermia, the immediate energy expenditure of the torpid brain is low, since there are no action potentials and many biochemical processes of synthesis are non-functional yet the synthesis of lactate and export to the ECF and utilisation in the TCA cycle for the production of GLU, GABA and GLN is clearly a major metabolic objective. The apparent emphasis on lactate production of the torpid hamster brain may be derived from replacement of a temporally displaced metabolic deficit incurred by neurons during the entrance into torpor. Alternatively or in addition, the brain of the torpid hamster may be mildly hypoxic. No measurements of brain tissue oxygen concentrations are available for torpid hibernators and it has been assumed on the basis of blood oxygen measurements that brain tissue oxygenation is preserved in the normoxic range with the low cerebral blood flow measured during torpor (Frerichs et al. 1994; Osborne and Hashimoto 2003) being adequate to balance the low metabolic requirements of cerebral tissue. However, the rapid synthesis of intracellular lactate and alanine (faster in forebrain than hindbrain) and the decreased ECF pyruvate:lactate ratio in torpor relative to cenothermia are biochemical responses to increased cytosolic NADH/NAD+ ratio that is, at cenothermia, associated with mild hypoxia (Ben-Yoseph et al. 1993; McKenna et al. 2006). Chronic mild hypoxia during torpor could be described as a form of natural endogenous preconditioning to

ischemia and rationalises the fact that brain tissues from hibernating hamsters and ground squirrels are remarkably tolerant of oxygen and nutrient deficiency (Ross et al. 2006).

TCA cycle metabolism during torpor.

In torpid hamsters the ^{14}C label from 1-^{14}C glucose or ^{14}C lactate dialysed into the ECF or ventricular space respectively, was incorporated into tissue GABA, GLU, GLN by reactions dependent upon TCA cycle rotations. These same cerebral amino acids were labelled after IV infusion of 1-^{13}C glucose. Consistent with dormice (Mandel et al. 1966) and ground squirrels (Henry et al. 2007), the hamster brain tissue content of amino acids is clearly different between the cenothermic and torpid states (Table 1). However, there was no evidence of a relationship between the tissue content of any amino acid and the duration of torpor. This was true for amino acids that were not enriched with the ^{14}C or ^{13}C label and also for alanine, GABA, GLU, GLN that were isotopically enriched. This implies that the denovo synthesis and degradation of brain tissue amino acids that results in these global changes in tissue amino acid content probably occurs during the entrance into torpor not during the torpor phase of the bout of hibernation. The allocation of these ATP consuming reactions to the entrance to the torpor phase is consistent with the very low energy expenditure measured during torpor.

In glia at cenothermia, enrichment of ^{14}C or ^{13}C label in the absence of accumulation of tissue levels of alanine, GABA, GLU, GLN implies recycling of labelled substrate, the mechanisms of which cannot be clarified by these experiments, but can involve either inhibition of glial anaplerotic pathways (Hyder et al. 2006) and/or pyruvate recycling (Haberg et al. 1998). In GABAergic and glutamatergic neurons, which lack pyruvate carboxylase (Yu et al. 1983), GLU and GABA are labelled without accumulation after the passage of pyruvate into the TCA cycle via pyruvate dehydrogenase. In the case of GABA this requires the reintroduction of labeled GABA into the TCA cycle at succcinylCoA via the GABA shunt (Hassel and Sonnewald 1995). In the brain of the torpid hamster, the ^{13}C label of glucose was transferred to GABA with the pattern of enrichment being C2>C3=C4, in the absence of extracellular release of GABA. This indicates that the TCA cycle in GABAergic neurons completed multiple full turns and that GABA containing ^{13}C labelled C2 was reintroduced into the TCA cycle at succcinylCoA to allow for the subsequent labelling of C3 and C4 carbons of GABA. Interestingly, aspartate that is co-localized in GABAergic neurons (Kaneko and Mizuno 1994) was not labelled by either ^{14}C or ^{13}C at any time despite the high enrichment of GABA. The absence of label in aspartate indicates that the conversion from oxaloacetate by aspartate transferase is of very low activity in both neurons and astrocytes, implying torpor specific inactivation of the malate-aspartate shuttle that may be influenced by the decreased brain tissue contents of aspartate and GLU or by hypoxia.

The ^{13}C label of glucose was transferred to GLU with the pattern of enrichment being C4>C3=C2 by 24 h after IV infusion indicating GLU labelling occurs predominantly in glutamatergic neuron TCA cycle via the bidirectional transamination between alpha KG and GLU (Hertz and Zielke 2004). A component of this GLU enrichment pattern may be derived from GABAergic neurons, where GLU is a substrate for glutamate decarboxylase and a precursor of GABA synthesis (Roberts and Frankel 1950). The exclusively glial localization of glutamine synthetase (Norenberg and Martinez-Hernandez 1979), determines that incorporation of ^{14}C and ^{13}C label into GLN is evidence of glial function during torpor, the nature of which cannot be determined by these experiments.

