Physiol. Res. 62 (Suppl. 1): S115-S124, 2013

Daily Profile of *glut1* and *glut4* Expression in Tissues Inside and Outside the Blood-Brain Barrier in Control and Streptozotocin-Treated Rats

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Received March 15, 2013
Accepted June 24, 2013

Summary

Glucose is molecule usually studied in relation to metabolism. Except for this traditional view, it is known that under certain conditions glucose can serve as a signal molecule for the circadian system. The circadian system is entrained by relevant synchronizing cues that can be tissue-dependent. Central oscillator is synchronized mainly by light-dark cycle, while peripheral oscillators can be entrained by food intake. Glucose transport in the organism is controlled by insulin dependent and independent mechanism. Therefore, we employed streptozotocin-induced diabetes to elucidate the influence of metabolic changes on glucose transporter (*glut1, glut4*) 24-h expression profile in peripheral oscillators in tissues, inside (frontal cortex, cerebellum) and outside (heart) the blood–brain barrier. Diabetes was induced by streptozotocin injection. Seventeen days later, sampling was performed during a 24-h cycle. Gene expression was measured using real-time PCR. We observed down-regulation of *glut1* and *glut4* expression in the heart of diabetic rats. The expression of *glut1* and *glut4* in brain areas was not down-regulated, however, we observed trend to phase advance in *glut1* expression in the cerebellum. These results may indicate higher glucose levels in diabetic brain, which might influence regulation of clock gene expression in different manner in brain compared to periphery.

Key words

Frontal cortex • Heart • Cerebellum • Glucose transporter • Circadian

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Introduction

Circadian system is entrained by relevant environmental cues, such as light-dark cycle, to synchronize internal biological oscillations with external daily changes. Proper synchronization allows organisms to adapt to upcoming changes in advance and achieve the appropriate physiological response according to the current daytime (Aschoff 1981).

Central circadian oscillator of hierarchically organized mammalian circadian system resides in the suprachiasmatic nuclei (SCN) of the hypothalamus. Central oscillator synchronizes peripheral oscillators located in other tissues and organs. Beside the light-dark cycle, the circadian system can be synchronized by additional relevant cues, including humoral signals and metabolites (Challet 2010).

Circadian oscillators generate circadian rhythms *via* tightly regulated changes in clock gene expression with subsequent posttranscriptional and posttranslational modifications. Clock gene expression is regulated by mechanism of feedback loop, where protein products of some genes (*per*, *cry*) inhibit their own transcription. The transcriptional factors BMAL1 and CLOCK play a key

role in this loop. Protein heterodimer CLOCK/BMAL1 initiates expression of target genes, including the clock genes *period* (*per1*, *per2*, *per3*) and *cryptochrome* (*cry1*, *cry2*), by binding to their E-box regulatory enhancer sequence. Negative feedback is achieved by PER/CRY heterodimer interaction with the CLOCK/BMAL1 complex, which results in inhibition of *per* and *cry* transcription. As a result, the formation speed of PER and CRY proteins cannot prevail over PER phosphorylation by casein-kinase 1 delta and casein-kinase 1 epsilon. Proteins PER and CRY are subsequently swiftly degraded by β-TrCP1 and FbXL3e3 ubiquitin ligase complexes. Lack of presence of PER/CRY repressor complex leads to the activation of a new transcriptional cycle by the CLOCK/BMAL1 heterodimer (Shearman *et al.* 2000, Albrecht 2006, Takahashi *et al.* 2008).

