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Non-invasive quantification of brain glycogen absolute concentration

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Abstract

The only currently available method to measure brain glycogen *in vivo* is ^{13}C NMR spectroscopy. Incorporation of ^{13}C-labeled glucose (Glc) is necessary to allow glycogen measurement, but might be affected by turnover changes. Our aim was to measure glycogen *absolute* concentration in the rat brain by eliminating label turnover as variable. The approach is based on establishing an increased, constant ^{13}C isotopic enrichment (IE). ^{13}C-Glc infusion is then performed at the IE of brain glycogen. As glycogen IE cannot be assessed *in vivo*, we validated that it can be inferred from that of *N*-acetyl-aspartate IE *in vivo:* After [1-^{13}C]-Glc ingestion, glycogen IE was 2.2 ± 0.1 fold that of *N*-acetyl-aspartate (n = 11, R^2 = 0.77). After subsequent Glc infusion, glycogen IE equaled brain Glc IE (n = 6, paired t-test, p = 0.37), implying isotopic steady-state achievement and complete turnover of the glycogen molecule. Glycogen concentration measured *in vivo* by ^{13}C NMR (mean ± SD: 5.8 ± 0.7 μmol/g) was in excellent agreement with that *in vitro* (6.4 ± 0.6 μmol/g, n = 5). When insulin was administered, the stability of glycogen concentration was analogous to previous biochemical measurements implying that glycogen turnover is activated by insulin. We conclude that the entire glycogen molecule is turned over and that insulin activates glycogen turnover.

Keywords

^{13}C; insulin; nuclear magnetic resonance; turnover

Glycogen is a macromolecule (~10^3-10^4 kDa); a polymer of glucosyl units present in a substantial amount in the brain relative to free glucose (Glc) (Gruetter 2003; Watanabe and Passonneau 1973). Interestingly, in the adult brain, glycogen is found only in astrocytes. Although under normal physiology it seems that this store is metabolized actively but comparatively slowly (Oz *et al.* 2007), glycogen support of astrocyte metabolism (Dienel and Cruz 2004), neuronal metabolism (Gibbs *et al.* 2006; Suh *et al.* 2007), or both could be important in prolonging neuronal activity (Brown *et al.* 2005) and limiting neuronal death. We have previously shown that the brain can store more than a few minutes' supply of Glc equivalent as glycogen (Morgenthaler *et al.* 2006). Therefore, glial glycogen may act as a substantial source of Glc equivalents during acute insulin-induced hypoglycemia (Choi *et al.* 2003; Gruetter 2003; Morgenthaler *et al.* 2006) or during hypoxia-ischemia (Allen *et al.*

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2005; Gruetter 2003; Nordstrom and Siesjo 1978; Rossi *et al.* 2007). Recently, it has been suggested that glycogen might be involved in learning in young chickens (Gibbs *et al.* 2006, 2008a, 2007). Glycogen may also be neuro-protective (Swanson and Choi 1993). One possible neuro-protective mechanism of glial glycogen might be to provide lactate as fuel for neurons (Magistretti and Pellerin 1996) and to provide ATP to allow continuous uptake of excitotoxic glutamate from the synaptic cleft (Gruetter 2003). However, such neuroprotection might interfere with proper recognition of hypoglycemia by interfering with Glc-sensing neurons. Therefore, it has also been suggested that brain glycogen might be implicated in the mechanism of hypoglycemia unawareness syndrome observed clinically in patients with type I diabetes (Choi *et al.* 2003; Cryer 2005, 2006; Gruetter 2003). Hypoglycemia unawareness is a very dangerous state characterized by the lack of warning symptoms of hypoglycemia prior to cognitive dysfunction, and rapid progression to severe hypoglycemia with confusion and coma.

There is normally an ongoing simultaneous utilization and resynthesis of brain glycogen with an active turnover resulting in consistent levels of brain glycogen under resting conditions (Brown and Ransom 2007; Choi *et al.* 2003; Watanabe and Passonneau 1973). Interestingly, glycogen turnover is increased during activation of brain tissue (Dienel *et al.* 2007; Dienel and Cruz 2003, 2004; Shulman *et al.* 2001; Swanson 1992). Moreover, extracellular Glc concentration (Brown *et al.* 2003; Morgenthaler *et al.* 2006), as well as numerous neurotransmitters (Sorg and Magistretti 1992) have been reported to influence glycogen content in the brain. For example, insulin increases brain glycogen metabolism (Choi *et al.* 2003; Morgenthaler *et al.* 2006).

^{13}C Nuclear Magnetic Resonance (NMR) is the only currently available method capable of measuring brain glycogen non-invasively. As ^{13}C natural abundance is low, ^{13}C NMR studies rely on measuring the incorporation of ^{13}C-labeled Glc into the glycogen molecule to enhance the signal. Therefore, the observed increase in ^{13}C signal over time might not necessarily be reflecting changes in concentration alone, but also an increased incorporation of the ^{13}C label. We have previously shown that changes in glycogen concentration measured by ^{13}C NMR were in excellent agreements with biochemical measurements (Lei *et al.* 2007; Morgenthaler *et al.* 2006). However, the increase in glycogen concentration after insulin infusion was smaller when studied with biochemical methods (Morgenthaler *et al.* 2006) as compared to *in vivo* ^{13}C NMR studies (Choi *et al.* 2003). This difference might be because of an effect of insulin on glycogen turnover.

The aim of this study was to develop a new approach that allows the measurement of *in vivo* brain glycogen *absolute* concentration. The pre-requisite consisted of minimizing the influence of turnover - a potential confounding factor - in the ^{13}C NMR measurement of glycogen. The strategy was to pre-label the rat and achieve a near steady-state Isotopic Enrichment (IE) using long term [1-^{13}C]-Glc administration. Glycogen IE was measured *in vivo* and this IE was matched by the subsequent ^{13}C-labeled Glc infusion, thus maintaining isotopic steady-state throughout the experiment. Consequently, the effects of insulin on brain glycogen concentration could be directly studied.

Materials and methods

`Rationale'

In vivo ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy of glycogen is based on observing the incorporation of ^{13}C label into glycogen (Choi and Gruetter 2003; Choi *et al.* 2003, 1999; Gruetter *et al.* 2003; Karelson *et al.* 2003; Oz *et al.* 2003). Therefore an increase in ^{13}C signal over time is not necessarily reflecting an increase in glycogen absolute concentration [as illustrated in the scheme of Fig. 1a(i-iii)].

The major aim of this study was to measure glycogen *absolute* concentration and second to eliminate label turnover (exchange with the glycogen molecule) as a potential confounding factor. Our approach is based on `pre-labeling' rat brain glycogen by long-term [1-^{13}C]-labeled Glc administration until steady-state of ^{13}C enrichment (i.e. constant over time) was reached. Infusion of [1-^{13}C]-labeled Glc then continues with the same Isotopic Enrichment (IE, see eqn 1) as brain glycogen (isotopic steady-state) [Fig. 1b(i)].

$$IE = \frac{^{13}C}{(^{13}C + ^{12}C)} \quad (1)$$

Subsequently, observed changes in glycogen signal intensity only reflect changes in absolute concentration and not label turnover into the glycogen molecule [Fig. 1b(i-iii)].

