

## RAPID REPORT

## Brain glycogen decreases during prolonged exercise

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**Non-technical summary** Energy sources for the brain include not only blood glucose, but also astrocytic glycogen, especially when the blood-borne glucose supply is short (e.g. hypoglycaemia). Although untested, it is hypothesized that during prolonged exercise that induces hypoglycaemia, the resultant hypoglycaemia may cause a decrease in brain glycogen. Here, we tested this hypothesis and provide evidence that brain glycogen decreases during prolonged exercise with hypoglycaemia. Furthermore, in the cortex, we show that the decrease in brain glycogen levels during prolonged exercise is associated with activation of monoamine metabolism, which could be a factor inducing central fatigue. Since the discovery of muscle glycogen depletion as a candidate of peripheral fatigue during prolonged exercise, this is the first study to our knowledge to show that brain glycogen can decrease with prolonged exercise. These findings may provide a clue towards understanding the mechanisms related to central fatigue.

**Abstract** Brain glycogen could be a critical energy source for brain activity when the glucose supply from the blood is inadequate (hypoglycaemia). Although untested, it is hypothesized that during prolonged exhaustive exercise that induces hypoglycaemia and muscular glycogen depletion, the resultant hypoglycaemia may cause a decrease in brain glycogen. Here, we tested this hypothesis and also investigated the possible involvement of brain monoamines with the reduced levels of brain glycogen. For this purpose, we exercised male Wistar rats on a treadmill for different durations (30–120 min) at moderate intensity (20 m min<sup>-1</sup>) and measured their brain glycogen levels using high-power microwave irradiation (10 kW). At the end of 30 and 60 min of running, the brain glycogen levels remained unchanged from resting levels, but liver and muscle glycogen decreased. After 120 min of running, the glycogen levels decreased significantly by ~37–60% in five discrete brain loci (the cerebellum 60%, cortex 48%, hippocampus 43%, brainstem 37% and hypothalamus 34%) compared to those of the sedentary control. The brain glycogen levels in all five regions after running were positively correlated with the respective blood and brain glucose levels. Further, in the cortex, the levels of methoxyhydroxyphenylglycol (MHPG) and 5-hydroxyindoleacetic acid (5-HIAA), potential involved in degradation of the brain glycogen, increased during prolonged exercise and negatively correlated with the glycogen levels. These results support the hypothesis that brain glycogen could decrease with prolonged exhaustive exercise. Increased monoamines together with hypoglycaemia should be associated with the development of decreased brain glycogen, suggesting a new clue towards the understanding of central fatigue during prolonged exercise.

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**Abbreviations** 5-HIAA, 5-hydroxyindoleacetic acid; HPLC, high-performance liquid chromatography; 5-HT, 5-hydroxytryptamine; MI, microwave irradiation; MHPG, methoxyhydroxyphenylglycol; NA, noradrenaline; PCA, perchloric acid.

## Introduction

Glycogen could be an important energy source for the brain. In the brain, the glucose storage molecule glycogen is located entirely in astrocytes (Wender *et al.* 2000). Astrocytic glycogen is degraded to provide neuroprotection primarily under conditions that induce glucose deprivation such as hypoglycaemia (Herzog *et al.* 2008). Furthermore, even under normal physiological conditions, astrocytic glycogen is degraded to support axonal function in response to sudden increases in energy demand during neurotransmission (Swanson *et al.* 1992). Astrocytic glycogen is broken down into lactate, as fuel for activated neurons, via increased noradrenaline (NA), histamine, 5-hydroxytryptamine (5-HT) and vasoactive intestinal peptide (Benington & Heller, 1995; Brown, 2004).

Physical exercise impacts nearly every system of the body, including muscles and the brain (Secher *et al.* 2008); thus, the energy supply available from immediate reserve sources for these organs plays a crucial role in maintaining their increased functions. During exercise, muscle glycogen decreases in an activity-dependent manner (exercise intensity and/or duration), and in turn supplies energy for muscle activity (Gollnick *et al.* 1974). Furthermore, during prolonged exhaustive exercise, rats become hypoglycaemic with marked depletion in muscular and liver glycogen at exhaustion (Winder *et al.* 1987). Although the brain increases glucose utilization as an energy source during exercise (Vissing *et al.* 1996), it remains unknown whether glycogen is used in the brain as an energy source during exercise.