It is well known that under certain pathophysiological conditions, including metabolic disorders, disturbances in function of the circadian system occur (Barnard *et al.* 2008, Laposky *et al.* 2008). Diabetes is characteristic with massive changes in plasma glucose, ketones and fatty acid levels, which can affect functioning of peripheral oscillators (Kohsaka *et al.* 2007, Oishi *et al.* 2009). During experimental streptozotocin-induced diabetes, plasma glucose levels are markedly elevated (Young *et al.* 2002, Oishi *et al.* 2004). Streptozotocin-induced diabetes is commonly used model of diabetes, where streptozotocin specifically damages pancreatic β-cells, leading to permanent inhibition of insulin secretion (Šoltésová and Herichová 2011). Diurnal rhythm of energy metabolism in diabetic animals differs from control animals. The highest level of carbohydrate utilization occur at the middle of the light phase in diabetic rats compared to maximal levels achieved at the end of the dark phase in the control group (Ichikawa *et al.* 2000). It was observed, that daytime feeding schedule caused more efficient phase shifts of circadian clock genes expression in diabetic rats than in control animals. Entrainment of the activity rhythm of diabetic rats was accomplished after 5 days of daytime feeding regimen, while the complete reversion of the activity pattern in the control group was still not observed (Wu *et al.* 2012). Daytime restricted feeding shifted the acrophase of rhythmic profile of plasma glucose levels in diabetic rats to antiphase compared to control animals. This could possibly indicate smaller stability of circadian system during diabetes and its higher sensitivity to feeding regime (Wu *et al.* 2012).

Glucose alone can influence the responsiveness of the circadian system to photic signals. It was reported that a reduced glucose availability inflicted by intraperitoneal injection of 2-deoxy-D-glucose is associated with attenuation of the light-induced phase resetting of the mouse circadian clock (Challet *et al.* 1999).

Addition of glucose solution to the culture medium can induce circadian rhythm in rat-1 fibroblast cell culture, followed by transient down-regulation of *per1* and *per2* expression (Hirota *et al.* 2002). Furthermore, continuous glucose infusion is able to affect the expression of *per2* in the SCN and to shift phase of *per2* expression in the rat liver (Iwanaga *et al.* 2005). It was previously observed, that diabetic animals exhibit altered daily profile of clock gene expression in the heart, liver and kidney (Young *et al.* 2002, Oishi *et al.* 2004, Herichová *et al.* 2005).

Glucose plasma levels in healthy individuals exhibit a clear-cut circadian rhythm with peak values occurring immediately before the onset of the active phase (La Fleur *et al.* 1999). Daily changes in glucose tolerance were also observed. The percentage of glucose removed from plasma per minute is higher at the beginning of the active phase of the 24-hour cycle compared to the values of glucose uptake achieved at the end of the active period. During the active period, glucose tolerance is gradually declining (Lee *et al.* 1992, La Fleur *et al.* 2001). These endogenous daily rhythms are driven and maintained by the SCN that control glucose plasma levels *via* innervations of the paraventricular nucleus, arcuate nucleus and the autonomic nervous system (La Fleur 2003, Kalsbeek *et al.* 2004, Cailotto *et al.* 2008, Froy 2010).

Glucose uptake by tissues differs between peripheral oscillators. Glucose transport in neural tissues is mediated by GLUT1 and GLUT3 glucose transporters independently of insulin levels. This implies unaffected glucose accessibility in the brain in insulin-dependent diabetes, characterized with insulin deficiency. Other tissues e.g. muscle and liver, are dependent on insulin-mediated glucose transport, mainly provided by insulin-regulated GLUT4 glucose transporter (Ebeling *et al.* 1998, Duelli and Kuschinsky 2001, Arble and Sandoval 2013).

Because of these substantial changes in glucose plasma levels and metabolism during diabetes, we focused on the expression of glucose transporters. The aim of present study was to ascertain daily profile of *glut1* and *glut4* expression in peripheral oscillators inside

(cerebellum, frontal cortex) and outside (heart) of the blood-brain barrier in control rats and rats with streptozotocin-induced diabetes and to consider involvement of *glut1* and *glut4* in diabetes-dependent changes in circadian system.

Methods

Male Wistar rats (n=52) were at the age of 16-weeks obtained from the Dobra Voda breeding facility (Slovak Republic). Animals (275-300 g body weight) were housed under controlled conditions (22±2 °C) and for two weeks synchronized to a 12:12 light:dark (LD) cycle, with lights on from 23:00 h (Zeitgeber time (ZT) 0) to 11:00 h (ZT 12). The Zeitgeber time is a relative measure of time reflecting the LD regimen in the animal facility, where ZT 0 is defined as the beginning of the light phase of the 24-h cycle and the dark phase starts at ZT 12. Food and water were provided *ad libitum*. After the acclimatization period, control and experimental groups of animals were established.