Amino Acid Neurotransmission during torpor.

Although the torpid hamster brain lacks action potentials, previous systemic and ICV injection studies demonstrate that many central receptors retain a stimulatory function capable of inducing arousal from hibernation (Lyman and O'Brien 1988; Hermes et al. 1993; Tamura et al. 2005). Our contention was that endogenous intracellularly synthesised GLU or GABA

from cortex, midbrain and brainstem tissues would be communicated to the ECF over the course of the torpor phase and the accumulating ECF amino acid neurotransmitter levels would ultimately act to stimulate arousal from torpor by activation of distant receptors by volume transmission. This did not occur. In contrast, there was no evidence of the ^{14}C enriched intracellular GLU and GABA being communicated to the ECF by 60h after synthesis. This was not a constraint of low temperature on diffusion since ECF applied ^{14}C label had diffused to the entire brain within this same time interval (See Figure S2 supplementary material). Contrary to expectation, low levels of newly synthesised GLN were communicated to and taken up from the CSF, possibly as a means of ammonia detoxification or as a substrate for the synthesis of GABA destined for vesicular release (Fricke et al. 2007). Surprisingly, the ECF levels of all amino and organic acids were constant and lower in torpor than during cenothermia (Figure 1) irrespective of the changes in tissue or plasma amino acid content between these two states (Table 1). Low ECF amino acid levels in torpid hamsters is consistent with a earlier study demonstrating that ECF GABA was lower in torpor than during cenothermia in ground squirrels (Osborne et al. 1999). ECF levels of all amino acids, including amino acid neurotransmitters, increased as the hamster aroused from torpor along a similar profile to body temperature rather than metabolic rate. The only exception was urate which reflected the profile of metabolic rate and has been demonstrated to be xanthine oxidoreductase dependently synthesised during arousal (Osborne and Hashimoto 2007).

The torpid brain is capable of monitoring internal body and external environmental stimuli and orchestrating a limited number of responses to these stimuli by as yet undetermined neural mechanisms. Electrophysiological recordings of torpid hamsters consistently demonstrate that recognisable cerebral action potentials are not recorded below 14°C (Chatfield et al. 1951; Chatfield and Lyman 1954; Gabriel et al. 1998) and recent histological studies demonstrate global dendritic regression during torpor (Magarinos et al. 2006; von der Ohe et al. 2006). In ground squirrels, immunohistological studies demonstrate a 50-65% loss of synapses over the entire brain during entry into torpor (von der Ohe et al. 2007). These measurements when combined with the lack of newly synthesised amino acid transmitter efflux into the ECF and constant, low ECF amino acid neurotransmitter levels during torpor demonstrated by these microdialysis studies suggest that torpor is maintained and the initial stages of arousal are coordinated in the absence of electrophysiological, anatomical or biochemical correlates of synaptic neurotransmitter activity. This raises two possibilities for intercellular cerebral communication in torpid animals. The first, being that the torpid brain communicates via an evasive network of numerically few neurons that retain classical cenothermic function at low temperatures. The increasingly compelling alternative is that functional communication between neurons during torpor is via non-synaptic mechanisms.

Recent histological studies on the brains of ground squirrels demonstrate a 35%-40% global decrease in cerebral cell body area in torpor that is reversed by arousal to cenothermia (von der Ohe et al. 2006). The resultant decrease in cerebral cell volume during torpor must result from the movement of intracellular water into the ECF that, if not totally equilibrated with the blood, will increase the cerebral ECF volume. However, the plasma cannot function as a reservoir for the efflux of cerebral water because plasma volume is decreased and urine formation is greatly reduced (Osborne et al. 2004) during torpor in hamsters. When viewed from this hydrodynamic perspective, the cause of the uniform increase in ECF levels of all amino and organic acids measured late in arousal from torpor, is unlikely to be the result of increased synthesis or transport across membranes since many metabolites would require compensatory decreases in ECF levels but more probably reflects a decrease in cerebral ECF volume that occurs as cerebral cells take up water and return to normal volume as part of the restoration of cenothermic brain water homeostasis that occurs late in arousal from torpor.

Torpor specific alteration of brain water homeostasis and the shrinking of cerebral cells during entrance into torpor may have both biochemical and biophysical effects. Intracellular dehydration may decrease the activity of intracellular enzymes by altering local substrate concentrations or pH. Biophysical effects of an enlarged ECF may emphasize new physical connections between specific cerebral cells thereby modifying the efficiency of neuronal signal transduction and communication. These physical changes when acting in conjunction with synaptic regression (Magarinos et al. 2006) may produce a torpor specific neural network that functions to co-ordinate torpor and the onset of arousal, possibly by mechanisms independent of classical synaptic activity. In addition to inducing a global increase in the level of cerebral ECF amino and organic acids, the reversal of the putatively enlarged ECF by uptake of water into cerebral cells late in arousal to cenothermia is also temporally consistent with the temperature threshold for the reappearance of measurable action potentials (Chatfield et al. 1951; Chatfield and Lyman 1954; Gabriel et al. 1998), the onset of mRNA and protein synthesis during arousal from torpor to cenothermia (van Breukelen and Martin 2001; Osborne et al. 2004) and the re-initiation of behaviour. This suggests that cerebral intracellular dehydration, the enlargement of the cerebral ECF and the biochemistries associated with brain water homeostasis may have a crucial and previously unappreciated role in regulating the physiology of mammalian hibernation.

