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Alteration of brain glycogen turnover in the conscious rat after 5 h of prolonged wakefulness

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ABSTRACT

Although glycogen (Glyc) is the main carbohydrate storage component, the role of Glyc in the brain during prolonged wakefulness is not clear. The aim of this study was to determine brain Glyc concentration ([]) and turnover time (τ) in euglycemic conscious and undisturbed rats, compared to rats maintained awake for 5 h.

To measure the metabolism of [1-^{13}C]-labeled Glc into Glyc, 23 rats received a [1-^{13}C]-labeled Glc solution as drink (10% weight per volume in tap water) *ad libitum* as their sole source of exogenous carbon for a "labeling period" of either 5 h (n = 13), 24 h (n = 5) or 48 h (n = 5). Six of the rats labeled for 5 h were continuously maintained awake by acoustic, tactile and olfactory stimuli during the labeling period, which resulted in slightly elevated corticosterone levels. Brain [Glyc] measured biochemically after focused microwave fixation in the rats maintained awake (3.9 ± 0.2 μmol/g, n = 6) was not significantly different from that of the control group (4.0 ± 0.1 μmol/g, n = 7; t-test, $P > 0.5$). To account for potential variations in plasma Glc isotopic enrichment (IE), Glyc IE was normalized by N-acetyl-aspartate (NAA) IE. A simple mathematical model was developed to derive brain Glyc turnover time as 5.3 h with a fit error of 3.2 h and NAA turnover time as 15.6 h with a fit error of 6.5 h, in the control rats. A faster τ_{Glyc} (2.9 h with a fit error of 1.2 h) was estimated in the rats maintained awake for 5 h.

In conclusion, 5 h of prolonged wakefulness mainly activates glycogen metabolism, but has minimal effect on brain [Glyc].

1. Introduction

Glycogen (Glyc) is the main carbohydrate storage component in the body and acts as a depot into which glucose (Glc) can be stored and rapidly released upon demand (Rao et al., 2006). Brain Glyc can be an emergency fuel reservoir during acute insulin-induced hypoglycemia (Choi et al., 2003; Gruetter, 2003; Morgenthaler et al., 2006) or during hypoxia–ischemia (Allen et al., 2005; Gruetter, 2003; Nordstrom and Siesjo, 1978; Rossi et al., 2007). Moreover, lines of evidence indicate that brain Glyc can be tapped for fuel without overt or prolonged energy deficits (Gibbs et al., 2006, 2008, 2007). The analysis of the utilization and metabolic fate of brain Glyc will help to understand its functional role in brain energetics (Dienel et al., 2007).

In the adult brain, Glyc is found mainly in astrocytes. Under resting conditions, brain Glyc level is stable and its turnover is slow (Choi et al., 2003; Watanabe and Passonneau, 1973). It has been suggested that neurons, unlike astrocytes, have no energy reserves and may rely on astrocytes for the supply of energy during acute changes in energy demands (Pellerin and Magistretti, 1994). It has been proposed that the abrupt energy demand for clearance of glutamate from the synaptic cleft and its conversion to glutamine create a stimulation of astrocytic glucose utilization (Dienel and Hertz, 2005; Pellerin and Magistretti, 1994). Moreover, it has been shown that glycogenolysis can increase substantially during brain activation (Cruz and Dienel, 2002; Dienel and Cruz, 2003, 2004; Swanson, 1992; Swanson et al., 1992). Glycogenolysis occurs in retina of the intact honeybee during light stimulation (Tsacopoulos and Evequoz, 1980), in the chick brain during early memory consolidation (Gibbs et al., 2006) and in rat somato-sensory cortex *in vivo* during vibrissae stimulation (Swanson, 1992; Swanson et al., 1992). On the other hand, it has been suggested that glial Glyc

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Abbreviations: F-1,6-bisP, fructose 1,6-bisphosphate; GA-3-P, glyceraldehyde-3-phosphate; Glc, glucose; Glyc, glycogen; IE, isotopic enrichment; NAA, N-acetyl-aspartate; SEM, standard error of mean; τ, turnover time.

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tends to accumulate during conditions of depressed neuronal activity (Cruz and Dienel, 2002).

Increased glucose metabolism in astrocytes can be achieved by increasing glucose uptake through glucose transporters (GLUTs) or by glycogenolysis, and both processes are affected by adrenergic receptor stimulation (Sorg and Magistretti, 1991; Subbarao and Hertz, 1990). For example, it has been shown that adrenergic receptors, as well as Glyc, play an important role in memory formation in the chick (Hutchinson et al., 2008). Glycogenolysis in cultured astrocytes and brain slices assayed *in vitro* under normoglycemic conditions is initiated by many signals arising from neuronal activity, including the widespread locus coeruleus noradrenergic arousal–stress–response systems (Hertz et al., 2007; Magistretti et al., 1981). However, data obtained in primary cultures of astrocytes indicate that noradrenaline, in addition to its rapid (within minutes) glycogenolytic effects (Subbarao and Hertz, 1990), trigger Glyc resynthesis beginning 60 ± 90 min after application and reaching a maximum after 9 h (Sorg and Magistretti, 1992). Similarly, sleep deprivation has been reported to elicit complex responses in Glyc synthesis and breakdown (Gip et al., 2002; Kong et al., 2002). It has been suggested that this may reflect an intricate and delicate balance between the ratio and rate of Glyc synthesis and degradation, influenced by stress, neuronal activity, and other factors (Gip et al., 2004). In mice cerebral cortex, an increase of protein targeting to glycogen mRNA and the decrease of both Glyc synthase and Glyc phosphorylase have been observed after 6 h of enforced waking (Petit et al., 2002). The temporal relationships between Glyc synthesis and degradation during activation are difficult to measure, but prevention of the decrease in $CMRO_2$:CMR_{glc} ratio during activation by propranolol, a β-adrenergic blocker, supports a role for Glyc turnover during brain activation (Schmalbruch et al., 2002). Recently, inhibition of Glyc phosphorylase during brief sensory stimulation has implied a role for Glyc in brain activation (Dienel et al., 2007). The increased Glyc turnover during activation complicates interpretation of standard approaches that assess concentration ([]) and labeling changes to evaluate roles or utilization of Glyc under various conditions (Dienel et al., 2007). Finally, a restricted set of neurotransmitters potentiate glycogenolysis (Magistretti, 2006; Magistretti et al., 1981). They include norepinephrine, serotonin, and histamine that are released maximally during waking (Benington and Heller, 1995).