The experimental group received a single intraperitoneal injection of streptozotocin (STZ) (65 mg/kg body weight, Sigma, USA) dissolved in 0.1 M citrate buffer (pH 4.5) injected in a volume of less than 1 ml per rat (Rerup 1970). Animals in control group were injected with a vehicle (0.1 M citrate buffer with pH 4.5). Development of diabetes was monitored using PHAN® diagnostic test strips for urine analysis (Pliva Lachema, Czech Republic).

Samples were taken on day 17–18 after STZ injection throughout the 24-h cycle (3 to 6 animals per group and time point) at ZT 14, ZT 17, ZT 20, ZT 23, ZT 4 and ZT 8. Rats were anaesthetized by carbon dioxide and subsequently decapitated (3-6 animals per group and time point). Blood was collected into heparinized tubes, and plasma was stored at −20 °C until analysis. Red light with low intensity was used when collecting blood samples during the dark period. Samples of the heart were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The frontal cortex and cerebellum were dissected and frozen on dry ice and subsequently stored at −80 °C until RNA extraction.

The experimental protocol was approved by the Ethics Committee for the Care and Use of Laboratory Animals at Comenius University Bratislava. All experiments were performed in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes.

Plasma glucose levels were measured using a commercial kit (Glucosa Liquid 1000, Lachema, Czech Republic). Total RNA was isolated from tissue samples using the single-step method by acid guanidinium thiocyanate-phenolchloroform extraction (Chomczynski and Sacchi 1987) with the use of Tri reagent® (MRC, USA). First-strand cDNA synthesis was performed in RNA samples isolated from the heart and cerebellum using the ImProm-II™ Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. First-strand cDNA synthesis in RNA samples isolated from the frontal cortex was carried out using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA).

Aliquots of the cDNA (0.75-1 µl of RT product) were analyzed for gene expression with the appropriate primers in 20-µl real-time PCR reactions. The quantification of cDNA was performed by real-time PCR using a Step-One System (Applied Biosystems, USA).

Real-time PCR quantification of *glut1* and *glut4* expression was evaluated using a TaqMan® Fast Advanced Master Mix and TaqMan® Gene Expression Assays Rn01417099_m1 (*glut4*), 4331182 TaqMan® Gene Expression Assays Rn01752377_m1 (*glut1*) according to the manufacturer's instructions (Applied Biosystems, USA). Real-time PCR conditions were: activation of AmpliTaq® Fast DNA Polymerase (95 °C, 20 s), followed by 50 cycles of denaturation (95 °C, 1 s) and annealing (60 °C, 20 s). The fluorescent dye ROX served as an internal reference for normalization of the FAM fluorescent signal. Gene expression was normalized to the expression of *rplp1 (ribosomal protein, large, P1)*.

Daily profiles of gene expression in relative values were fitted to a cosinor curve with a 24-h period and when experimental data significantly matched the cosinor curve, its acrophases (peak time referenced to the time of lights on in the animal facility) were calculated with 95 % confidence limits. Goodness of fit (R value – correlation coefficient) of the approximated curve was calculated by ANOVA (Nelson 1979, Klemfuss and Clopton 1993). An unpaired Student t-test was used to compare variables between groups. The data on the graphs are presented as arithmetic mean and standard error of the mean (SEM).