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	Forebrain (µm/mg ww)		Midbrain (µm/mg ww)		Plasma (µM)	
Analyte	IBC	Torpor	IBC	Torpor	IBC	Torpor
Taurine	8053 ± 460	8215 ± 430	3356 ± 203	3377 ± 422	na	na
Glutamine	13440 ± 402	14582 ± 376↑↑	10536 ± 810	12279 ± 1099	434 ± 29	569 ± 60↑
Glutamate	9023 ± 251	5851 ± 135↓↓↓	7638 ± 300	5507 ± 588↓↓↓	203 ± 18	174 ± 13
GABA	2191 ± 74	2731 ± 70↑↑↑	3060 ± 152	4664 ± 293↑↑↑	bdl	bdl
Aspartate	1775 ± 107	902 ± 27↓↓↓	1833 ± 69	980 ± 122↓↓↓	na	na
Alanine	923 ± 61	2093 ± 107↑↑↑	531 ± 35	1078 ± 135↑↑↑	268 ± 24	147 ± 7↓↓↓
Glycine	1101 ± 116	1035 ± 106	1404 ± 46	1624 ± 203	na	na
Serine	628 ± 20	934 ± 49↑↑↑	459 ± 45	777 ± 97↑↑↑	130 ± 9	57 ± 2↓↓↓
Threonine[e]	194 ± 17	375 ± 19↑↑↑	167 ± 10	341 ± 16↑↑↑	92 ± 2	31 ± 2↓↓↓
Methionine	245 ± 20	208 ± 12	124 ± 14	169 ± 10↑	41 ± 4	19 ± 2↓↓↓
Valine[e]	97 ± 2	103 ± 6	96 ± 3	113 ± 4↑↑	247 ± 13	199 ± 5↓↓
Lysine[e]	113 ± 5	252 ± 10↑↑↑	148 ± 9	298 ± 9↑↑↑	190 ± 14	406 ± 20↑↑↑
Histine	93 ± 3	91 ± 5	110 ± 5	106 ± 13	69 ± 3	48 ± 2↓↓↓
Leucine[e]	64 ± 2	58 ± 4	56 ± 3	69 ± 4↑	132 ± 12	174 ± 5↑
Tyrosine	66 ± 4	38 ± 2↓↓↓	57 ± 5	30 ± 1↓↓↓	66 ± 7	25 ± 1↓↓↓
Ph.alanine[e]	28 ± 1	31 ± 1↓↓↓	29 ± 1	37 ± 1↑↑↑	42 ± 3	57 ± 2↑↑
Isoleucine[e]	17 ± 0	11 ± 1↓↓↓	19 ± 1	13 ± 1↓↓	59 ± 5	49 ± 2
Tyrptophan[e]	17 ± 1	8 ± 1↓↓↓	7 ± 3	bdl ↓	56 ± 5	27 ± 2↓↓↓
Ammonia	na	na	1.69 ± 0.14	1.54 ± 0.08	na	na

Table 1.
Comparison of brain tissue and plasma amino acids levels during torpor and cenothermia. Amino acid content of brain tissue (µm/mg wet weight) and plasma (µM) from cenothermic hamsters (3hr to 2 weeks after last arousing from torpor, IBC - interbout cenothermia, N=10) and hamsters hibernating for 29 to 62h (N = 8). Essential amino acids are denoted by superscript e. Mean ± SEM. Increase significantly different from IBC. ↑, $P<0.05$; ↑↑, $P<0.01$; ↑↑↑, $P<0.001$. Decrease significantly different from IBC. ↓, $P<0.05$; ↓↓, $P<0.01$; ↓↓↓, $P<0.001$. Na – not analysed. bdl – below detection limit.

Figure 1.