In contrast to radiotracer studies, NMR studies to date have not detected activation-induced turnover and glycogenolysis (unpublished results). As *in vivo* NMR of glycogen requires the infusion of ^{13}C-labeled Glc at high IE and thus necessitates anesthesia, we sought to use ^{13}C isotopes, but applied to the awake rat brain. The goal of this study was to measure brain [Glyc] and turnover (directly) in euglycemic rats maintained awake for 5 h.

2. Methods

2.1. Groups of animal studied

Four groups of rats (Sprague–Dawley rats, Charles River, France) were studied (n = 23; mean weight ± SEM: 247 ± 8 g). The first two groups of rats were labeled during 5 h (see below) and the effects of prolonged wakefulness on Glyc were studied. These groups consisted in the rats maintained awake during the labeling period (n = 6) and the corresponding control, i.e. undisturbed group (n = 7). With the two other groups of undisturbed rats, the ratio of Glyc isotopic enrichment (IE) over N-acetyl-aspartate (NAA) IE over time was modeled. The rats were therefore labeled for a longer period of time, either for 24 h (n = 5) or for 48 h (n = 5).

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

2.2. Brain Glyc labeling

Rats were fasted overnight with free access to water before studies were performed. The following day the only nutrient they received was a 10% weight per volume [1-^{13}C]-labeled Glc solution in tap water *ad libitum* for about 5 h (n = 13), 24 h (n = 5) or 48 h (n = 5). The amount of ^{13}C-labeled solution ingested by each rat was estimated by subtracting the amount of liquid left in the bottle at the end of the experiment to the amount that had been proposed to each rat. All 23 rats ingested a significant amount (at least 1.9 g) of [1-^{13}C]-labeled Glc solution during the labeling period. All of the rats labeled for 5 h (n = 13) and most of the rats labeled for 24 h (n = 3) received a 99% ^{13}C-enriched solution. The other rats labeled during 24 h (n = 2) and the rats labeled during 48 h (n = 5) received a 50% ^{13}C-enriched solution as labeling solution, to minimize the cost of the experiment.

To avoid brain activation in the control group of rats, they were labeled in a quiet and familiar environment in a 12:12 h light–dark cycle (see under).

2.3. Arousal paradigm

The animals were kept at ambient temperature (~22 °C) under a 12:12 h light–dark cycle and adapted to these conditions for at least 5 days prior to the experiment.

On the experimental day at 7:00 (light on), all rats (n = 13) received an identified amount of labeled Glc containing solution (see above). To minimize variability due to Glyc synthase and Glyc phosphorylase circadian variations (Petit et al., 2002), rat arousal always started at the same time. The rats maintained awake (n = 6) were moved into a separate room for about 5 h. They were constantly observed and whenever they exhibited signs of drowsiness or behavioral signs of falling asleep, they were aroused by acoustic stimuli, by tactile stimuli, by introducing objects into the cage, or by olfactory stimuli (such as being introduced to another cage with smells from other rats). These stimulations initially occurred approximately every half an hour and were progressively increased up to 15 min intervals.

2.4. Tissue analysis

At the end of each experiment, rats were anesthetized using isoflurane (isoflurane, Halocarbon Laboratories, 5% for induction and 1.5% for maintenance while withdrawing blood and inserting the rat into the microwave) in oxygen (O_2) gas. Blood was collected by tail bleed and immediately centrifuged to obtain plasma for subsequent analysis of [Glc] and [corticosterone] (see below). Then, the rats were sacrificed using a focused microwave fixation device irradiating the brain at 4 kW for 1.4–1.6 s (Gerling Applied Engineering, Inc., Modesto, CA, USA) as in previous studies (Morgenthaler et al., 2006, 2008; Lei et al., 2007). This procedure inactivates most brain enzymes before extraction or digestion, thereby minimizing possible *in vitro* Glyc 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.

2.5. Glyc assay

Biochemical measurement of brain Glyc was performed on brain extracts as previously described (Cruz and Dienel, 2002; Morgenthaler et al., 2006, 2008).

2.6. In vitro ^{1}H NMR: Glc IE, Glyc IE and NAA IE measurement

Brain Glc IE, Glyc IE and NAA IE were measured *in vitro* by ^{1}H NMR as previously described (Morgenthaler et al., 2008), using a Bruker Avance-DRX 600 (14.1 T, 600 MHz) spectrometer (Bruker BioSpin SA, Fällanden, Switzerland).