Prolonged exercise increases monoamine (i.e. NA and 5-HT) levels in the brain (Newsholme *et al.* 1992; Pagliari & Peyrin, 1995). Moreover, brain glycogen breakdown occurs during exercise via a  $\beta$ -adrenergic receptor-mediated mechanism as shown by measuring the concentration differences in arterial–jugular venous glucose, lactate and oxygen, and their ratios (Ide *et al.* 2000; Larsen *et al.* 2008). Based on these findings, it has been hypothesized that exercise induces depletion of brain glycogen through hypoglycaemia and activation of metabolism of monoamines in the brain (Nybo & Secher, 2004).

This hypothesis, however, remains to be tested because of technical difficulties in determining levels of brain glycogen during exercise with accuracy and precision. Indeed, brain glycogen is rapidly depleted by the activation of glycogenolysis enzymes under hypoxic-ischaemic conditions that occur after the animals are killed. Postmortem instability of glycogen in the brain prevents traditional biochemical methods of measurement (Kong *et al.* 2002). Therefore, we first established an accurate method for determining brain glycogen levels using high-power (10 kW), focused microwave irradiation

(MI), which momentarily inactivates glycogenolytic and glycosynthetic enzymes. Furthermore, we designed experiments to examine the effects of exercise on glycogen levels in different brain loci along with checking the involvement of blood glucose and monoamine levels using MI, which allowed us to test the hypothesis that exercise induces brain glycogen depletion through increased monoamines and under hypoglycaemic conditions. Our results have shown, for the first time, that exercise results in a decrease in brain glycogen due to hypoglycaemia and to an increase of NA and 5-HT metabolism in the cortex.

## Methods

The present study consisted of four major steps. In Pilot Study 1, we examined brain glycogen levels with and without anaesthesia, in order to determine valid conditions for analysing brain glycogen levels. In Pilot Study 2, we also checked the validity of brain glycogen determination, examining insulin-induced hypoglycaemia on cerebellum glycogen levels (Herzog *et al.* 2008). Then, the effects of prolonged exercise on glycogen levels in nine discrete brain loci (third step) and the effects of various durations of exercise on glycogen levels in five brain loci (fourth step) were examined, respectively. Details of Pilot Studies 1 and 2 are presented in the online Supplemental material.

## Materials

All chemicals, including amyloglucosidase, hexokinase, NADP<sup>+</sup>-dependent glucose-6-phosphate dehydrogenase, NADP<sup>+</sup>, ATP, EDTA, MgSO<sub>4</sub>, glucose, glucose-6-phosphate, KOH, imidazole, perchloric acid and Tris-HCl, were purchased from Sigma (St Louis, MO, USA) and Nacalai tesque (Kyoto, Japan).

Adult male Wistar rats (250–270 g; SLC Inc., Shizuoka, Japan) were housed and cared for in an animal facility, fed a standard pellet diet (MF, Oriental Yeast Co., Ltd, Ibaraki, Japan), and given water *ad libitum*. The room temperature was maintained between 22 and 24°C under a 12 h light:12 h dark cycle (lights on: 07:00–19:00). All experimental protocols were conducted in accordance with University of Tsukuba Animal Experiment Committee guidelines. Our experiments comply with the policies and regulations of *The Journal of Physiology* (Drummond, 2009).

## Habituation to treadmill running

After a 1 week acclimatization period, rats were habituated to run on a treadmill (SN-460, Shinano, Tokyo, Japan) for a total of five sessions over 6 days. The running duration

was 30 min day<sup>-1</sup>, and the running speed was gradually increased from 5 to 25 m min<sup>-1</sup> (Nishijima & Soya, 2006).

### Surgery

Surgery was performed according to methods described by Soya *et al.* (2007a). After habituation to treadmill running, the rats were anaesthetized with sodium pentobarbital (50 mg kg<sup>-1</sup>, i.p.), and a silicone catheter was inserted into the jugular vein and fixed with a silk thread (32 mm). The external distal end of the catheter was fixed at the animal's nape.

### Prolonged exhaustive exercise

Two days after the surgery, rats fasted before exercise for 2 h to obtain stable glycogen levels in the brain, and exercised by treadmill running at 20 m min<sup>-1</sup> for 120 min ( $n = 5-11$  rats). A sedentary group of animals ( $n = 11$  rats) was placed on a stationary treadmill for 120 min. Fatigue during prolonged exercise of an intensity between 65 and 85% maximum oxygen uptake ( $\dot{V}_{O_{2,max}}$ ) is associated with glycogen depletion in active skeletal muscles (Gollnick *et al.* 1974). Brooks & White (1978) and Soya *et al.* (2007a) have reported that running at 20 m min<sup>-1</sup> on the treadmill is equivalent to 50–70%  $\dot{V}_{O_{2,max}}$  for rats. Thus, prolonged exercise at moderate intensity in this study should be sufficient to induce depletion of glycogen in active muscles, warranting its validity as exhaustive exercise.