Results

The plasma glucose levels in diabetic rats (32.50±2.03 mmol/l) were significantly higher than in

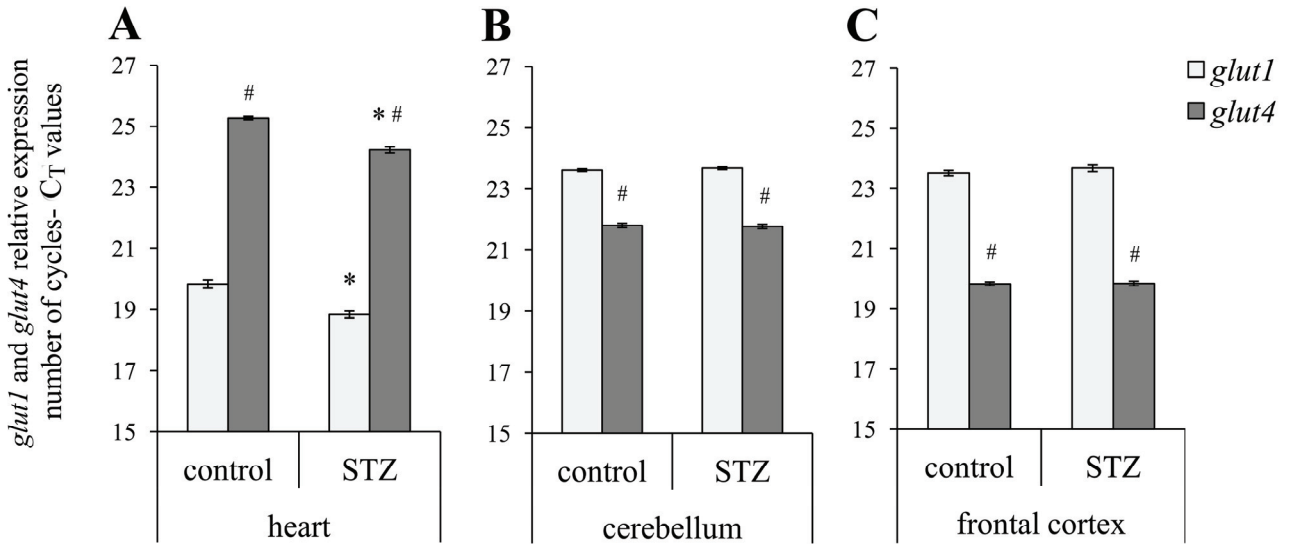

Fig. 1. Relative expression of *glut1* (light columns) and *glut4* (dark columns) in the heart (**A**), cerebellum (**B**) and frontal cortex (**C**) of control and diabetic rats (STZ). Relative expression is presented as threshold cycle values deducted from the total number of PCR cycles. C_T represents the threshold cycle: the cycle number of PCR at which the fluorescence reaches the threshold in the amplification plot. Number of PCR cycles was 50. Values are presented as mean ± SEM (n=19-25). Unpaired t-test: * significant difference between control and diabetic group (P<0.05), # significant difference between *glut1* and *glut4* expression (P<0.001)

Table 1. Cosinor analysis of gene expression in the heart, frontal cortex and cerebellum of control and diabetic rats.

Tissue	Gene	Control			STZ			Significant difference in rhythm between groups
		Acrophase ± SEM [hh:mm]	R	P<	Acrophase ± SEM [hh:mm]	R	P<	
Heart	*glut1*		0.19	ns		0.17	ns	down-regulation (t-test, P<0.0001)
	glut4		0.43	ns		0.22	ns	down-regulation (t-test, P<0.0001)
Frontal cortex	*glut1*		0.25	ns		0.24	ns	
	glut4		0.09	ns		0.07	ns	
Cerebellum	*glut1*	14:43±0:57	0.65	0.01	11:17±1:07	0.56	0.05	
	glut4	13:52±1:03	0.60	0.01	12:49±0:53	0.68	0.001	

Results of cosinor analysis describe the best-fitting cosinor curve significantly reflecting experimental data (P<0.05, ANOVA). Acrophase represents time at which the fitted cosinor curve reached its maximum value in relation to the beginning of the light phase. Time is given as a Zeitgeber time (ZT, ZT 0 is related to beginning of the light phase). control – control rats, STZ – streptozotocin-treated rats, SEM – standard error expressed in hours and minutes, P – level of significance, ns – not significant, R – goodness of fit, down-regulation – down-regulated expression (t-test)

control animals (10.35±0.34 mmol/l) on day 17 after STZ administration. The body weight of control animals (341.49±4.04 g) was significantly higher compared to that in diabetic animals (282.41±5.51 g) 15 days after STZ administration.