The approach depends on continuing the infusion at the IE of glycogen. As brain glycogen IE cannot be assessed *in vivo*, we investigated its indirect estimation through measuring *N*-acetyl-aspartate (NAA) IE, which can be directly measured *in vivo*. NAA was chosen because of the excellent correlation between the amount of labeled Glc incorporated into brain glycogen and into NAA previously described; as well as the similar turnover time of these two molecules (Choi and Gruetter 2003). Therefore, once the isotopic steady-state is reached, the IE of glycogen should be mimicked by that of NAA.

Together with the known brain glycogen IE, the reliable quantification of ^{13}C-labeled glycogen implies that brain glycogen absolute concentration can be calculated by dividing the glycogen ^{13}C NMR signal by glycogen IE.

Rat `pre-labeling'

Rats were fasted overnight with free access to water before studies were performed. The following day they received a 10% w/v [1-^{13}C]-labeled Glc solution (99% [1-^{13}C]-enriched) *ad libitum* as their sole source of exogeneous carbon for 24 h (n = 11) or 48 h (n = 6). Most of the rats labeled for 24 h received a 99% ^{13}C-enriched solution (n = 9), but rats pre-labeled during 48 h received a 50% ^{13}C-enriched solution as labeling solution, to optimize the cost of the experiment.

To keep the ratio between glycogen IE and NAA IE stable and allow the calculation of glycogen IE from NAA IE, rats were pre-labeled in a quiet and familiar environment as glycogen turnover has been reported to be increased during brain activation (Dienel *et al.* 2007; Dienel and Cruz 2003, 2004; Shulman *et al.* 2001; Swanson 1992).

Animal preparation

The study was performed in accordance with the local and federal guidelines and was approved by the local ethics committee.

Two main groups of rats were studied. The first group (n = 11, mean weight ± SD: 240 ± 27 g, Sprague-Dawley rats, Harlan, Madison, WI, USA) was used for validation of the method for indirect estimation of glycogen IE via NAA IE measurement. The rats were pre-labeled for either 24 h (n = 5)or 48 h (n = 6). They were anesthetized using isoflurane (isoflurane, Halocarbon laboratories, 5% for induction and 1.8-2.0% for maintenance) in a 70 : 35 mixture of nitrous oxide (N_2O) and oxygen (O_2) gases for preparation. Then, rats were intubated and ventilated with a pressure-driven ventilator (Kent Scientific, Litchfield, CT, USA) for the rest of the experiment. They were placed into the 9.4 T magnet and their body temperature was maintained at 37.5 ± 0.5°C with circulating heated water (RTE-101 bath circulator, Thermo NESLAB, Portsmouth, NH, USA). *In vivo* measurement of NAA IE was performed. The

animal was then killed and *in vitro* measurements (NAA IE for validation, glycogen concentration and glycogen IE) were performed on the brain extracts (see below).

With the second group of rats ($n = 6$), brain glycogen absolute concentration was measured *in vivo* (see below), as well as the effects on brain glycogen of insulin infusion over time. Glycogen IE was calculated at the beginning of the experiment from the *in vivo* measurement of NAA IE. Male Sprague-Dawley rats (mean weight ± SD: $n = 6$, 247 ± 38 g, Charles River, France) were pre-labeled during 24 h and prepared as described above and previously (Morgenthaler *et al.* 2006). Respiratory rate and pattern were monitored during experiments with a respiratory pillow (SA Instruments, Stony Brook, NY, USA). Preparation consisted of inserting catheters into both femoral arteries for blood gases and Glc analysis, and into both femoral veins for i.v. infusion of α-chloralose (97%, Acros Organics, Geel, Belgium), [1-^{13}C]-Glc (Cambridge Isotope Laboratories Inc., Andover, MA, USA) mixed with D-Glc (Sigma, St Louis, MO, USA), and insulin (Humulin R, Eli Lilly and Company, Indianapolis, IN, USA).

Immediately after preparation, anesthesia was switched to α-chloralose, which was administered i.v. as follows: 15 min after a 80 mg/kg bolus injection, a continuous i.v. infusion was started (26.7 mg/kg/h) to maintain a light anesthesia state according to previous report (Ueki *et al.* 1992). All solutions used for i.v. infusion were diluted in Dulbecco's Phosphate-Buffered Saline (without calcium chloride and without magnesium chloride, Sigma).

Arterial blood was withdrawn from the femoral artery through a polyethylene tubing line (PE50, Becton-Dickinson, Franklin Lakes, NJ, USA) roughly every 30 min. Around 0.05 mL of blood was used to measure physiological parameters with a pH/blood gas analyzer (AVL Compact 3 BGA, Roche Diagnostics, Rotkreuz, Switzerland) while the rest of the blood (~0.15 mL) was immediately centrifuged to obtain plasma. Plasma Glc concentration was measured using the Glc oxidase method in a Glc analyzer (GM7 Micro-stat, Analox Instruments, London, UK). Physiologic parameters (respiration rate and breathing volume) were adjusted throughout the experiments to maintain pCO_2 and pH within normal physiological ranges (mean pH ± SD = 7.31 ± 0.04; mean pCO2 ± SD = 43.5 ± 1.8 and mean body temperature ± SD = 38.1 ± 0.2) (Morgenthaler *et al.* 2006).

The infusion rate of Glc (20% weight/volume in Dulbecco's Phosphate-Buffered Saline) was continuously adjusted based on the measured plasma Glc concentrations to maintain target glycemia. The IE of the infusate was calculated from the *in vivo* measurement of NAA IE at the beginning of each experiment (see below). Glc infusion rate was adjusted to maintain plasma Glc levels during insulin infusion (Morgenthaler *et al.* 2006).

In vivo magnetic resonance spectroscopy

In vivo magnetic resonance spectroscopy experiments were performed on an actively shielded 9.4 T/31 cm magnet (Magnex Scientific, Abingdon, Oxon, UK) with actively shielded gradients (400 mT/m in 120 ms, 12 cm inner diameter) connected to a Varian INOVA console (Varian, Palo, Alto, CA, USA). A quadrature 14 mm ^{1}H coil combined with a three-turn linearly polarized ^{13}C coil 10 mm diameter was used for radio frequency transmission and signal reception. A sphere containing 99% ^{13}C-enriched formic acid (FA) was located at the center of the ^{13}C coil as an external reference, and the coil was placed on the animal's head as described previously (Choi *et al.* 2001, 2000, 1999; Lei *et al.* 2007). FASTMAP (Gruetter and Tkac 2000) was applied in 100-150 μL volumes of interest to adjust magnetic field inhomogeneity. A Single-shot Inversion Recovery based Non-Echo sequence with optimized outer volume suppression (Choi *et al.* 2000) was applied for 3D localization (inversion time for glycogen was 0.12 s) to ensure elimination of signals from non-cerebral tissue (Choi *et al.* 2001, 2000, 1999; Lei *et al.* 2007). Bilevel WALTZ-16 RF pulses were applied at the ^{1}H water frequency

to generate a nuclear Overhauser effect and to decouple during acquisition (Choi *et al.* 2000; Lei *et al.* 2007). The robustness of localization was verified from the absence of the natural abundance lipid signal (30.5 ppm) in spectra optimized for the amino acid region by placing the carrier frequency at 30.5 ppm and thus minimizing the chemical shift displacement error (20-40 ppm) as lipid signals are not detectable in the normal brain *in vivo* (Choi and Gruetter 2003).