### Exercise of various time durations

Two days after the habituation period, rats fasted for 3 h, and ran on a treadmill at 20 m min<sup>-1</sup> for 0 (pre-exercise), 30, 60 or 120 min ( $n = 5-6$  rats per group).

### Tissue preparation

Immediately after exercise or rest, rats were anaesthetized with isoflurane (a mixture of 30% vol/vol isoflurane in propylene glycol; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) in a bell jar and killed using focused microwave irradiation (MI) (NJE-2603, New Japan Radio Co., Ltd., Tokyo, Japan; 10 kW, 1.2 s). Following the MI, nine brain loci (cortex, septum, striatum, hippocampus, thalamus, hypothalamus, mid-brain, cerebellum and brainstem) were collected using a method modified from Hirano *et al.* (2006). Two skeletal muscles (soleus and plantaris), the liver and blood samples were also collected. All specimens were stored at  $-80^{\circ}\text{C}$  for subsequent biochemical analysis.

### Blood glucose and lactate assays

Blood lactate and glucose were measured using an automated glucose–lactate analyser (2300 Stat Plus, Yellow Springs Instruments, USA).

### Brain glucose and glycogen assays

Glucose and glycogen measurements were taken according to methods described by Kong *et al.* (2002). Tissues were homogenized (Polytron, Kinematica, Kriens-Luzern, Switzerland; setting 6; 60 s) in ice-cold 6% perchloric acid (PCA) containing 1 mM EDTA. For tissue glycogen content measurements, glycogen was hydrolysed to glucose in 100  $\mu\text{l}$  aliquots of homogenate that were removed and incubated for 1 h at 37°C with 1 ml of 0.2 M sodium acetate, 20  $\mu\text{l}$  of 1.0 M KHCO<sub>3</sub> and 20 U ml<sup>-1</sup> of amyloglucosidase. The addition of 0.5 ml PCA solution stopped the reaction. After centrifugation (14,000 g for 10 min at 4°C), supernatants were neutralized with a KOH solution consisting of (in M): 3 KOH, 0.3 imidazole and 0.4 KCl. The supernatants were then centrifuged (16,000 g for 10 min at 4°C) and assayed for glucose content. To measure endogenous (background) glucose levels, non-hydrolysed samples were obtained by centrifuging the homogenates (14,000 g for 10 min at 4°C) and adjusting the pH of the supernatants to a final level of 6–8 with KOH solution. Neutralized samples were mixed thoroughly, centrifuged (16,000 g for 10 min at 4°C), and assayed for endogenous glucose levels. The glucose content assay was performed in 96-well plates using a coupled enzyme assay method modified from a previous study (Passonneau & Lauderdales, 1974). A total of 200  $\mu\text{l}$  of a reaction solution containing 50 mM Tris-HCl (pH 8.1), 0.5 mM ATP, 0.5 mM NADP<sup>+</sup>, 5 mM MgSO<sub>4</sub> and 0.1 U ml<sup>-1</sup> glucose-6-phosphate dehydrogenase was added to each well. The plate was then placed in a fluorescence plate reader (Arvo, Perkin Elmer, Groningen, the Netherlands) and shaken, and measurements of the resultant NADPH were taken at 350 nm excitation and 450 nm emission. After the addition of hexokinase (0.3 U) to each well, plates were shaken, and measurements were taken after a 30 min incubation period. Tissue glycogen levels, indicated as glucose units, were calculated by subtracting the final micromolar concentration of glucose per gram of wet weight of the non-hydrolysed tissue sample from the micromolar concentration of glucose per gram of wet weight of the hydrolysed tissue sample. Our samples showed that the averaged hypothalamic glucose levels of sedentary rats were 2.8  $\mu\text{mol g}^{-1}$  (baseline levels), which correspond well with previous findings from microdialysis studies of extracellular fluid in the ventromedial nucleus of the hypothalamus (de Vries *et al.* 2003). It was the same in the hypoglycaemic levels induced by insulin (Herzog *et al.* 2008). As for the brain glycogen levels