The effect of arousal from torpor to cenothermia on the profile of changes in striatal ECF levels of amino and organic acids sampled by slow perfusion microdialysis. A) urate - solid circle; lactate - x dotted line; pyruvate - open circle; GLU - open diamond; alanine - solid triangle; GLN - open square. B) lysine - open circle; taurine - open square; aspartate - solid triangle. Profile of glycine, histidine, isoleucine, leucine, methionine, phenyalanine, serine, tyrosine, tyrptophan and valine were indistinguishable and contained in lines denoted as {Amino acids 1-10}. (C) Profile of changes in brain temperature (°C) (filled black squares) and (D) metabolic rate (filled black circles) during collection of microdialysis samples. Brain temperature and metabolic rate determined from previous publication based upon measurement of cheek pouch temperature, heart and respiratory rate (Osborne and Hashimoto 2003). Torpor (Tor) and non-shivering thermogenesis (NST) dialysates collected from discontinuous sampling. Shivering thermogenesis (ST), cenothermia (CEN 1h and CEN 2h) dialysates collected from continuous sampling. Basal striatal ECF dialysate levels of each analyte are presented in Supplementary Table S1. Mean ± SEM (n=5-13). *P<0.05. SEM are often smaller than symbols.

Figure 2.

Steady state labelling of newly synthesised striatal ECF lactate during torpor.
Striatal ECF levels of total lactate (µm) (filled X), and ^{14}C incorporation into lactate (dpm x1000/(µm) (open X), in 12 µl samples during 36h of perfusion of striatum with 1-^{14}C glucose at 1 µL/h. Mean ± SEM (n=7). *P<0.05

Figure 3.

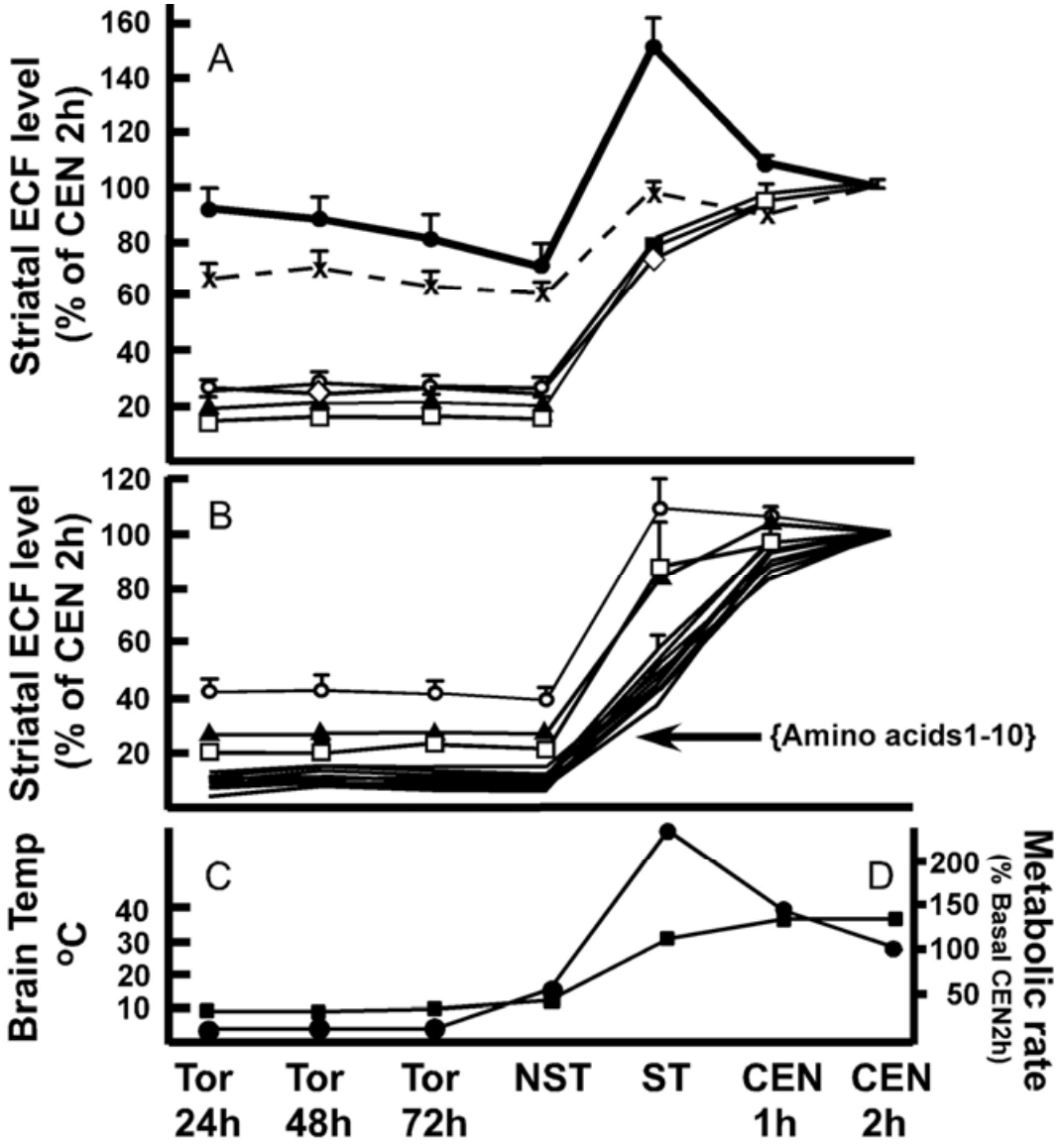
Incorporation of radioactive label into tissue amino acids increases over the torpor phase.
Total striatal tissue content (µm/mg wet weight) of GLN (filled square with solid line), GABA (filled circle with solid line), GLU (filled diamond with dotted line) and alanine (filled triangle with dotted line) from torpid hamsters killed after microdialysis perfusion with 1-^{14}C glucose at 1 µL/h for 24h, 48h and 60h. Radioactive ^{14}C content (dpm/µm/mg wet weight) incorporated into striatal tissue GLN (open square), GABA (open circle), GLU (open diamond) and alanine (open triangle) after microdialysis perfusion with 1-^{14}C glucose at 1 µL/h for 24h, 48h and 60h. Mean ± SEM (n=4 striata per perfusion time). Different from 24h *P<0.05