In vivo NAA IE measurement using ^{1}H NMR

N-acetyl-aspartate IE was measured by ^{1}H NMR using a localized ACED-STEAM sequence (Pfeuffer *et al.* 1999a). 256 scans (repetition time = 4 s; echo time TE = 7.9 ms; mixing time TM = 25 ms) were acquired in 17 min. In the ^{13}C-edited ^{1}H spectrum, only ^{1}H bound to ^{13}C in different metabolites were thus observed. The two interleaved recorded spectra were apodized by 5-Hz exponential line broadening, zero-filled and Fourier transformed. They were then subtracted and yielded the ^{1}H-^{13}C resonances and edited for J_{CH} = 129 Hz. The non-edited ^{1}H spectrum (Fig. 2a, upper spectrum) enabled the measurement of the [6-^{12}C]-NAA ^{1}H signal at 2.01 ppm (Pfeuffer *et al.* 1999b), while the ^{13}C-edited ^{1}H spectrum (Fig. 2a, lower spectrum) allowed to measure the doublet of the labeled [6-^{13}C]-NAA molecule. As the downfield satellite at 2.17 ppm [6-^{13}C]-NAA ^{1}H signal was slightly overlapped by glutamate, only the 1.85 ppm [6-^{13}C]-NAA ^{1}H signal was used for measurement. NAA IE was thus obtained by dividing twice the intensity of the 1.85 ppm [6-^{13}C]-NAA ^{1}H signal, by twice the intensity of the 1.85 ppm [6-^{13}C]-NAA ^{1}H signal plus the [6-^{12}C]-NAA ^{1}H signal intensity.

In vivo brain glycogen concentration measurement using ^{13}C NMR

Glycogen and Glc signals were acquired in 34 min (2048 scans, repetition time = 1 s) using the Single-shot Inversion Recovery based Non-Echo sequence. They were summed, apodized by 25-Hz exponential line broadening, zero-filled, and Fourier transformed. Two blocks of spectra were acquired before insulin infusion (as baseline) and seven blocks after insulin infusion.

The glycogen C1 signal intensity was fitted by a Gaussian line shape with a fixed linewidth using the spectrometer software (Varian Inc.). The linewidth was obtained from the fit to a spectrum summing all acquisitions. Quantification was performed using the external reference method (Gruetter *et al.* 1991; Lei *et al.* 2007), based on the measurement of the signal from a phantom containing 500 mM of natural abundance oyster glycogen (Sigma). Phantom measurements were performed under identical experimental conditions (temperature, number of scans, position in the magnet, localized volume) to the *in vivo* studies.

In vivo ^{13}C absolute glycogen concentration $[Glyc]_{invivo}$ was calculated by comparing the *in vivo* NMR signal I_{glyc}^{invivo} with the phantom signal I_{glyc}^{phan} while taking the known phantom concentration $[Glyc]_{phan}$ and *in vivo* IE of glycogen IE_{Glyc} into account as in previous studies (Choi *et al.* 1999; Lei *et al.* 2007). A correction for the difference in coil loading was made by multiplying by the ratio of the formic acid reference signal (I_{FA}) in both cases, and by an experimentally determined correction factor ($\alpha = 0.98$) for the slightly different magnetic relaxation behavior of rat and oyster glycogen under a short T1 and nuclear Overhauser effect. The final equation was:

$$[Glyc]_{invivo} = \frac{I_{glyc}^{invivo}}{I_{glyc}^{phan}} \times \frac{I_{FA}^{phan}}{I_{FA}^{invivo}} \times \frac{1.1\%}{IE_{glyc}} \times \alpha \times [Glyc]_{phan} \quad (2)$$

Natural abundance in the oyster phantom solution was assumed to be 1.1% and $[Glyc]_{phan}$ was 500 mM.

Tissue analysis

At the end of each experiment, rats were killed using a focused microwave fixation device by applying 4 kW onto a 2 mL volume for 1.4-1.6 s (Gerling Applied Engineering, Inc., Modesto, CA, USA) which inactivates most brain enzymes before extraction or digestion, thereby minimizing possible *in vitro* glycogen loss (Kong *et al.* 2002). The anterior parts of the brain (excluding cerebellum) were dissected, and immediately placed into liquid nitrogen and manually reduced to powder with a pestle and mortar. Brain powder was stored at -80°C until further processing.

Glycogen assay

Biochemical measurement of brain glycogen was performed on brain extracts as previously described (Cruz and Dienel 2002; Morgenthaler *et al.* 2006). Briefly, 0.03 M HCl (500 μL) was carefully added to frozen brain powders (~200 mg) in tubes on dry ice. Brain powders were homogenized (ultrasonic processor, Cole-Parmer Instruments, Vernon Hills, IL, USA), heated to 90°C for 45 min and homogenized again. For the glycogen assay, sodium acetate (Sigma) was added to 200 μL samples in 0.03 M HCl. Well-mixed portions of each sample were incubated at 37°C for at least 2 h with gentle mixing in a water bath (Julabo SW 22, Milian, Gahanna, OH, USA) in two parallel aliquots, one containing amylo-α-1,4-α-1,6-glucosidase (24 μg/mL, Roche) to convert glycogen to Glc and the other without amylo-α-1,4-α-1,6-glucosidase (this latter sample allowed brain Glc concentration as well as IE measurements). Glc levels were determined using the same Analox instrument as above. Glycogen levels per gram wet weight were calculated as Glc released by amylo-α-1,4-α-1,6-glucosidase, that is, Glc level after incubation with amyloglucosidase minus that without the enzyme (Ghajar *et al.* 1982).

In vitro ^{1}H-NMR: Glc IE, glycogen IE and NAA IE measurement

^{13}C IE (see eqn 1) of NAA, Glc and digested glycogen were further determined *in vitro* by ^{1}H NMR, using a Bruker Avance-DRX 600 (14.1 T, 600 MHz) spectrometer (Bruker BioSpin SA, Fällanden, Switzerland). Samples obtained from the glycogen assay were filtered (Ultrafree-CL filters, Millipore Corporation, Bedford, MA, USA) to remove high molecular weight molecules. They were lyophilized in a speedVac (DNA 120, SpeedVac Concentrator, Thermo electron corporation) and resuspended into 500 μL D_2O (deuterium oxide, 99.9 atom % D, Aldrich, Germany).

For [1-^{13}C]-Glc IE measurement, ^{1}H spectra were acquired at 44°C using a delay time of 15 s. The ^{1}H NMR spectra were calibrated with H_2O (HDO) fixed at 4.6 ppm upfield to tetramethylsilane (internal standard for NMR calibration). The level of [1-^{13}C]-Glc was determined by appropriate integration of the ^{1}H signals (bound to Glc C1) of the α-anomer. ^{1}H signals consisted of a doublet for the unlabeled molecule (chemical shift: ~5.28 ppm, $^3J_{H1,H2}$ = ~3.7 Hz) and a doublet of doublets for the C1-labeled molecule [the $^1J_{C1,H1}$ coupling (~170 Hz) superimposed on the smaller $^3J_{H1,H2}$ coupling (~3.7 Hz)].