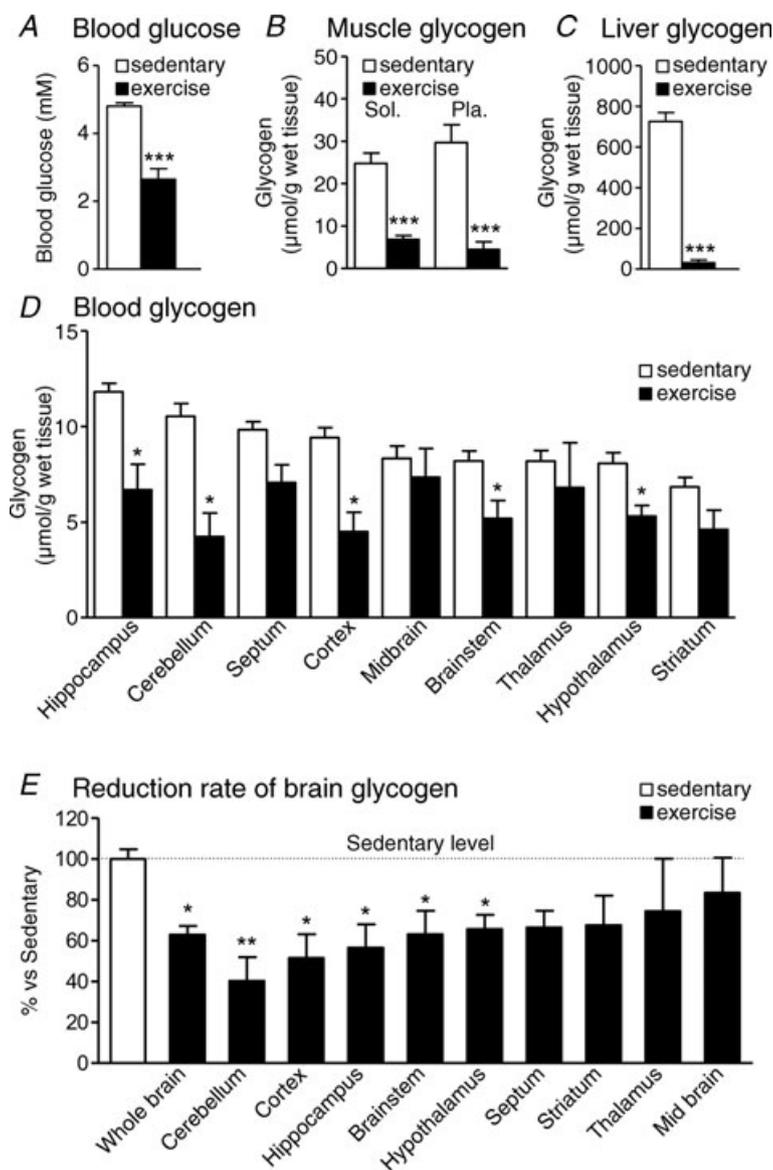
indicating methodological validity, sedentary control levels of glycogen in the hippocampus and cerebellum were approximately  $10 \mu\text{mol (g tissue)}^{-1}$  (Fig. 1D), which is consistent with previous findings using high-power MI (9.5 kW, 1.7 s; 10 kW, 1.2 s) (Kong *et al.* 2002; Herzog *et al.* 2008).

### Brain lactate assays

Lactate measurements were taken according to the method described by Passonneau & Lauderdale (1974). Our average hypothalamic lactate levels of sedentary animals were  $1.1 \mu\text{mol g}^{-1}$ , which correspond well with previous findings from microdialysis studies of extracellular fluid in the hypothalamus (Wang *et al.* 2002), indicating methodological validity.

### Monoamine assays

Measurements of monoamines NA, 5-HT and dopamine (DA), and their metabolites MHPG, 5-HIAA and DOPAC, respectively, were taken using high-performance liquid chromatography (HPLC) with electrochemical detection according to methods described by Takeda *et al.* (1990). To detect the brain monoamines more quickly, easily, and selectively, we used this system with a series of three-coulometric working electrodes. In addition, the detection mode consisted of the combination of REDOX and SCREEN. Briefly, 0.5 ml of a 0.05 M PCA solution containing 2.0 mM sodium bisulfate and 0.02 mM EDTA.2Na was added to the samples of cortical tissue. After homogenization followed by centrifugation at 12,000 g for 5 min, the supernatants were used to determine the levels of each of the above molecules



**Figure 1. Blood glucose and glycogen levels in muscles, the liver and several brain loci immediately after treadmill running exercise or sedentary behaviour for 120 min**

A, blood glucose levels; B, muscle glycogen levels; C, liver glycogen levels; D, glycogen levels in 11 brain loci; and E, reduction rate of brain glycogen. Data represent the mean  $\pm$  SEM ( $n = 5-11$  rats). \* $P < 0.05$ ; \*\*\* $P < 0.0001$  compared to sedentary rats (unpaired  $t$  test).