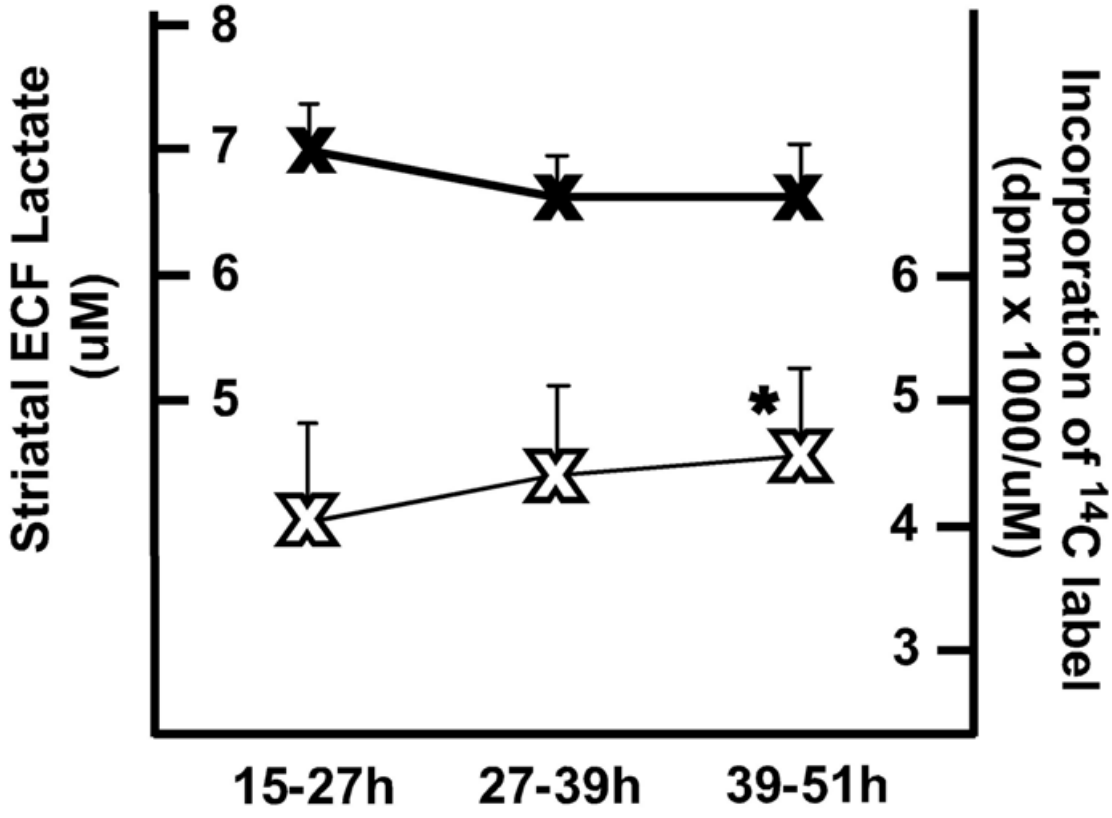
Figure 4.

Plasma glucose levels over the torpor phase in response to no infusion, IV infusion of glucose (200mg/kg) and IV infusion of ^{13}C glucose (200mg/kg). Open circles – no infusion (n=5), also mean ± SEM; open triangle - 1h after IV infusion of ^{12}C glucose (200mg/kg) (n=3), also mean ± SEM; solid triangle 1h (n=3); asterisk 24h (n=3); cross 48h (n=3); solid circles 60h (n=3) after IV infusion of ^{13}C glucose (200mg/kg). Glucose in 160 µl of saline was infused at 5-10 µl/min 20h after the start of torpor (indicated by arrow).