In vitro the IE of Glc was calculated from applying eqn 1 to the integrals obtained from the *in vitro* spectrum. Brain Glc IE was directly measured with the high resolution 600 MHz magnet. Brain glycogen IE was calculated according to eqn 3 after having processed the brain extracts with the glycogen assay so that the glycogen molecule was digested into Glc molecular units (Lei *et al.* 2007).

$$IE_{glyc} = \frac{(IE_{TotalGlc} \times [TotalGlc]) - (IE_{BrainGlc} \times [BrainGlc])}{[Glyc]} \quad (3)$$

Total Glc (*TotalGlc*) represents brain Glc (*BrainGlc*) plus digested brain glycogen, i.e. measurement performed in samples subjected to amyloglucosidase digestion. Total Glc and brain Glc IE were obtained by high field ^{1}H-spectroscopy, and brain Glc and glycogen concentrations were obtained by biochemical measurement.

After having measured brain Glc and `total Glc' IE, the pH of the samples was lowered to 1.8 with 0.1 M DCl. ^{1}H NMR spectra were acquired at 25°C with a repetition time of 9 s. They were calibrated with H_2O (HDO) fixed at 4.8 ppm upfield to tetramethylsilane. The level of [6-^{13}C]-NAA IE was determined by appropriate integration (see above) of the ^{1}H signals bound to NAA C6. ^{1}H signals consisted of a single peak for the unlabeled molecule (chemical shift: ~2.01 ppm) and a doublet for the C6-labeled molecule ($^{1}J_{C1,H1}$ coupling: ~129 Hz). *In vitro* NAA IE was calculated from eqn 1 (Fig. 2b).

Results

During the `pre-labeling period', the rats ingested significant amounts of [1-^{13}C]-labeled Glc solution either for 24 h [mean Glc solution (10% w/v) ingested ± SD: 122 ± 27 mL, n = 11] or for 48 h (170 ± 68 mL, n = 6).

With the first group of rats (n = 11), our pre-labeling method was validated as follows: There was no significant difference between NAA IE measured *in vivo* and subsequently *in vitro* in the same brains (Fig. 2c, n = 9, paired t-test, p = 0.62) confirming the validity of the *in vivo* measurement of NAA IE. NAA IE was correlated (n = 11, R^2 = 0.82) with the amount of [1-^{13}C]-Glc ingested by each rat (Fig. 3) and no significant difference could be observed between 24 or 48 h of pre-labeling. There was also less variability in the amount of ^{13}C-labeled Glc solution ingested by the rats after 24 h than after 48 h. We therefore decided to pre-label the rats only during 24 h for the rest of the experiments. The biochemical measurements of glycogen concentration in the post-mortem brain extracts yielded 4.5 ± 1.0 μmol/g (mean ± SD). Finally, the *in vitro* measurement of glycogen IE allowed us to measure the relationship between glycogen IE and NAA IE; glycogen IE was 2.2 ± 0.1 times that of NAA IE (mean ± SD, n = 11, Fig. 4b) as expected from the breakdown of [1-^{13}C]-Glc into two molecules of acetyl-CoA. Taken together these results (see Figs 2c and 4b in particular) showed that brain glycogen IE can be inferred from the *in vivo* measurement of NAA IE after 24 h of pre-labeling.

With the second group of rats (n = 6), we measured brain glycogen absolute concentration *in vivo* after 24 h of pre-labeling (Fig. 5). From the NAA IE (mean percentage ± SD: 15.3 ± 2.3%, n = 6), the IE of the Glc infusion solution was adjusted to 36 ± 6% based on the 2.2-fold difference in IE (see above). Nine blocks of glycogen spectra were then acquired. The first two blocks were taken as reference. Insulin infusion (24 IU/kg) was then started and plasma Glc was maintained at 10 mM (mean plasma Glc ± SD: 10.7 ± 0.5 mM, n = 6) by adjusting the infusion rate of the Glc solution. The brain ^{13}C-glycogen signal (Fig. 5b) was typically detected with a high signal to noise ratio on the order of 8 : 1. Absolute glycogen concentration obtained *in vivo* (mean ± SD: 5.8 ± 0.7 μmol/g, n = 6)by ^{13}C NMR was in excellent agreement with biochemical determination (mean ± SD: 6.4 ± 0.6 μmol/g, n = 5, paired t-test, p = 0.12, Fig. 5c). At the end of 7 h of Glc infusion, brain Glc IE was similar to the IE of the infused Glc solution (n = 6, paired t-test, p = 0.52, data not shown) and glycogen IE (n = 6, paired t-test, p = 0.37, Fig. 6).

Finally, the effects of insulin infusion on brain glycogen concentration were investigated *in vivo* and over time. The infusion of insulin together with Glc over 4.5 h did not significantly affect brain glycogen concentration measured by *in vivo* NMR ($n = 5$ rats, paired t-test between the last measurement before insulin infusion and the last measurement during insulin infusion, $p = 0.5$) (Fig. 7).

Discussion

We show for the first time that the non-invasive measurement of brain glycogen concentration is feasible. This is possible by accurate quantification of the labeled proportion of the glycogen molecule and therefore of the total brain glycogen content. Matching the IE of the Glc infusate solution to the IE of glycogen allowed us to perform time-resolved measurement of brain glycogen concentration.

The *in vivo* brain glycogen concentration obtained by ^{13}C NMR spectroscopy in the present study was similar to subsequent biochemical measurement in the same brain and to previous biochemical measurements of brain glycogen concentration (mean ± SD: 6.2 ± 0.6 μmol/g). Furthermore, the *in vitro* measurements of brain glycogen concentration of the first group of rats were in perfect agreement with previous measurement made in rats after short isoflurane anesthesia (Morgenthaler *et al.* 2006). As expected, the pre-labeling protocol did not alter brain glycogen concentration, which is consistent with Glc being sufficient for brain metabolism under these conditions.

That brain Glc IE was similar to the IE of the Glc infusate at the end of the time course experiments was expected from the rapid equilibration of plasma Glc across the blood brain barrier (Choi *et al.* 2001; Gruetter *et al.* 1998). Moreover, the stability of the IE of glycogen over 7 h of Glc infusion equaling that of brain Glc suggests near complete turnover of the glycogen molecule after a 24 h pre-labeling period. As a consequence, glycogen turnover is unlikely to be a confounding effect after 24 h of pre-labeling. Since 24 h of pre-labeling seem sufficient to nearly reach isotopic steady-state, we conclude that the slower turnover time of limit dextrin (Watanabe and Passonneau 1973) is sufficiently fast to lead to near-complete turnover in 24 h. As the pre-labeling period of 24 h is longer than glycogen (Choi *et al.* 1999; Oz *et al.* 2007; Watanabe and Passonneau 1973) and NAA (Choi and Gruetter 2003) turnover times, the IE of both of these molecules should be close to steady-state. In other words, both turnover curves are expected to evolve in a similar manner.