after filtration through a 0.45  $\mu\text{m}$  filter (Millipore, MA, USA). An LC/ECD (electron capture dissociation) system equipped with a guard cell (Model 5020, ESA, USA) and a high sensitive analytical cell (Model 5011, ESA, USA) connected with an electrochemical detector (Model 5100A, ESA, USA) was used for all measurements. The detector conditions were as follows: Guard cell potential,  $E$ , 450 mV; Analytical cell potentials,  $E_1$ , -50 mV and  $E_2$ , 450 mV; and Sensitivity, 2  $\mu\text{A}$ . Separation was carried out using a 5  $\mu\text{m}$  MCM C18 column (4.6  $\phi \times 150$  mm; MC Medical Inc., Japan) column at 20°C under isocratic conditions with a mobile phase of acetonitrile, methanol and 0.1 M phosphate buffer adjusted to pH 3.0 with phosphoric acid containing 0.05 M citrate, 4.0 mM sodium 1-heptasulfonate and 0.1 mM EDTA.2Na. The flow rate and injection volume were set to 1.0 ml min<sup>-1</sup> and 50  $\mu\text{l}$ , respectively. A 20  $\mu\text{l}$  aliquot of 1000 ng ml<sup>-1</sup> isoproterenol hydrochloride was added to each sample as an internal standard, and 30  $\mu\text{l}$  of the same solution was used to wash the injection syringe. The concentrations of monoamines and their metabolites in cortical samples were determined based on their chromatographic peak heights relative to that of the internal standard (isoproterenol) using appropriate calibration curves in the Chromeleon software (Dionex, CA, USA).

### Statistics

Data are expressed as mean  $\pm$  standard error (SEM) and were analysed using Prism 5 (MDF Co., Ltd., Tokyo, Japan). Comparisons of each exercise group *versus* the sedentary group were performed using Student's *t* test for unpaired data. Group comparisons were performed using a one-way ANOVA with Dunnett's or Tukey's *post hoc* tests. Simple correlations were calculated using Pearson's product-moment correlations. Statistical significance was assumed at *P* values < 0.05.

## Results

### Brain glycogen levels after exercise

The blood glucose level was 45% lower in the exercise group as compared to the sedentary group ( $F = 16.65$ ;  $P < 0.0001$ ; Fig. 1A). The glycogen levels in the liver and skeletal muscles (slow type, m. soleus; fast type, m. plantaris) were found to be depleted by exercise (Fig. 1B and C). Moreover, glycogen levels decreased significantly by ~37–60% with exercise in five discrete brains loci: the cerebellum 60%, cortex 48%, hippocampus 43%, brainstem 37%, and hypothalamus 34% ( $P < 0.05$ ; Fig. 1D and E). Conversely, glycogen levels in the septum, mid-brain, thalamus and striatum were unchanged by exercise (Fig. 1D and E).