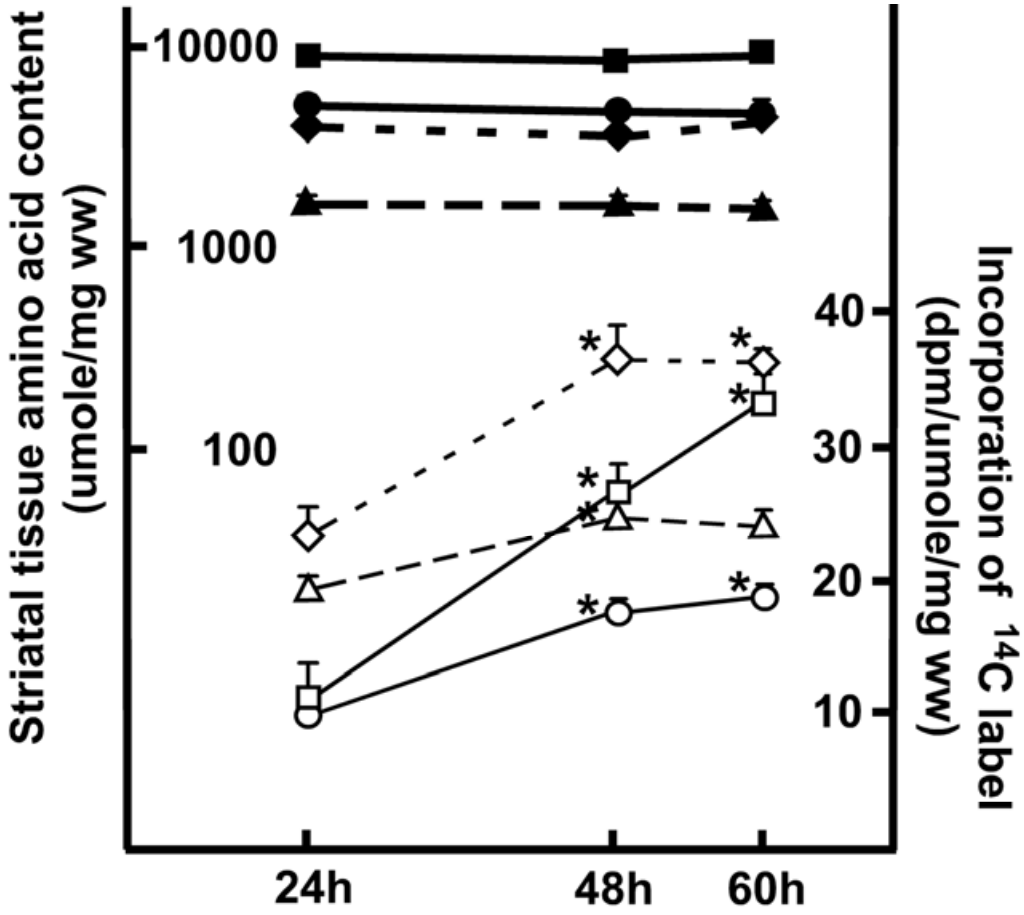
Figure 5.
^{13}C NMR spectra of deproteinated homogenate of hindbrain from torpid hamsters.
A) Endogenous ^{13}C resonances 1h after IV infusion of ^{12}C glucose (200mg/kg). B - 1h; C - 24h; D - 48h; E - 60h after IV infusion of ^{13}C glucose (200mg/kg).
1- alanine C3- 16.9 ppm; 2- lactate C3- 20.81; 3- GABA C3-24.2; 4- pyroglutamate C3-25.8; 5- GLN C3-27.1; 6- GLU C3-27.6; 7- pyroglutamate C4-30.3; 8- GLN C4-31.5; 9- GLU C4-34.0; 10- GABA C2-35.0; 11- taurine N-36.1; 12- aspartate C3-37.7; 13- GABA C4-39.9; 14- N-acetylaspartate C3- 40.2; 15- taurine S- 48.6; E- ethanol external standard- 49.6; 16- aspartate C2-53.8; 17- GLN C2-54.5; 18- GLU C2-55.1; 19- pyroglutamate C2-58.7; 20- dihydroxyacetone phosphate-68.2 or lactate C2 - L2- 69; 21- inositols (C4, C6)-71.5; 22- inositols (C1, C3)-72.9; 23- inositols (C5)-74.7; 24- glucose C1apha-92.4; 25- glucose C1beta-96.3.