Brain Glyc IE was calculated according to the following Eq. (1) after having processed the brain extracts with the Glyc assay so that the Glyc molecule was digested into Glc molecular units (Lei et al., 2007; Morgenthaler et al., 2008).

$$IE_{Glyc} = \frac{(IE_{Total\,Glc} \times [Total\,Glc]) - (IE_{BrainGlc} \times [Brain\,Glc])}{[Glyc]} \quad (1)$$

Total Glc (TotalGlc) represents brain Glc (BrainGlc) plus digested brain Glyc, 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 [Glyc] were obtained by biochemical measurement.

2.7. Plasma analysis

Plasma [Glc] was determined using the same Analox instrument as above.

Plasma [corticosterone] was measured by immunoassay (Correlate-EIA, corticosterone, Assay Designs, Ann Arbour, MI, USA).

2.8. Model of ^{13}C incorporation into Glyc and NAA after [1-^{13}C]-Glc ingestion

The chemical pathways of Glc that are involved in the ^{13}C label incorporation into Glyc and NAA after [1-^{13}C]-Glc ingestion are in Fig. 1. In this model, we only consider the chemical pools with a high concentration (NAA, Glyc) and the pools of the network localized at biochemical branch points (Fig. 1). The following mono-exponential Eq. (2) describes the IE of Glyc over time, where the turnover time (τ) of

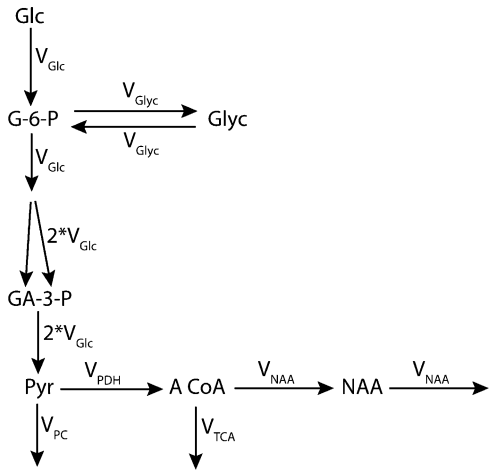

Fig. 1. Chemical pathways used for Glc metabolism modeling. The mathematical model considers metabolic steady-state conditions and is simplified in order to consider only the chemical pools with high concentration, such as NAA and Glyc, as well as the pools located at chemical branch points. The carbon chain, composed of 6 carbons, is split into 2 chains of three carbons at the level of F-1,6-bisP resulting in the production of 2 molecules of GA-3-P. Therefore, the total flux of molecules (in micromol/g/min) is doubled. Subsequently to a labeling procedure with [1-^{13}C]-labeled Glc, only one carbon in the F-1,6-bisP molecule is labeled, and only one of the two produced GA-3-P is labeled. Therefore, a dilution of the labeling by a factor of 2 is observed after F-1,6-bisP. Thus, the labeling flux remains the same as the one entering the pool of F-1,6-bisP (i.e. V_{Glc}). Glc, Glyc, G-6-P, F-1,6-bisP, GA-3-P, Pyr, TCA, A CoA and NAA stand for glucose, glycogen, glucose-6-phosphate, fructose 1,6-bisphosphate, glyceraldehyde-3-phosphate, pyruvate, tricarboxylic acid cycle, acetyl-CoA, and N-acetyl-aspartate, respectively. V_{Glc} represents the rate of Glc metabolism via glycolysis and V_{Glyc} the exchange rate with the Glyc molecule. V_{PDH} represents the flux of pyruvate into acetyl-CoA catalysed by pyruvate dehydrogenase (PDH), and V_{PC} the rest of the efflux from pyruvate to oxalo-acetate than to the pyruvate carboxylase (PC). The TCA cycle flux is represented by V_{TCA}.

Glyc, τ_{Glyc}, is defined by Glyc/V_{Glyc} (see Appendix 1A):

$$IE_{Glyc}(t) = \frac{^{13}Glyc}{Glyc} = IE_{Glc}\left(1 - \exp\left(-\frac{t}{\tau_{Glyc}}\right)\right) \quad (2)$$

As $V_{Glyc} << V_{Glc}$ (Choi and Gruetter, 2003; Watanabe and Passonneau, 1973), there is a negligible influence of Glyc labeling on the labeling equations of pyruvate (Pyr) (see Appendix 1B). Accordingly, this implies that the label incorporation of NAA is largely unaffected by glycogen metabolism, regardless of the specific NAA synthesis rate. Thus, we also obtain a mono-exponential time course for NAA IE (Eq. (3), where τ_{NAA} is defined by NAA/V_{NAA}):

$$IE_{NAA}(t) = \frac{^{13}NAA}{NAA} = \tfrac{1}{2}IE_{Glc}\left(1 - \exp\left(-\frac{t}{\tau_{NAA}}\right)\right) \quad (3)$$

The factor 1/2 takes into account the fact that the 6-carbon glucose is split into two 3-carbon units of which only one carbon is labeled. This labeling dilution implies that the maximum IE reachable for NAA is 50% of the IE of the infused Glc solution. And, the ratio of Glyc IE over NAA IE becomes Eq. (4) by dividing Eq. (2) by Eq. (3):

$$\frac{IE(Glyc)}{IE(NAA)} = (t) = 2\frac{1 - \exp(-t/\tau_{Glyc})}{1 - \exp(-t/\tau_{NAA})} \quad (4)$$

Finally, plotting Glyc IE/NAA IE over time suggests that there might be 3 different curves depending on τ_{Glyc} and τ_{NAA} times (Fig. 2).