N-acetyl-aspartate was chosen as a `reporter molecule' to estimate glycogen IE for two main reasons. First, NAA has a similar turnover time to glycogen (Choi and Gruetter 2003) and second NAA is synthesized early in the Glc metabolic pathway, just after glycolysis. The ratio close to 2 : 1 between glycogen IE and NAA IE observed here suggests that Glc is the major source of NAA biosynthesis.

The effect of insulin on brain glycogen metabolism measured previously by ^{13}C NMR suggested a several fold increase in glycogen concentration or metabolism (Choi *et al.* 2003). However, when measured with biochemical methods (Morgenthaler *et al.* 2006) the increase in glycogen concentration after insulin infusion was less than 1 μmol/g over 4.5-5 h of insulin (6 units/kg/h) infusion. The present study did not show a significant increase of brain glycogen concentration over time, however, the average SEM of 0.5 μmol/g corresponding to a 95% confidence interval of about ± 1 μmol/g allows for such small increases. These results suggest that insulin has a modest effect on glycogen concentration (around 1.0 μmol/g over 4.5 h). Therefore, the present study is within experimental error in very good agreement with the previous biochemical determination of glycogen concentration (Morgenthaler *et al.* 2006) rather than the previous ^{13}C NMR measurements with rats that had not been pre-labeled (Choi

et al. 2003). Together with the previous NMR observation of several-fold increased ^{13}C incorporation into glycogen (Choi *et al.* 2003), the apparent stability of the glycogen signal in the current study implies that insulin mainly increases glycogen turnover rather than glycogen concentration. The modest effect of insulin on glycogen concentration and the more pronounced effect on turnover are consistent with insulin crossing the blood brain barrier (Banks 2004) and affecting insulin sensitive pathways such as glycogen metabolism.

We have previously shown that acute hypoglycemia promoted glycogenolysis at an enhanced rate (Choi *et al.* 2003, 1999). This increased glycogenolysis was not compensated by enhanced glycogen synthesis as it resulted in net reduction of glycogen concentration (Morgenthaler *et al.* 2006). Previous studies have reported changes in glycogen concentration during physiological activation (Cruz and Dienel 2002; Dienel *et al.* 2007; Swanson *et al.* 1992), which were performed at euglycemia and in awake animals, unlike the present study. It is quite possible that insulin acts on glycogen by a different mechanism than activation, likely mediated in the latter case by noradrenergic mechanisms (Gibbs *et al.* 2008b; Sorg and Magistretti 1991).

We conclude that pre-labeling a rat with [1-^{13}C]-labeled Glc for 24 h is adequate to obtain nearly total turnover of the glycogen molecule, to measure glycogen absolute concentration, and to monitor glycogen concentration changes over time. Moreover, this protocol shows that insulin activates brain glycogen synthesis and breakdown simultaneously.

We further conclude that brain glycogen absolute concentration can be measured *in vivo* in a time-resolved manner, which should allow important insight into the role of glycogen in glial cells.

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Abbreviations used

IE, isotopic enrichment; NAA, *N*-acetyl-aspartate.

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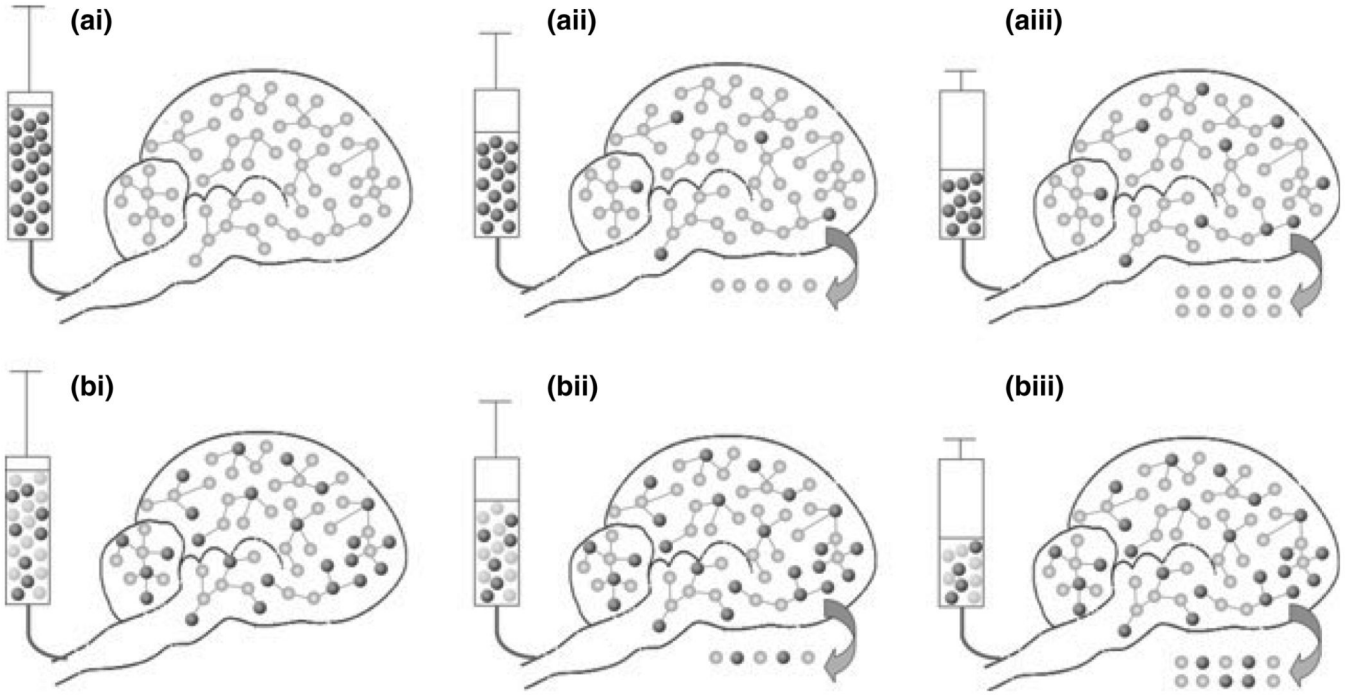

Fig. 1.

Schematic illustration of the effect of label turnover on glycogen quantification. (a) Brain glycogen is labeled by infusing a highly enriched solution of ^{13}C-labeled Glc (dark spheres) into an unlabeled rat (a-i) femoral vein. Light spheres represent ^{12}C-Glc (so-called unlabeled Glc). An increase in ^{13}C signal can be observed over time (represented by an increase in the number of dark spheres), even in the absence of changes in brain glycogen concentration (the total number of spheres does not change over time). The ^{13}C signal augmentation is thus because of an increase in glycogen IE over time. (b) The new method uses long term administration of ^{13}C-labeled Glc prior to the measurement to achieve significant brain glycogen IE. After measuring this IE *in vivo* (using NAA IE as an indirect method), the same IE of the infused Glc solution is matched to that of brain glycogen IE and the ^{13}C-NMR glycogen measurement is started. By subsequently infusing a Glc solution whose IE is similar to that of glycogen, glycogen IE remains stable over time and therefore no change in signal intensity is observed unless the absolute glycogen concentration changes (total number of spheres). Due to glycogen turnover, represented by the arrow, the glycogen concentration in the rat brain remains constant (schematized here as a constant number of spheres) despite the infusion of glucose. N.B.: ^{13}C natural abundance (1.1%) has been neglected (especially in a-i).