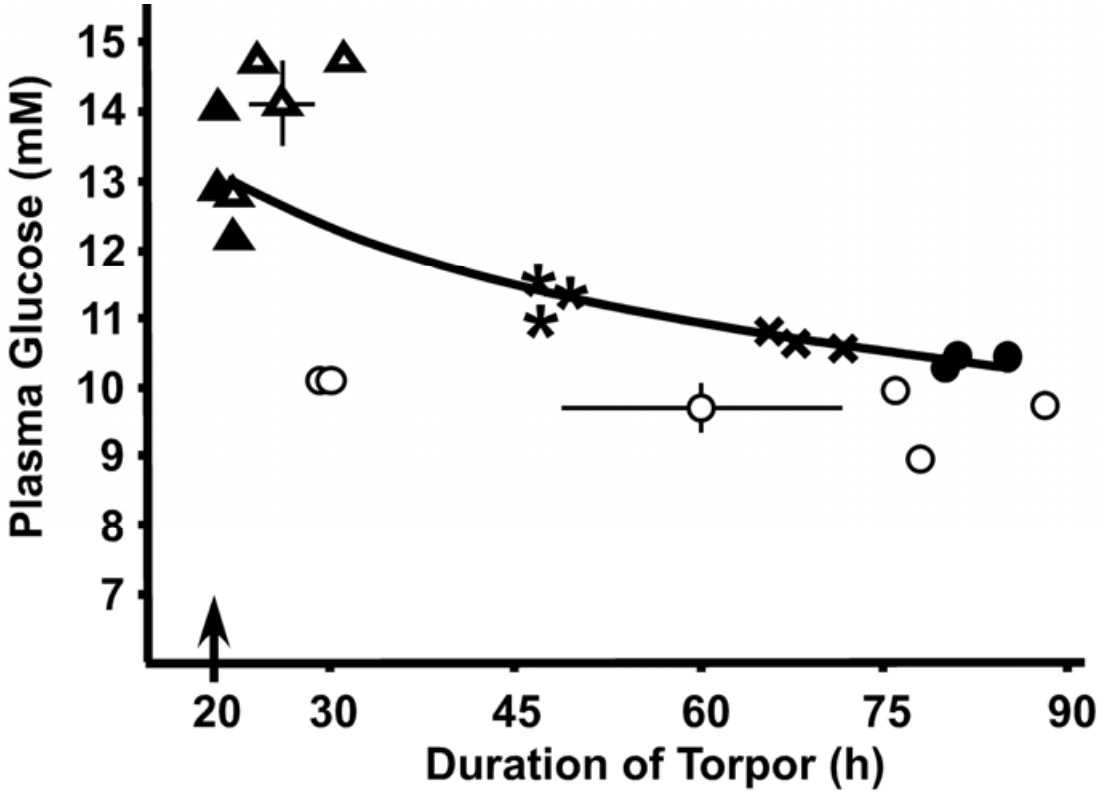
Figure 6.
Mean specific ^{13}C enrichment of metabolites in forebrain and hindbrain tissue from torpid hamsters. Histograms listed left to right. Black and white histogram - No infusion killed at 20 h hibernation. Cross hatched histogram - 1h after IV infusion of ^{12}C glucose (200mg/kg). Open histogram - 1h; Left diagonal histogram - 24h; Grey and white histogram - 48h; solid histogram - 60h after IV infusion of ^{13}C glucose (200mg/kg).