3. Results

In order to measure brain [Glyc] and turnover in euglycemic rats after several hours of prolonged wakefulness, conscious rats were ingesting a [1-^{13}C]-Glc solution (10% weight per volume) *ad libitum*. There was no significant difference in the amount of labeled Glc solution ingested by the rats maintained awake (mean Glc ingested ± SEM: 3.1 ± 0.4 g, n = 6) or the control rats (3.4 ± 0.4 g, n = 7; two-tailed t-test, P = 0.59).

Maintaining the rats awake for 5 h resulted in an increase in the plasma corticosterone levels ([mean] ± SEM: 97 ± 9 ng/mL, n = 6) compared to the control group (66 ± 21 ng/mL, n = 7) of rats (Table 1). This increase in corticosterone levels was not significant (two-tailed t-test, P = 0.21) due to one particular rat in the control group who had an unusually high corticosterone level of 160.3 ng/mL.

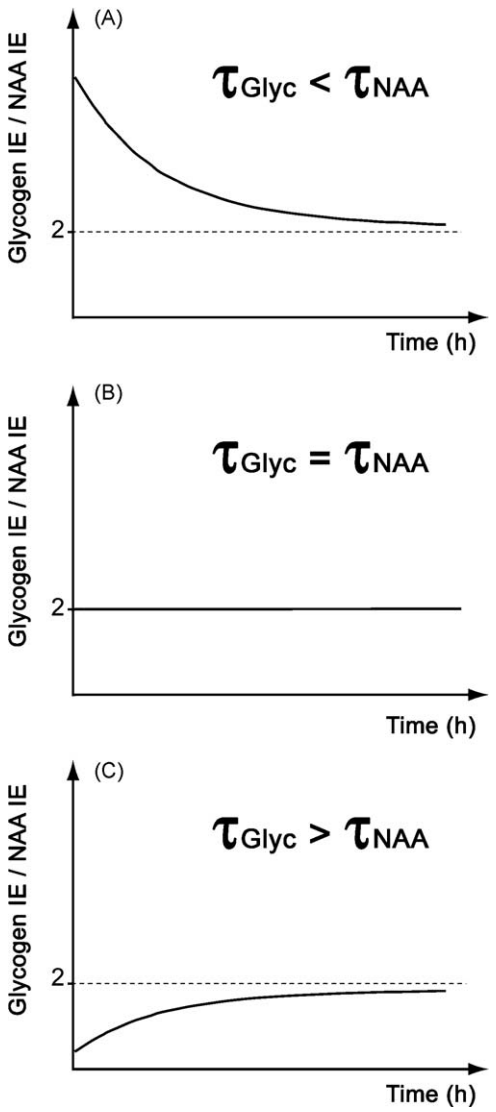

Fig. 2. The 3 different curves that could be obtained by plotting Glyc IE/NAA IE over time using Eq. (4). These curves depend on the values of τ_{Glyc} and τ_{NAA}. The 3 different case figures are shown in (A) if $\tau_{Glyc} < \tau_{NA}$, in (B) if $\tau_{Glyc} = \tau_{NAA}$ or in (C) if $\tau_{Glyc} > \tau_{NAA}$.

Brain [Glyc] (Table 1) of the rats maintained awake for ~5 h (mean ± SEM: 3.9 ± 0.2 μmol/g, n = 6) was not significantly different from that of the control group (4.0 ± 0.1 μmol/g, n = 7; two-tailed t-test, P = 0.65).

In order to evaluate changes in Glyc turnover due to prolonged wakefulness, NAA IE was used as an internal reference to minimize the impact of potential variations in the enrichment of plasma glucose (precursor). Fig. 3 shows significant increase of Glyc IE/NAA IE in the "group of rats maintained awake"

Table 1
Effects of 5 h of prolonged wakefulness on rat plasma corticosterone levels and brain [Glyc]. Mean ± SEM are shown.

	Control rats (n = 7)	Rats maintained awake (n = 6)
Plasma [corticosterone] (ng/mL)	66 ± 21	97 ± 9
Brain [Glyc] (μmol/g)	4.0 ± 0.1	3.9 ± 0.1

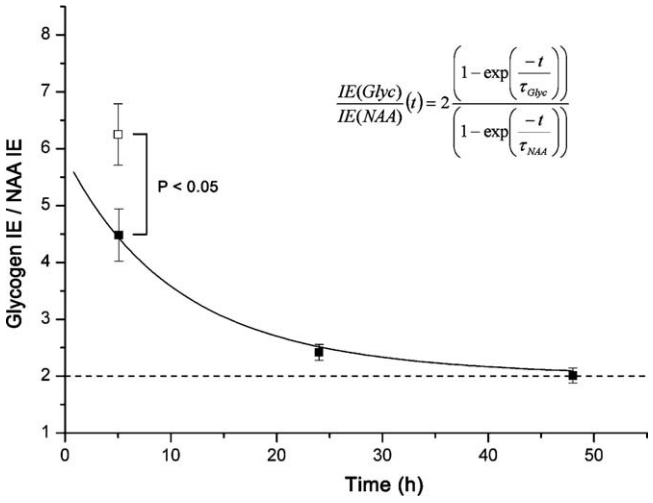

Fig. 3. Graph representing Glyc IE/NAA IE over time. Shown are the mean values ± SEM for each group of rats (black squares: control rats, white square: rats maintained awake for 5 h). The ratio of brain Glyc IE over NAA IE is significantly increased ($P < 0.05$) in the brain of the rats maintained awake for 5 h as compared to the corresponding control group (5 h labeled) of rats. Eq. (4) is fitted to the data obtained from each individual control – undisturbed – rats. This plot enables to calculate a τ of 5.3 h with a fit error of 3.2 h and 15.6 h with a fit error of 6.5 h for Glyc and NAA, respectively.