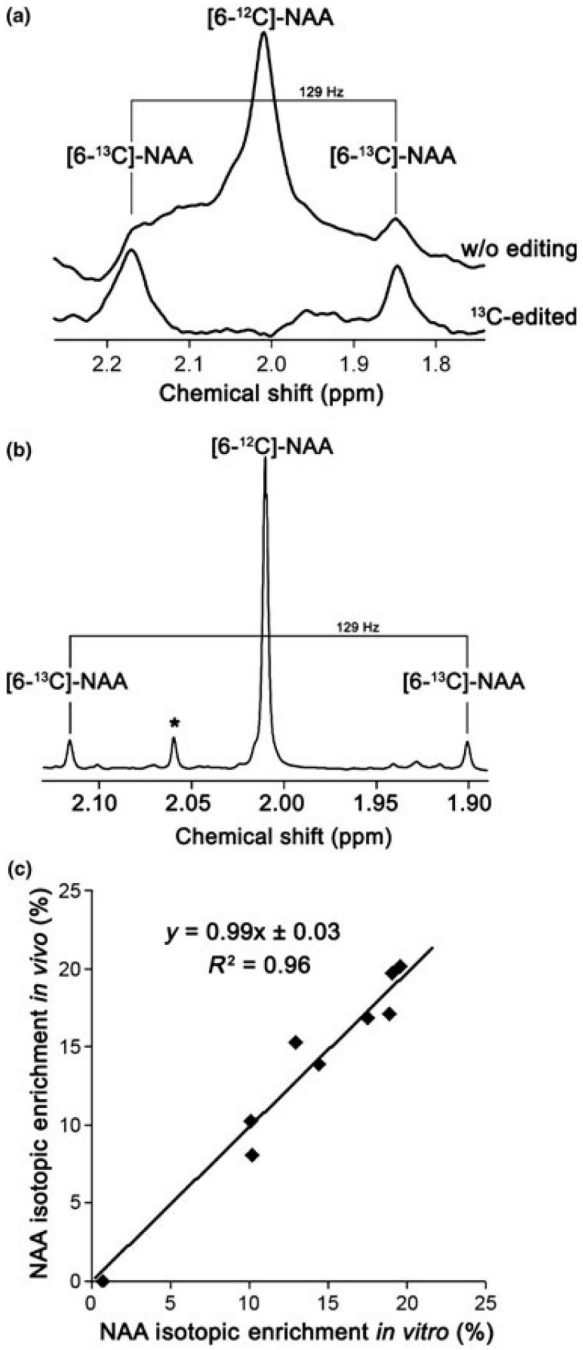

Fig. 2.

Comparison of *in vivo* measurement of NAA Isotopic Enrichment (IE) with *in vitro* measurement. (a) Example of *in vivo* NAA spectra with and without editing. It can be observed that the 2.17 ppm ^{13}C peak satellite is slightly larger because of glutamate contamination. (b) Example of *in vitro* spectrum. Signals are much better separated. *NAA-G. (c) *In vivo* measurement of [6-^{13}C]-NAA IE is identical to *in vitro* measurement (n = 9 rats, paired t-test, p = 0.62). The result of linear regression is shown.

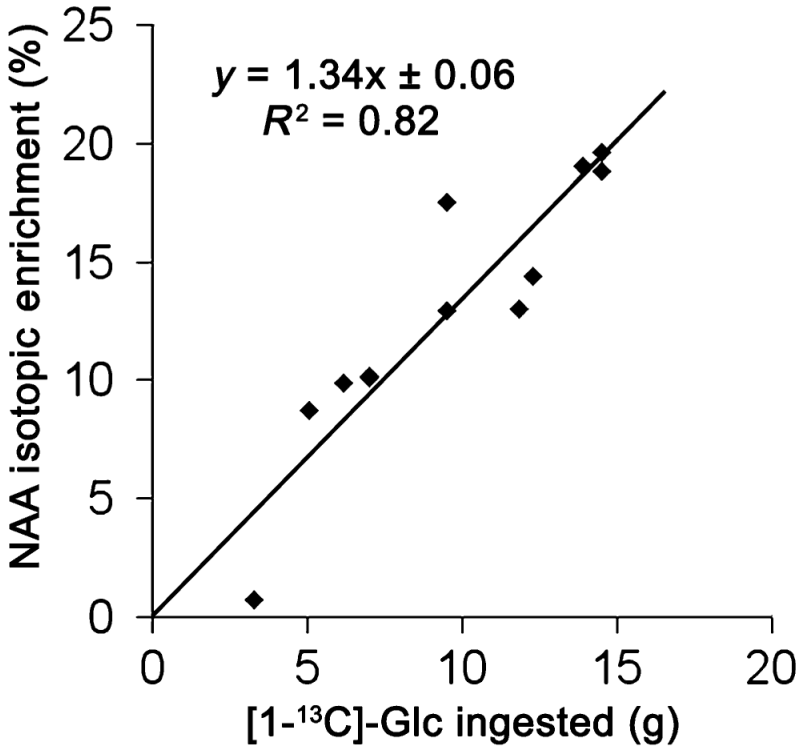

Fig. 3.

NAA IE is correlated with the amount of [1-^{13}C]-Glc ingested by the rat. [6-^{13}C]-NAA IE as a function of the amount of [1-^{13}C]-Glc ingested (in grams) by the rat (n = 11). Shown is the result of linear regression.