We examined the expression of *glut1* and *glut4* glucose transporters. The ratio of *glut1* mRNA over *glut4* mRNA was significantly higher in brain tissues (cerebellum and frontal cortex) than in the peripheral organs. *Glut4* was more expressed in the heart compared to *glut1* (Fig. 1).

Both *glut1* and *glut4* transporters showed an arrhythmic expression profile in the heart and frontal cortex of control and STZ-induced animals (Table 1,

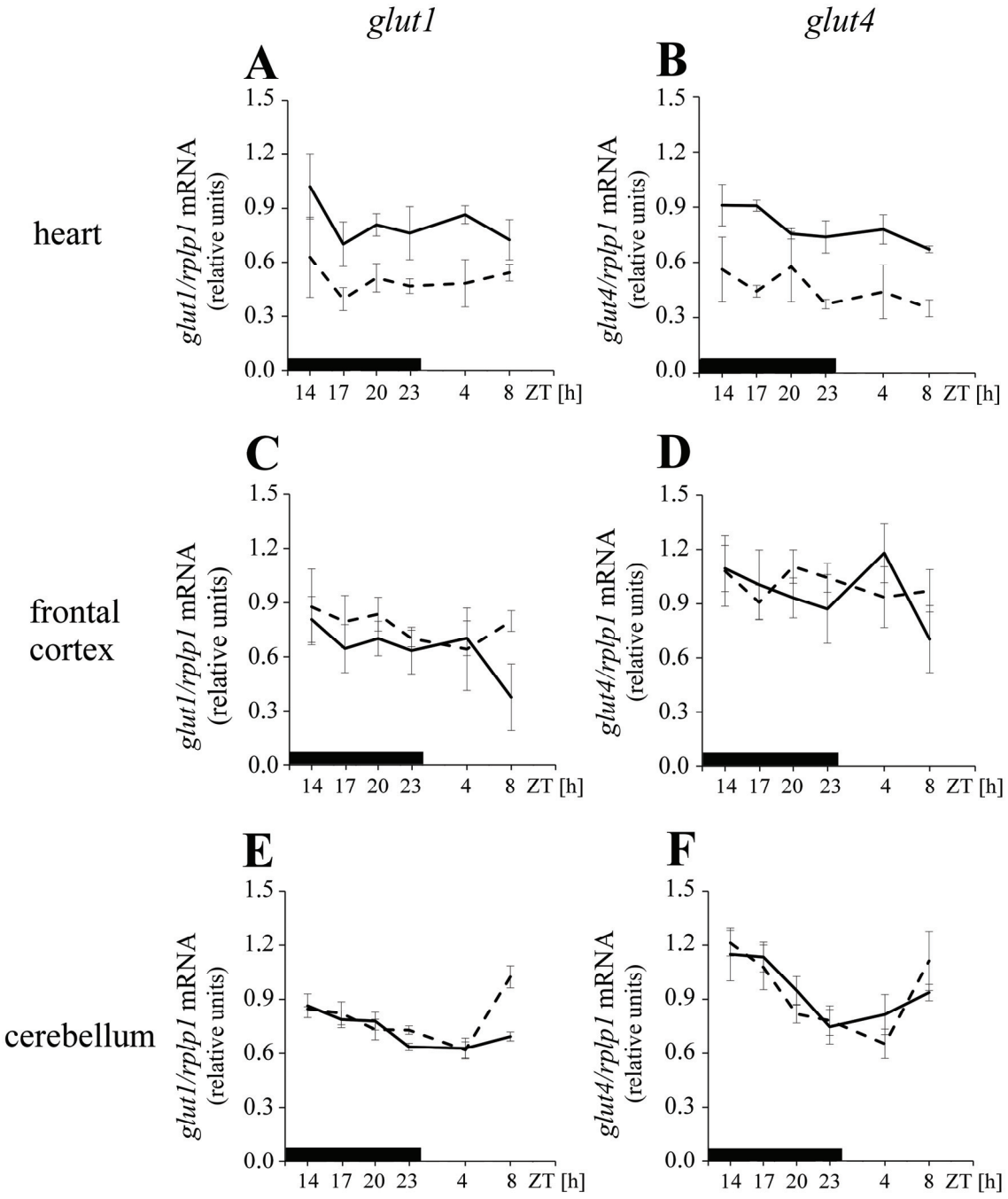

Fig. 2. Daily profile of *glut1* (**A, C, E**) and *glut4* (**B, D, F**) expression in the heart (**A, B**), frontal cortex (**C, D**) and cerebellum (**E, F**) of control rats (continuous line) and rats with streptozotocin-induced diabetes (dashed line). Glucose transporter expression is normalized to expression of housekeeping gene *rplp1*. Animals were synchronized to 12:12 LD cycle. Time is expressed as a Zeitgeber time (ZT) with beginning of the light phase at ZT 0. Black bar at the bottom of the graph represents the dark period. Values are presented as mean ± SEM (n=3-6).

Fig. 2). Expression of *glut1* and *glut4* was rhythmic only in the cerebellum of control and STZ-induced rats. Maximal levels of *glut1* and *glut4* expression were achieved at the beginning of the dark phase of the 24-h cycle (ZT 14:43, cosinor, P<0.01; ZT 13:52, cosinor, P<0.01, respectively) in the cerebellum of control rats (Table 1, Fig. 2E, 2F). STZ-induced diabetes caused trend to phase advance in cerebellar *glut1* expression (Table 1, Fig. 2E).

We observed significant down-regulation of the expression of *glut1* and *glut4* glucose transporters in the heart of STZ-induced diabetic rats (t-test, P<0.0001) (Table 1, Fig. 2A, 2B). STZ treatment did not reduce the mean values of *glut1* and *glut4* expression in the frontal cortex and cerebellum (t-test, Fig. 2).

Discussion

Our study showed different effect of STZ-induced diabetes on expression of *glut1* and *glut4* glucose transporters in brain tissues protected by blood-brain barrier compared to periphery. Glucose transporter expression in the heart of STZ-diabetic rats exhibits down-regulation, however this effect was not observed in *glut1* and *glut4* expression in the frontal cortex and cerebellum. Among examined tissues, only cerebellum showed rhythmic daily profile of *glut1* and *glut4* expression.