(mean ± SEM: 6.3 ± 0.5, $n = 6$) as compared to the control group of rats (4.5 ± 0.5, $n = 7$; two-tailed t-test, $P = 0.03$) after ~5 h of ^{13}C-labeled Glc ingestion. The decrease of Glyc IE/NAA IE over time in control rats (Fig. 3) implied that the τ_{Glyc} was smaller than the τ_{NAA}, as shown from the case simulation in Fig. 2. As we show in Appendix 2 (Eq. (A17)), the value of IE(Glyc)/IE(NAA) at time close to 0 provides an estimate of $2(\tau_{NAA}/\tau_{Glyc})$. At time t close to zero, the ratio of Glyc IE/NAA IE has to be higher than the value of the control rats labeled for 5 h (Fig. 3), i.e. 4.5, because IE(Glyc)/IE(NAA) is a decreasing function of time (case A in Fig. 2). This means that $2(\tau_{NAA}/\tau_{Glyc}) > 4.5$ and consequently that $\tau_{Glyc} < 44\%$ of τ_{NAA}. Furthermore, fitting the time course of IE(Glyc)/IE(NAA) using Eq. (4) allowed to estimate brain τ_{Glyc} and τ_{NAA} as 5.3 h with a fit error of 3.2 h and 15.6 h with a fit error of 6.5 h, respectively (Fig. 3). Finally, a faster τ_{Glyc} of 2.9 h (with a fit error of 1.2 h) was calculated in the rats maintained awake, assuming that τ_{NAA} was not altered by the arousal paradigm. This corresponds to an increase in Glyc turnover and it was calculated by isolating τ_{Glyc} in Eq. (4) using the mean measured ratio of IE(Glyc)/IE(NAA) (i.e. 6.3 ± 1.0 for the rats maintained awake) as well as the mean of the measurement time (4.5 h). It is worth noting that there was no significant difference in brain Glc IE of the control rats (mean ± SEM: 78 ± 7%, $n = 7$) as compared to the rats maintained awake for 5 h (83 ± 3%, $n = 6$; two-tailed t-test, $P = 0.60$).

4. Discussion

The present study shows that maintaining rats awake for 5 h mainly activates Glyc turnover, but does not impact brain [Glyc] substantially.

Our arousal paradigm was not very stressful. The rise in plasma corticosterone levels was lower than in other studies where rats were kept awake by gentle handling for a similar period of time (Gip et al., 2004). Moreover, no significant increase in either brain Glc (Gip et al., 2004) or plasma Glc levels (Dienel et al., 2007) was observed in our study (not shown). Glucocorticoids, such as corticosterone, are adrenal steroid hormones released systematically in response to stressors (Tobler et al., 1983). It has been shown that glucorcorticoids themselves seem to affect regional brain Glyc stores (Gip et al., 2004). In cultured astrocytes, glucocorticoids decrease Glyc stores (Allaman et al., 2004; Tombaugh et al., 1992). Similarly, adrenalectomy can either increase brain Glyc levels (Passonneau et al., 1971) or have no effect in the whole brain (Goldberg and O'Toole, 1969) or cortex (Plaschke et al., 1996).

The present study did not reveal any significant decrease in brain [Glyc] among the rats that were maintained awake for ~5 h as compared to the control rats. It has been suggested that promotion of glycogenolysis during waking might provide a continual supplementary source of glucose to prevent transient impairment of astrocytic maintenance of the cellular milieu in the nervous system during waking behavior, thereby optimizing the capacity of the nervous system to respond to stimuli (Benington and Heller, 1995). Significant decrease of 11% (Dienel et al., 2002; Madsen et al., 1999) to 22% (Dienel et al., 2002; Madsen et al., 1999) in [Glyc] after stimulation of sensory, motor and whisker-to-barrel pathways has been reported. In a more recent study, Dienel et al. (2007) observed a concentration decrease of 14% during a mixed-modal generalized sensory, acoustic and visual stimulation. Our study is consistent with a milder stimulus precluding changes in [Glyc] < ~8% over 4.5 h (corresponding to a 95% confidence interval) which is in agreement with another study using a similar stimulation paradigm (Kong et al., 2002).

The calculated τ_{NAA} of 15.6 ± 6.5 h and τ_{Glyc} of 5.3 ± 3.2 h were comparable to previous reports (Choi and Gruetter, 2003, 2004; Moreno et al., 2001; Oz et al., 2007; Xin et al., 2008). A similar τ_{NAA} has been reported in anesthetized rats (Choi and Gruetter, 2004) and in human (Moreno et al., 2001). A slightly longer τ_{Glyc} of about 10 h has been reported in anesthetized rats (Choi and Gruetter, 2003). Moreover, we have previously shown that the slower turnover time of limit dextrin (Watanabe and Passonneau, 1973) is sufficiently fast to lead to near-complete Glyc turnover in 24 h (Morgenthaler et al., 2008). The faster Glyc turnover in our control group of rats is consistent with τ_{Glyc} being affected by stimulation of the adrenergic system, largely suppressed by anesthesia (Dienel et al., 2007). However, it is of interest to note that in awake humans, τ_{Glyc} is about 1 day (Oz et al., 2007). As humans are well-known to generally have a slower brain energy metabolism than awake rats, this probably reflects species difference.