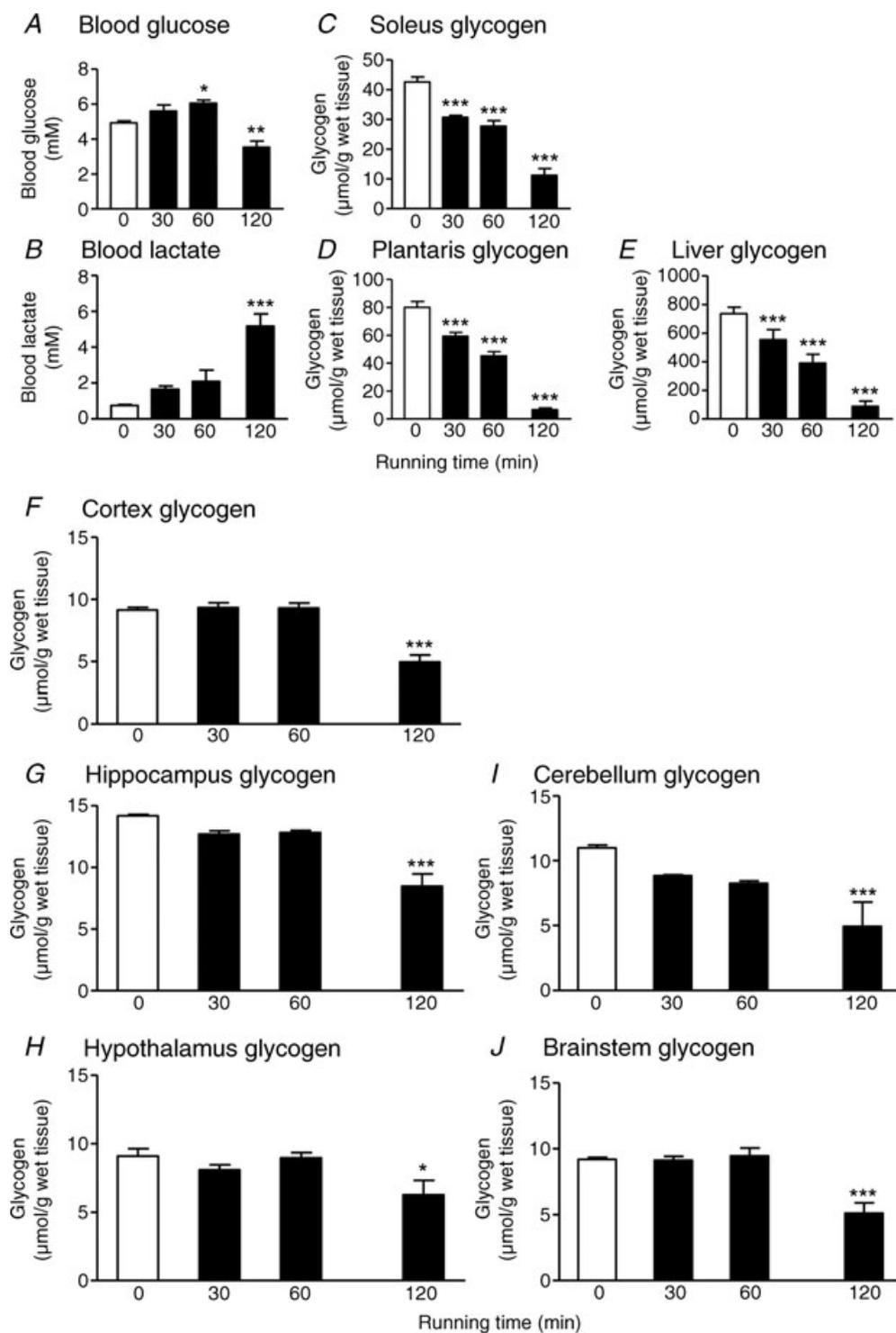
### Spatio-temporal changes in brain glycogen levels during exercise

At the end of 30 and 60 min of running, blood glucose and lactate levels were unchanged compared to pre-exercise levels. At the end of 120 min of running, however, blood glucose was 46% lower, and blood lactate was five-fold higher than before exercise and under sedentary conditions at 120 min ( $P < 0.01$ ; Fig. 2A; data not shown). Similar to blood glucose levels, after 30 and 60 min of running, glycogen levels in the cortex, hippocampus, hypothalamus, cerebellum and brainstem did not significantly decrease from those before exercise and sedentary conditions (data not shown) at 120 min; however, they did decrease after 120 min of exercise ( $P < 0.05$ ; Fig. 2F–J). Glycogen levels in the skeletal muscles and liver were significantly decreased after 30 min of running and further decreased after 60 and 120 min of running (Fig. 2C–E), while those of the skeletal muscles and liver under sedentary conditions (data not shown) at 120 min remained unchanged throughout experiments (Fig. 2C–E).

Glucose levels in the cortex, hippocampus, hypothalamus, cerebellum and brainstem were significantly decreased, and lactate levels in the cortex, hippocampus, hypothalamus, cerebellum and brainstem were significantly increased following 120 min of exercise (Table 1). Furthermore, the decrease in brain glycogen levels was positively correlated with the decrease in blood ( $r = \sim 0.55\text{--}0.70$ ;  $P < 0.05$ ; Fig. 3A) and brain ( $r = \sim 0.59\text{--}0.91$ ;  $P < 0.001$ ; Fig. 3B) glucose levels, and was negatively correlated with the increase in brain lactate levels excluding the hypothalamus ( $r = -0.47\text{--}-0.65$ ;  $P < 0.05$ ; Fig. 3C).

### Cortical monoamine metabolic levels after exercise

Figure 4A–F shows the levels of monoamines and their metabolites in the cortex for different exercise durations. Monoamines NA, 5-HT and DA levels in the cortex increased with exercise but were not significantly different from pre-exercise levels (Figure 4A–C). After 60 min of running, MHPG levels in the cortex were increased from pre-exercise levels ( $P < 0.05$ ; Fig. 4D) and increased further after 120 min of running ( $P < 0.05$ ; Fig. 4D). 5-HIAA levels in the cortex after 60 and 120 min of running were increased from pre-exercise levels ( $P < 0.05$ ; Fig. 4E). 3,4-dihydroxyphenylacetic acid (DOPAC) in the cortex increased with exercise but was not significantly different from pre-exercise levels (Fig. 4F). Finally, the increases in cortical MHPG and 5-HIAA levels were negatively correlated with the decrease in cortical glycogen levels ( $r = \sim -0.66\text{--}0.68$ ;  $P < 0.01$ ; Fig. 4G and H).