These results partially agree with previous data showing the impact of diabetes on brain tissues. Elevated plasma glucose concentration in diabetic animals is linked with a rise in cerebral extracellular glucose levels (Silver and Erecińska 1994, Jacob *et al.* 2002, Puchowicz *et al.* 2004). In our study expression of *glut1* and *glut4* transporters in the frontal cortex of STZ-treated rats did not differ from that in control rats. Similarly, in previous studies increased glucose levels did not influence the expression of GLUT1 and GLUT4 proteins in the cerebral cortex of STZ-diabetic rats as well as in the brain of hyperglycemic *db/db* mice (Vannucci *et al.* 1997, Vannucci *et al.* 1998, Badr *et al.* 2000, Tang *et al.* 2000). On the other hand, in spontaneously hyperglycemic non-obese diabetic mice the blood-brain-barrier glucose permeability and GLUT1 transporter velocity was reduced (Cornford *et al.* 1995). Altered expression of *glut1* mRNA and GLUT1 proteins in the brain in diabetic animals was reported in some cases, but the extent of changes varied and no measurements were done specifically in the frontal cortex. It was observed, that the expression of *glut1* mRNA in the brain of *db/db* mice was regionally increased compared to control animals (Vannucci *et al.* 1997). In brain capillary of rats with STZ-induced diabetes, the level of *glut1* mRNA was increased in comparison to control group (Lutz and Pardridge 1993). The expression of both *glut1* mRNA and GLUT1 protein was markedly reduced in the whole brains of rats with STZ-induced diabetes compared to expression levels in control group (Hou *et al.* 2007). The local densities of glucose transporters GLUT1 in the brain of rats with STZ-induced diabetes were lowered in 12 out of 28 investigated brain structures; the GLUT1 densities in remaining examined structures were unchanged (Duelli *et al.* 2000). However, the measured local cerebral glucose utilization in frontal cortex of STZ-treated rats showed a significant increase (Duelli *et al.* 2000).