Our results suggest that prolonged wakefulness increases Glyc turnover. Although Glyc synthase and Glyc phosphorylase have opposite effects on Glyc content, a number of studies have shown parallel variations of these enzymes relative to changes in Glyc levels (David et al., 1990; Melendez et al., 1999; Mulmed et al., 1979; Petit et al., 2002). Moreover, a number of neurotransmitters that potentiate glycogenolysis, including norepinephrine, serotonin, and histamine are released maximally during waking (Benington and Heller, 1995). Increases in Glyc turnover have been observed after shorter, but stronger stimulation paradigm (Dienel et al., 2007). It has been postulated that high Glyc turnover during sensory stimulation could arise if local neuronal activity triggers pulsatile Glyc degradation and resynthesis, particularly in astrocytic fine processes that surround synaptic structures, as well as their soma and endfeet (Dienel et al., 2007). The increased Glyc turnover does not preclude small transient fluctuations in [Glyc], as such transient fluctuations in [Glyc] might result in increased label incorporation.

In the aforementioned derivation of about a twofold reduced τ_{Glyc} with arousal, we assumed that τ_{NAA} was not affected. While an increase in τ_{NAA} (reflecting reduced NAA metabolism) is rather unlikely in situations of increased brain metabolism, the effects of a potential decrease in τ_{NAA} were investigated. In Appendix 3 Eq. (A22), we show that the measurement of IE(Glc)/IE(NAA) (at ~5 h in the control group of rats) can be used to estimate a minimum τ_{NAA} of 11.7 h. Replacing τ_{NAA} of 15.6 h with any value between 15.6 h and 11.7 h in Eq. (4) results in a τ_{Glyc} between 0 h

and 2.9 h, thus shorter than the value for τ_{NAA} = 15.6 h. Therefore, a calculated value of τ_{Glyc} in case of a shorter τ_{NAA} would anyway be smaller than the value found with an assumption of unchanged τ_{NAA}. Subsequently, the difference in τ_{Glyc} between control and aroused rats would be even bigger than reported.

In conclusion, we show that a short period of prolonged wakefulness induced by mild sensory-motor stimulation results in an increase in the turnover of brain Glyc without substantially affecting its concentration.

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Appendix A. Appendix

A.1. Enrichment of Glyc following exogenous administration of [1-^{13}C]-labeled Glc

To measure the IE enrichments of Glyc, we consider a standard step function as input for the [Glc], as well as a negligible amount of label at $t = 0$ (^{13}C natural abundance being ~1.1%). Moreover, we model a situation of steady-state, i.e. absolute concentrations remain constant over time. For example, no change in [Glyc] implies equal Glyc synthesis and degradation fluxes (represented by V_{Glyc} in Fig. 1). Since the concentration of glucose-6-phosphate (G-6-P) is low compared to Glyc, the labeling equation of Glyc corresponds to the labeling equation of a single pool system, in which the precursor is glucose and the product is Glyc. The rate of label incorporation into G-6-P is given by:

$$\frac{d(^{13}\mathrm{G}-6-\mathrm{P})}{dt} = V_{Glc}\frac{^{13}\mathrm{Glc}}{\mathrm{Glc}} + V_{Glyc}\frac{^{13}\mathrm{Glyc}}{\mathrm{Glyc}} - (V_{Glc} + V_{Glyc})\frac{^{13}\mathrm{G}-6-\mathrm{P}}{\mathrm{G}-6-\mathrm{P}} \quad \text{(A1)}$$

where the names without ^{13} represent the total amount of molecules (labeled + unlabeled). From this we can isolate the IE of G-6-P:

$$\frac{^{13}\mathrm{G}-6-\mathrm{P}}{\mathrm{G}-6-\mathrm{P}} = \frac{1}{(V_{Glc}+V_{Glyc})} \times \left[V_{Glc}\frac{^{13}\mathrm{Glc}}{\mathrm{Glc}} + V_{Glyc}\frac{^{13}\mathrm{Glyc}}{\mathrm{Glyc}} - \frac{d(^{13}\mathrm{G}-6-\mathrm{P})}{dt}\right] \quad \text{(A2)}$$

For Glyc, the labeling equation is the following:

$$\frac{d(^{13}\mathrm{Glyc})}{dt} = V_{Glyc}\frac{^{13}\mathrm{G}-6-\mathrm{P}}{\mathrm{G}-6-\mathrm{P}} - V_{Glyc}\frac{^{13}\mathrm{Glyc}}{\mathrm{Glyc}} \quad \text{(A3)}$$

Inserting Eq. (A2) into Eq. (A3), and rearranging the terms yields to Eq. (A4):

$$\frac{d(^{13}\mathrm{Glyc})}{dt} + \frac{V_{Glyc}}{(V_{Glc}+V_{Glyc})}\frac{d(^{13}\mathrm{G}-6-\mathrm{P})}{dt} = V_{Glyc}\frac{1}{V_{Glc}+V_{Glyc}}\left[V_{Glc}\frac{^{13}\mathrm{Glc}}{\mathrm{Glc}} + V_{Glyc}\frac{^{13}\mathrm{Glyc}}{\mathrm{Glyc}}\right] - V_{Glyc}\frac{^{13}\mathrm{Glyc}}{\mathrm{Glyc}} \quad \text{(A4)}$$