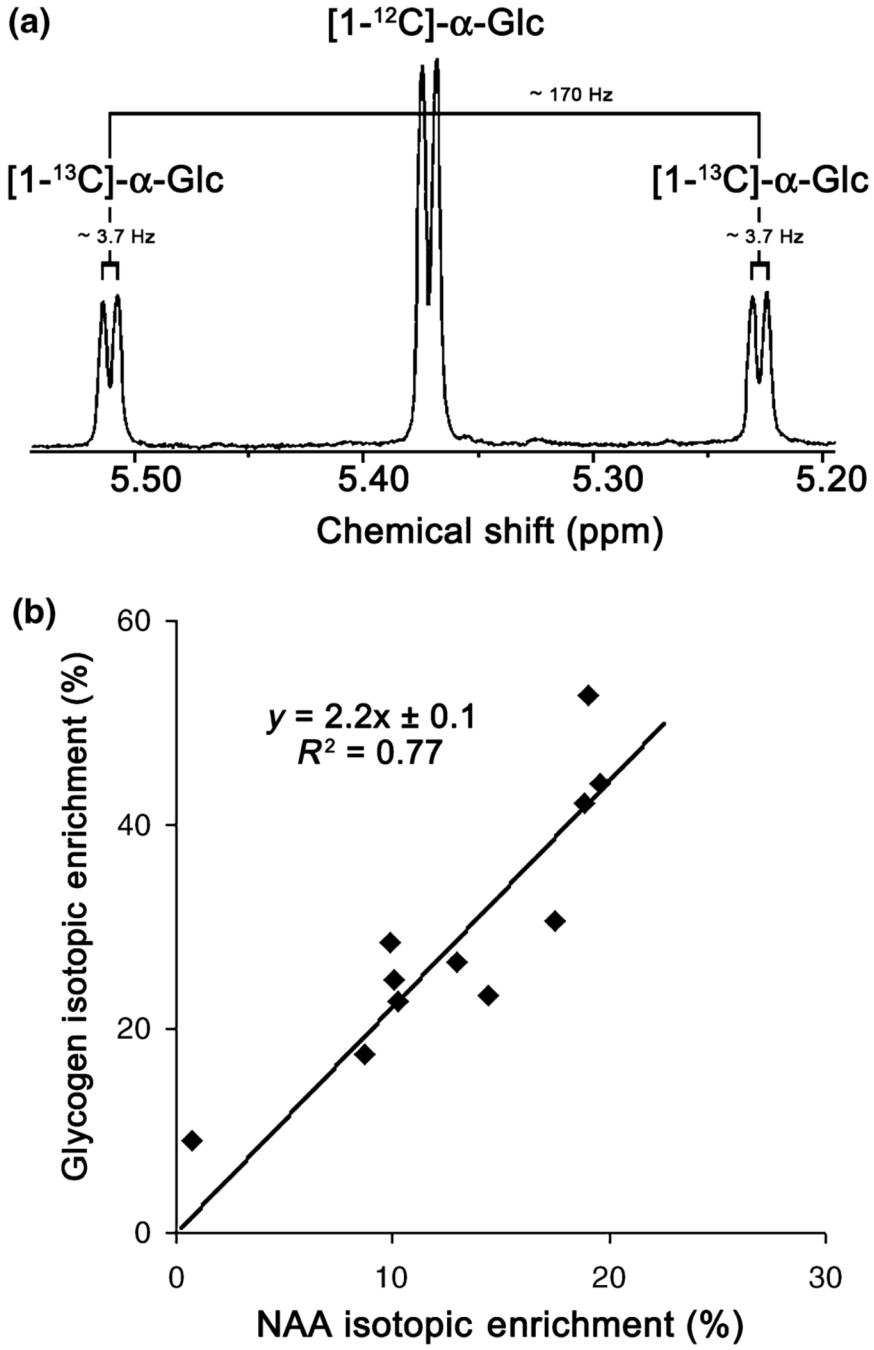

Fig. 4.

At isotopic near steady-state enrichment, NAA IE reflects glycogen IE. (a) Example of *in vitro* Glc ^{1}H resonance of the α-anomer at 5.28 ppm used for calculating brain Glc and glycogen IE. (b) *In vitro* measurement of ^{13}C-glycogen IE as a function of [6-^{13}C]-NAA IE after [1-^{13}C]-Glc ingestion (n = 11). Shown is the result of linear regression.

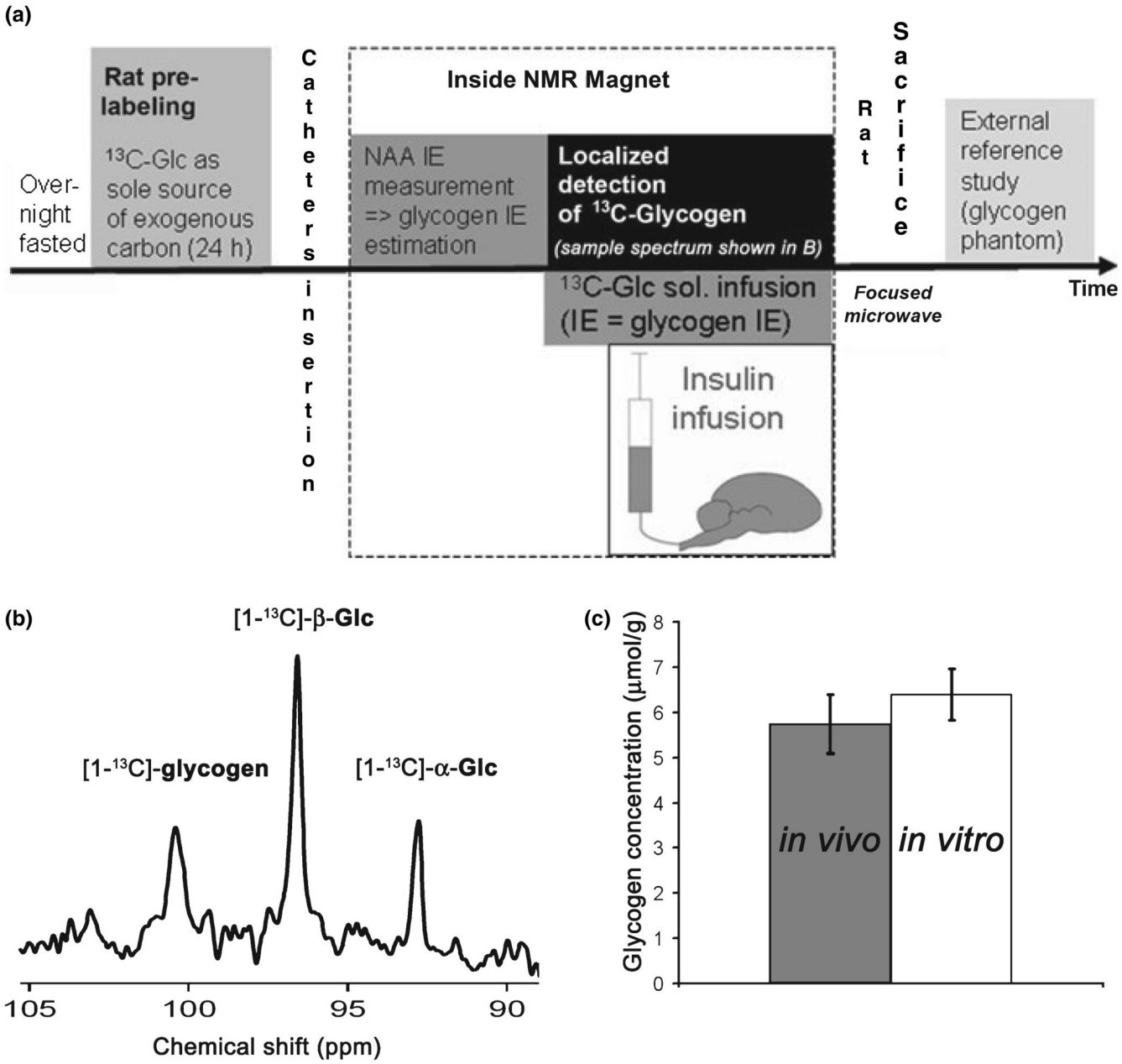

Fig. 5.