Differences in diabetic brain glucose transporter expression can be partially explained by the different duration and model of diabetes. Other factor contributing to diversity of measured glucose transporter expression values is the dissimilar sampling time.

The expression of GLUT4 protein was down-regulated in STZ-diabetic rat cerebellum (Vannucci *et al.* 1998). This is consistent with the trend for lower *glut4* mRNA expression values in the cerebellum of STZ-diabetic rats observed at certain time points of the 24-h cycle in our study.

Down-regulation of *glut1* and *glut4* expression outside the blood-brain barrier in the heart of STZ-treated rats in our study correlates with similar effect observed in peripheral tissues in previous studies. Strongly diminished GLUT4 protein expression was found in the heart of rats with STZ- induced diabetes (Camps *et al.* 1992, Hoenack and Roesen 1996). Down-regulated *glut1* and *glut4* expression was observed in the heart of rats with acute (7 days) as well as chronic (6 months) STZ-induced diabetes (Depre *et al.* 2000). Reduced *glut1* and *glut4* expression was detected in the heart, kidney and lung of STZ-diabetic rats; *glut1* was down-regulated also in the liver and pancreas of diabetic rats (Jurysta *et al.* 2013). In other work, STZ-induced diabetes caused increased *glut1* and *glut4* expression in the kidney and heart, increased *glut1* expression in muscle and *glut4* expression in the liver. The different character of changes of *glut1* and *glut4* expression when compared to our results and results of other authors might be explained as a consequence of overnight fasting before the tissue collection (Sokolovska *et al.* 2011). However, unlike our results, aforementioned authors did not examine the 24-h expression profile of glucose transporter expression.

It is likely that increased glucose and fatty acid levels during diabetes act directly on circadian oscillator functioning in the peripheral tissues (Hirota *et al.* 2002, Kohsaka *et al.* 2007). The expression levels of *glut1* and *glut4* are regulated by insulin in the periphery. This effect is mediated by AMP-activated protein kinase (Barnes *et al.* 2002, Karnieli and Armoni 2008, McGee *et al.* 2008). Furthermore, diabetes changes the daily rhythm of locomotor activity and synchronization capacity regulated by central oscillator (Shimazoe *et al.* 2000, Ramadan *et al.* 2006). Except for the direct effect of glucose on the molecular oscillator, glucose can affect function of brain oscillators *via* glucose sensing brain regions (hypothalamus, brain stem, nucleus of the solitary tract (Mizuno and Oomura 1984, Silver and Erecińska 1998,

Routh 2010, Thorens 2011). We demonstrated that *glut1* and *glut4* expression in cerebellum, frontal cortex and the heart strongly differ. Their role in regulation of peripheral oscillators requires further research.

To conclude, STZ-induced diabetes exhibits different effects in tissues located inside and outside the blood-brain barrier. In peripheral tissues such as the heart, the expression of *glut1* and *glut4* was down-regulated. In tissues protected by blood-brain barrier, STZ-induced diabetes does not cause decrease in mean levels of *glut1* and *glut4* transcription. We observed a daily rhythm in *glut1* and *glut4* expression in the cerebellum and diabetes caused trend to phase advance in *glut1* expression in this tissue. Expression of *glut1* and *glut4* in frontal cortex did not show a daily pattern and their expression was unaffected by diabetes. The reason why cerebellum shows a daily pattern of *glut* transporters expression, while *glut1* and *glut4* exerts tonic expression in frontal cortex, needs to be elucidated.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This study was supported by grant VEGA 1/1262/12.

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