Assuming that the temporal change of the isotopic enrichment of pools with a much smaller concentration can be neglected compared with pools with higher concentrations (Uffmann and Gruetter, 2007).

$$\text{e.g. } \frac{d(^{13}\mathrm{G}-6-\mathrm{P})}{d(^{13}\mathrm{Glyc})} << 1 \quad \text{(A5)}$$

Eq. (A4) is simplified to:

$$\frac{d(^{13}\mathrm{Glyc})}{dt} = \frac{V_{Glc}V_{Glyc}}{V_{Glc}+V_{Glyc}}\left(\frac{^{13}\mathrm{Glc}}{\mathrm{Glc}} - \frac{^{13}\mathrm{Glyc}}{\mathrm{Glyc}}\right) \quad \text{(A6)}$$

Eq. (A6) shows that the effective labeling flux of Glyc is a composite flux of V_{Glc} and V_{Glyc}. Nevertheless, glycolysis flux V_{Glc} is typically much faster than Glyc synthesis flux V_{Glyc} ($V_{Glyc} << V_{Glc}$) (Choi and Gruetter, 2003; Watanabe and Passonneau, 1973). Consequently, Eq. (A6) is simplified to:

$$\frac{d(^{13}\mathrm{Glyc})}{dt} = V_{Glyc}\left(\frac{^{13}\mathrm{Glc}}{\mathrm{Glc}} - \frac{^{13}\mathrm{Glyc}}{\mathrm{Glyc}}\right) \quad \text{(A7)}$$

Thus, the labeling equation of Glyc, solution of Eq. (A7), is:

$$^{13}\mathrm{Glyc}(t) = \mathrm{IE}_{Glc}\left(1 - \exp\left(-\frac{t}{\tau_{Glyc}}\right)\right) \quad \text{(A8)}$$

where IE_{Glc} = ^{13}Glc/Glc and τ_{Glyc} = Glyc/V_{glyc}.

A.2. Enrichment of NAA following injection of [1-^{13}C]-labeled Glc

The carbon chain, composed of 6 carbons, is split into 2 chains of three carbons at the level of fructose 1,6-bisphosphate (F-1,6-bisP) resulting in the production of 2 molecules of glyceraldehydes 3-phosphate (GA-3-P). Thus, one F-1,6-bisP molecule produces 2 molecules of GA-3-P. Therefore, the total flux of molecules (in micromol/g/min) is doubled (Fig. 1). Nevertheless, since only one carbon is labeled in the F-1,6-bisP, only one of the two produced GA-3-P is labeled and a dilution of the labeling by a factor of 2 is observed after F-1,6-bisP. Thus, the labeling flux remains the same as the one entering the pool of F-1,6-bisP (i.e. V_{Glc}).

The labeling of glucose-6-phosphate (G-6-P) is the following:

$$\frac{d(^{13}\mathrm{G}-6-\mathrm{P})}{dt} = V_{Glc}\frac{^{13}\mathrm{Glc}}{\mathrm{Glc}} + V_{Glyc}\frac{^{13}\mathrm{Glyc}}{\mathrm{Glyc}} - (V_{Glc} + V_{Glyc})\frac{^{13}\mathrm{G}-6-\mathrm{P}}{\mathrm{G}-6-\mathrm{P}} \quad \text{(A9)}$$

The time evolution of ^{13}Glyc, given by Eq. (A8), can be explicitly replaced in Eq. (A9). The solution for G-6-P labeling is then:

$$^{13}\mathrm{G}-6-\mathrm{P}(t) = \underbrace{^{13}\mathrm{Glc}/\mathrm{Glc}(\mathrm{G}-6-\mathrm{P})\left(1-e^{-V_{Glc}+V_{Glyc}/\mathrm{G}-6-\mathrm{P}\,t}\right)}_{\mathrm{A}} + \underbrace{^{13}\mathrm{Glc}/\mathrm{Glc}(\mathrm{G}-6-\mathrm{P})V_{Glyc}(V_{Glc}+V_{Glyc})\mathrm{Glyc}/(V_{Glc}+V_{Glyc})^2\mathrm{Glyc} - V_{Glc}V_{Glyc}(\mathrm{G}-6-\mathrm{P})\left(e^{-V_{Glc}+V_{Glyc}/(\mathrm{G}-6-\mathrm{P})\,t} - e^{-V_{Glc}V_{Glyc}/V_{Glc}+V_{Glyc}\,t/\mathrm{Glyc}}\right)}_{\mathrm{B}} \quad \text{(A10)}$$

Using the initial condition ^{13}G-6-P ($t = 0$) = 0.The term A in Eq. (A10) is the free turnover part that would characterize G-6-P if the pool of glycogen would not exist. The term B is a transitional part which represents the temporary dilution of G-6-P through glycogen, until glycogen reached its labeling steady-state corresponding to the IE of glucose. As the glycolysis flux V_{Glc} is much smaller than the glycogen flux V_{Glyc} (dilution of G-6-P) (Choi and Gruetter, 2003; Watanabe and Passonneau, 1973), the second term B is negligible and the expression of the labeling is a simple free exponential turnover (term A). This can be seen from the fact that the coefficient in front of the parentheses in term B approaches zero for $V_{Glc} >> V_{Glyc}$ (not shown).