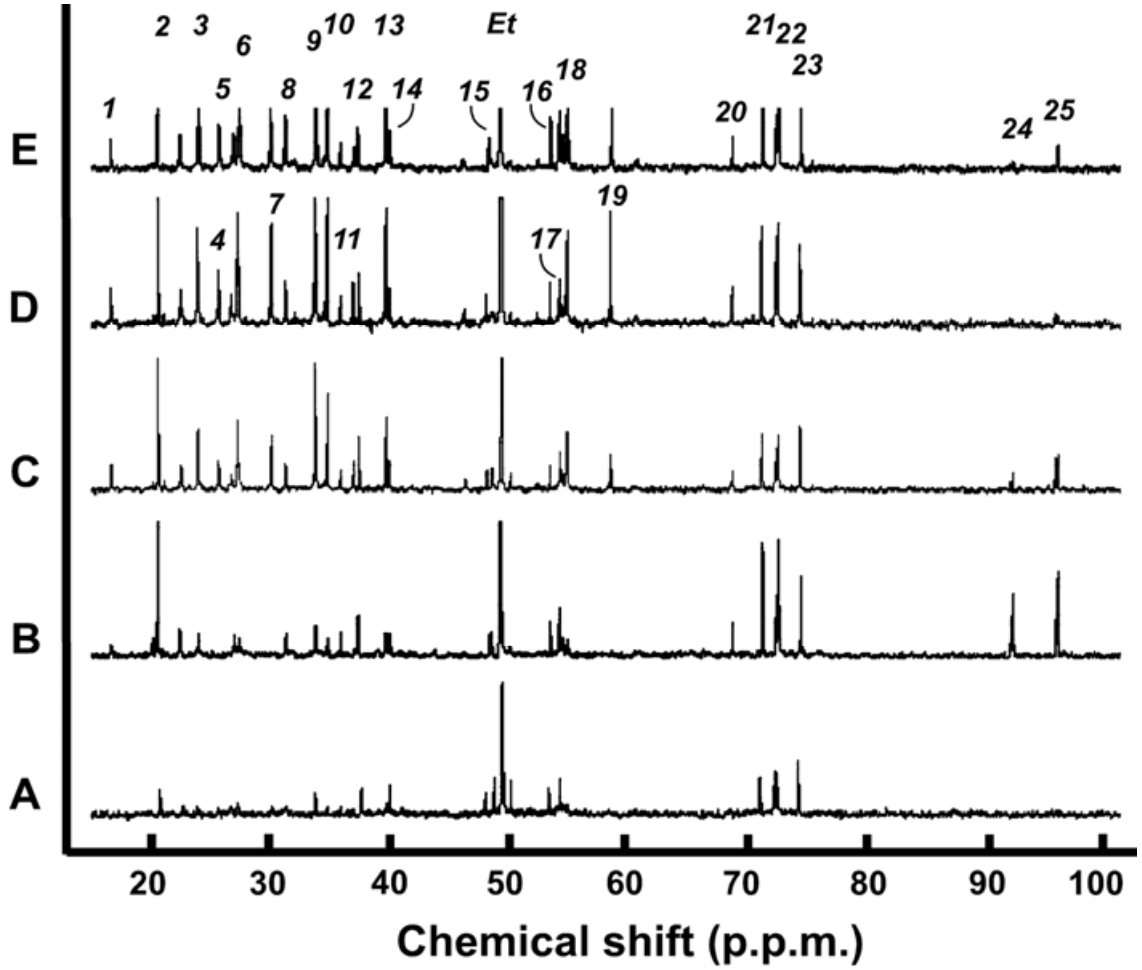

A
Striatal ECF level
(% of CEN 2h)
160
140
120
100
80
60
40
20
B
Striatal ECF level
(% of CEN 2h)
120
100
80
60
40
20
{Amino acids1-10}
C
Brain Temp
°C
40
30
20
10
D
Metabolic rate
(% Basal CEN2h)
200
150
100
50
Tor 24h
Tor 48h
Tor 72h
NST
ST
CEN 1h
CEN 2h

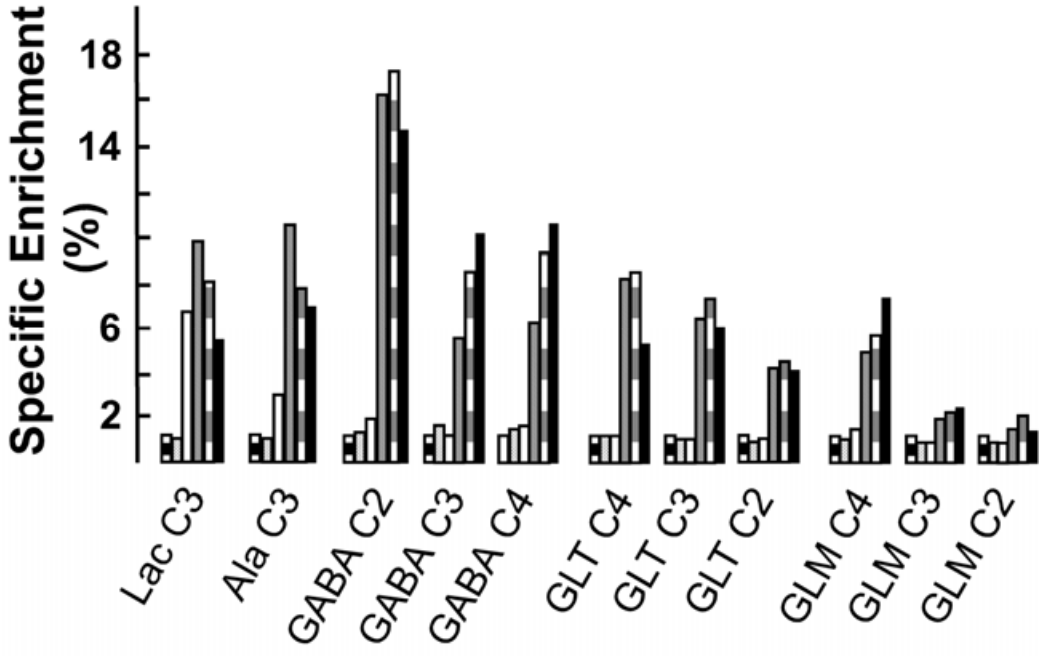


Time of collection and duration of *in-vivo* perfusion with ^{14}C glucose during torpor


Striatal tissue amino acid content
(umole/mg ww)
10000
1000
100
40
30
20
10
Incorporation of ^{14}C label
(dpm/umole/mg ww)
24h
48h
60h
Duration of *in-vivo* perfusion with
^{14}C glucose during torpor


Plasma Glucose (mM)
15
14
13
12
11
10
9
8
7
20
30
45
60
75
90
Duration of Torpor (h)


E
D
C
B
A
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
Et
20
30
40
50
60
70
80
90
100
Chemical shift (p.p.m.)


Specific Enrichment (%)
18
14
6
2
Lac C3
Ala C3
GABA C2
GABA C3
GABA C4
GLT C4
GLT C3
GLT C2
GLM C4
GLM C3
GLM C2