**Figure 2. Blood glucose and glycogen levels in muscles, the liver and several brain loci, immediately after treadmill running exercise for 0 (pre-exercise), 30, 60 and 120 min**

A, blood glucose levels; B, blood lactate levels; C, soleus glycogen levels; D, plantaris glycogen levels; E, liver glycogen levels; F, cortex glycogen levels; G, hippocampus glycogen levels; H, hypothalamus glycogen levels; I, cerebellum glycogen levels; and J, brainstem glycogen levels. Data represent the mean  $\pm$  SEM ( $n = 5-6$  rats). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.0001$  compared to pre-exercise (Dunnett's *post hoc* test).

**Table 1. Glucose and lactate levels in five brain loci immediately after treadmill running exercise for 0 (Pre-Exercise), 30, 60 and 120 min ( $\mu\text{mol g}^{-1}$  wet tissue)**

Brain region		0 min	30 min	60 min	120 min
Cortex	Glucose	2.6 $\pm$ 0.1	3.3 $\pm$ 0.1*	3.2 $\pm$ 0.2	1.3 $\pm$ 0.4*
	Lactate	1.1 $\pm$ 0.0	1.9 $\pm$ 0.3*	1.9 $\pm$ 0.3	2.5 $\pm$ 0.2***
Hippocampus	Glucose	2.8 $\pm$ 0.1	3.4 $\pm$ 0.1*	3.5 $\pm$ 0.1*	1.6 $\pm$ 0.3*
	Lactate	1.3 $\pm$ 0.0	2.2 $\pm$ 0.3	2.0 $\pm$ 0.3	2.7 $\pm$ 0.4**
Hypothalamus	Glucose	2.8 $\pm$ 0.1	3.2 $\pm$ 0.1	3.0 $\pm$ 0.1	1.9 $\pm$ 0.3*
	Lactate	1.1 $\pm$ 0.1	1.9 $\pm$ 0.3*	1.8 $\pm$ 0.3	2.4 $\pm$ 0.2***
Cerebellum	Glucose	3.1 $\pm$ 0.1	3.7 $\pm$ 0.2	3.4 $\pm$ 0.1	1.9 $\pm$ 0.4*
	Lactate	0.9 $\pm$ 0.1	1.5 $\pm$ 0.2*	1.5 $\pm$ 0.2	2.0 $\pm$ 0.2**
brainstem	Glucose	2.7 $\pm$ 0.1	3.2 $\pm$ 0.1	3.3 $\pm$ 0.2	1.6 $\pm$ 0.3*
	Lactate	1.1 $\pm$ 0.1	1.9 $\pm$ 0.3*	1.9 $\pm$ 0.3	2.5 $\pm$ 0.2***

Data are mean  $\pm$  SEM for 5–6 rats. \* $P < 0.05$ , \*\*\* $P < 0.0001$  vs. 0 min (Dunnett's *post hoc* test).

## Discussion

This is the first report testing the hypothesis that prolonged exhaustive exercise, which induces hypoglycaemia and muscle glycogen depletion, will result in decreased levels of brain glycogen, the principal energy store in brain, using a high-power MI (10 kW) which nearly immediately fixes brains, rendering glycogen-producing and -metabolizing enzymes inactive. We thus determined levels of brain glycogen, which decreased significantly by approximately 50% in brains of rats that underwent 120 min of running, compared to those of sedentary control rats (Fig. 1D): the cerebellum 60%, cortex 48%, hippocampus 43%, brainstem 37% and hypothalamus 34%. Further, changes in brain glycogen correlated well with changes in blood glucose levels, and negatively correlated with changes in brain lactate levels. These results support the hypothesis that brain glycogen could decrease with prolonged exhaustive exercise that induces hypoglycaemia. Further, in the cortex, MHPG and 5-HIAA levels, which are metabolites of NA and 5-HT, increased during prolonged exercise, and negatively correlated with decreased cortical glycogen, and it is possible that such a decrease in cortical glycogen is also associated with increased metabolism of NA and 5-HT in the cortex. These results suggest that increased metabolism of NA and 5-HT in the cortex, and probably in the whole brain, together with hypoglycaemia, should be associated with the development of decreased brain glycogen and could be a new clue towards our understanding of central fatigue.