Therefore, the rate of labeling of G-6-P is largely independent of the metabolic rate of glycogen. This will have a consequence on the labeling of the following metabolites, and notably NAA.

After this simplification, we have the following effective labeling equation of G-6-P:

$$\frac{d(^{13}\mathrm{G-6-P})}{dt} = V_{Glc}\frac{^{13}\mathrm{Glc}}{\mathrm{Glc}} - V_{Glc}\frac{^{13}\mathrm{G-6-P}}{\mathrm{G-6-P}} \tag{A11}$$

The labeling equation for GA-3-P is the following:

$$\frac{d(^{13}\mathrm{GA-3-P})}{dt} = V_{Glc}\frac{^{13}\mathrm{G-6-P}}{\mathrm{G-6-P}} - 2V_{Glc}\frac{^{13}\mathrm{GA-3-P}}{\mathrm{GA-3-P}} \tag{A12}$$

All the intermediates of the glycolysis are assumed to have a much smaller concentration than the NAA, and thus a smaller temporal change in IE, i.e.:

$$\frac{d(^{13}\mathrm{G-6-P})}{d(^{13}\mathrm{NAA})} << 1;\ \frac{d(^{13}\mathrm{GA-3-P})}{d(^{13}\mathrm{NAA})} << 1;\ \frac{d(^{13}\mathrm{Pyr})}{d(^{13}\mathrm{NAA})} << 1;\ \frac{d(^{13}\mathrm{A\,CoA})}{d(^{13}\mathrm{NAA})} << 1 \tag{A13}$$

With this assumption, we obtain a simplified differential equation for NAA labeling, similar to the one found for glycogen:

$$\frac{d(^{13}\mathrm{NAA})}{dt} = \frac{V_{NAA}}{2}\frac{^{13}\mathrm{Glc}}{\mathrm{Glc}} - V_{NAA}\frac{^{13}\mathrm{NAA}}{\mathrm{NAA}} \tag{A14}$$

The solution of Eq. (A14), assuming ^{13}NAA ($t = 0$) = 0, has the following time evolution:

$$^{13}\mathrm{NAA}(t) = \tfrac{1}{2}\mathrm{IE}_{Glc}\mathrm{NAA}\left(1 - \exp\left(-\frac{t}{\tau_{NAA}}\right)\right) \tag{A15}$$

where τ_{NAA} = NAA/V_{NAA}.

We can see that the factor 1/2 is only affecting the steady-state enrichment and not the dynamics of the exponential turnover, i.e. the characteristic time constant is still NAA/V_{NAA}.

Appendix B. Relation between τ_{Glyc} and τ_{NAA}

When time tends to zero, the following first order Taylor expansion can be used:

$$1 - \exp\left(-\frac{x}{\alpha}\right) \cong \frac{x}{\alpha}, \quad (\text{for } x << \alpha) \tag{A16}$$

From this we calculate that:

$$\lim_{t\to 0}\frac{IE(\mathrm{Glyc})}{IE(\mathrm{NAA})} = \lim_{t\to 0} 2\frac{1-\exp(-t/\tau_{Glyc})}{1-\exp(-t/\tau_{NAA})} = 2\frac{\tau_{NAA}}{\tau_{Glyc}} \tag{A17}$$

Appendix C. Lower bound for τ_{NAA}

From the labeling Eq. (4), we consider a measured value IE(Glyc)/IE(NAA) at a time point t_{exp} (in our case ~5 h). For simplicity of notation, we substitute IE(Glyc)/IE(NAA) (t_{exp}) by M.

$$M = 2\frac{(1-\exp(t_{exp}/\tau_{Glyc}))}{(1-\exp(t_{exp}/\tau_{NAA}))} \tag{A18}$$

Thus,

$$1 - \frac{M}{2} + \frac{M}{2}\exp\left(-\frac{t_{exp}}{\tau_{NAA}}\right) = \exp\left(-\frac{t_{exp}}{\tau_{Glyc}}\right) \tag{A19}$$

Which necessarily implies that

$$0 < 1 - \frac{M}{2} + \frac{M}{2}\exp\left(-\frac{t_{exp}}{\tau_{NAA}}\right) < 1 \tag{A20}$$

$$\Leftrightarrow 1 - \frac{2}{M} < \exp\left(-\frac{t_{exp}}{\tau_{NAA}}\right) < 1 \tag{A21}$$

$$\Leftrightarrow \frac{-t_{exp}}{\ln(1-2/M)} < \tau_{NAA} < \infty \tag{A22}$$

Since $M > 2$, Eq. (A22) can be computed. Thus, the knowledge of a single measurement (average) point makes it possible to find a lower bound to the value of τ_{NAA}, in the case of a decreasing Glyc IE/NAA IE curve (see Fig. 2A) (in the other situation as schematized in Fig. 2C, a similar calculation could be done for τ_{Glyc}).

Neglecting the error on the measured time, we obtain the following uncertainty on the lower limit of τ_{NAA} using noise error propagation calculation:

$$\Delta\tau_{NAA} = \frac{2t_{exp}}{M^2\ln(1-2/M)}\Delta M \tag{A23}$$

In the group of aroused rats, we have $M = 6.3 \pm 1$ and t_{exp} = 4.5 h, which gives a lower limit of τ_{NAA} of 11.7 ± 1.6 h.

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