In vivo measurement of brain glycogen absolute concentration. (a) Scheme summarizing the experimental protocol for `time course experiments'. (b) Example of *in vivo* ^{13}C NMR spectrum showing localized detection of [1-^{13}C]-glycogen and [1-^{13}C]-Glc (α and β anomers). (c) No significant difference between glycogen absolute concentration measured *in vivo* by NMR spectroscopy (last measurement before kill) and *in vitro* in the post-mortem brain extracts (n = 5 rats, paired t-test, p = 0.12). Error bars indicate SD.

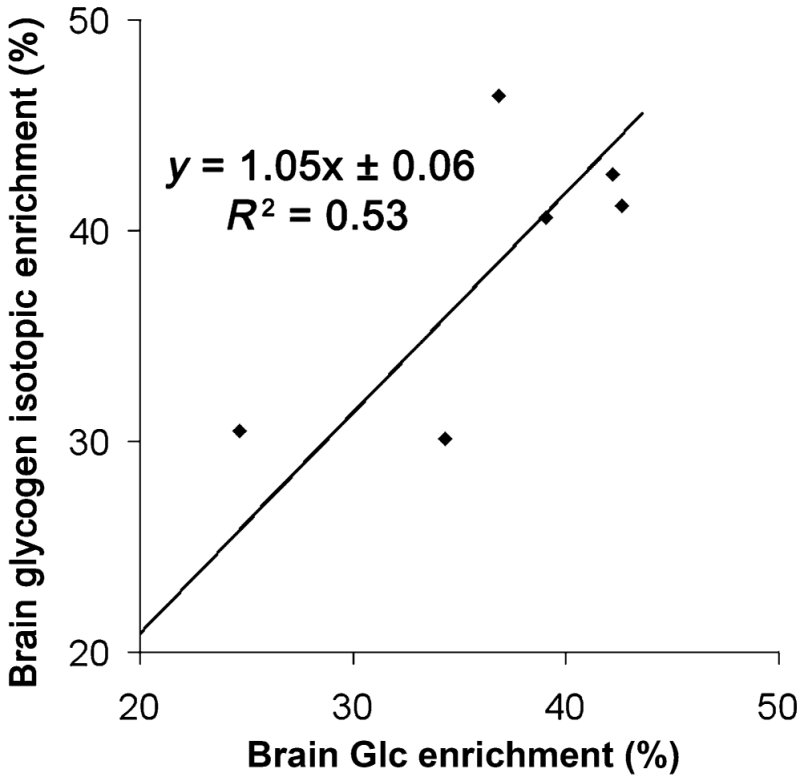

Fig. 6.

Glycogen turnover is complete. At the end of the time course experiment, brain glycogen isotopic enrichment was similar to the isotopic enrichment of brain Glc (n = 6 rats). Shown is the result of linear regression.

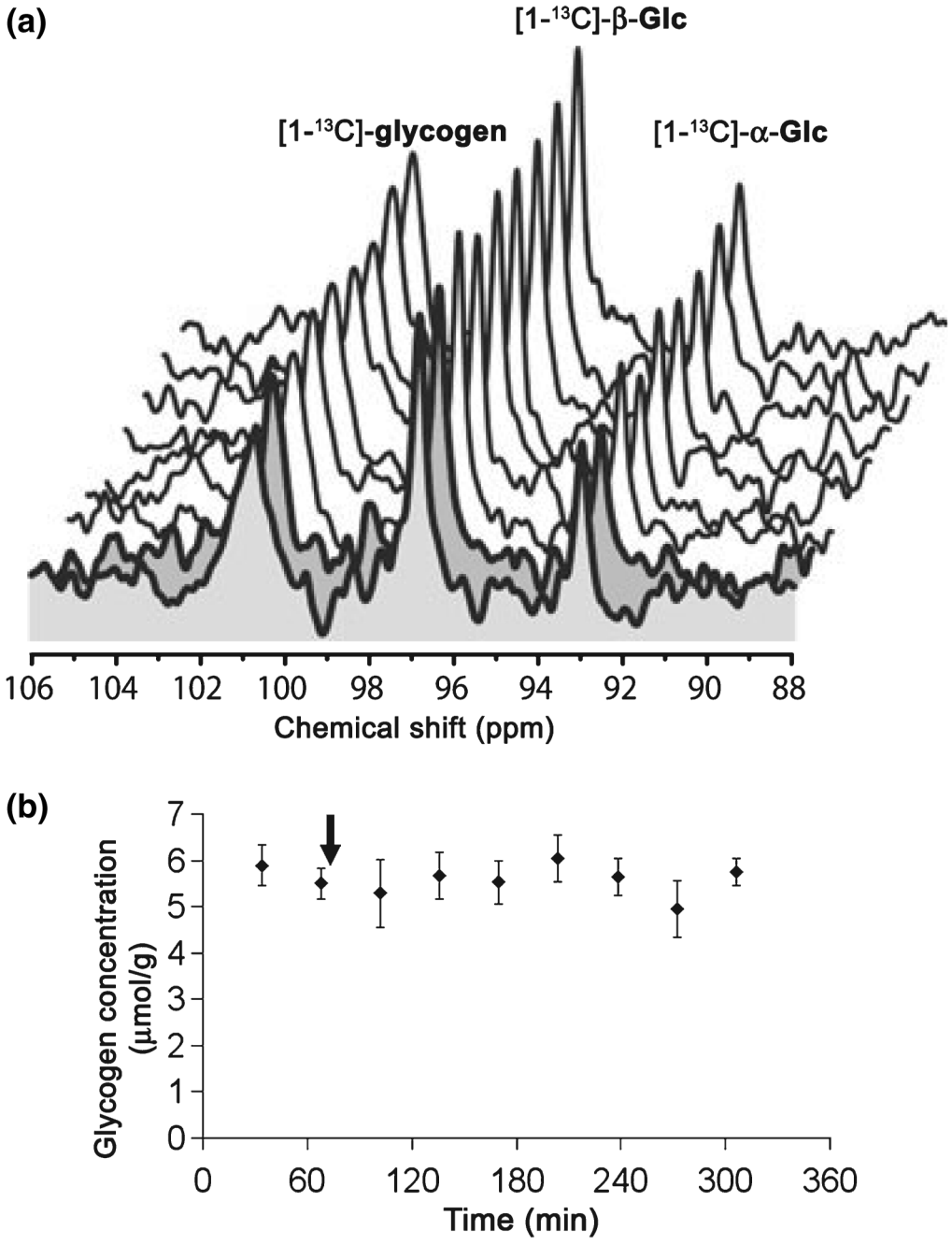

Fig. 7.

Apparent stability of the time-resolved NMR signal of glycogen during insulin infusion. (a) Time resolved stack plot (one spectrum each 34 min) of *in vivo* ^{13}C spectra acquired in one rat showing localized detection of [1-^{13}C]-glycogen and [1-^{13}C]-Glc (α and β anomers). Whereas [1-^{13}C]-Glc infusion was performed during the whole experiment (to maintain plasma glycemic levels around 10 mM) at an IE matching that estimated for glycogen, insulin infusion was started after the acquisition of the first two spectra (shown in grey). (b) Mean brain glycogen concentration (n = 5 rats) over time. n = 5 out of the six rats of this group; absolute quantification of glycogen was not possible in one rat because of instrument failure. The arrow indicates when insulin infusion started. Error bars indicate SEM.