An exhaustive run for 120 min decreased blood glucose and brain glycogen levels in the cortex, hippocampus, hypothalamus, cerebellum and brainstem. To examine the possibility that decreased brain glycogen is due to hypoglycaemia, we examined the effects of various durations of exercise on blood glucose and brain glycogen levels. Blood glucose levels remained unchanged after 30 or 60 min of exercise, but decreased after prolonged exercise (Fig. 2A). Similar to blood glucose levels, glycogen levels

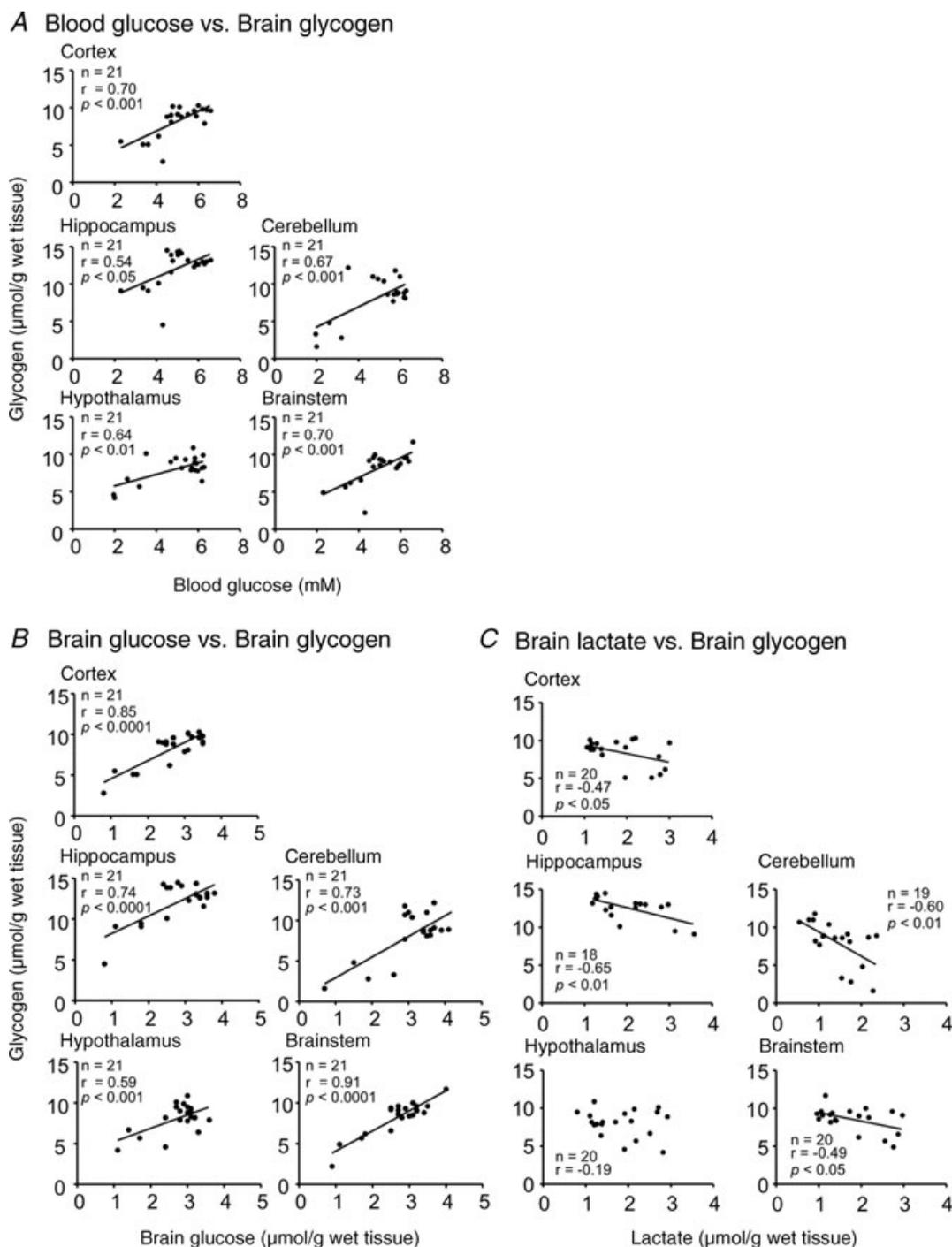
in the cortex, hippocampus, hypothalamus, cerebellum and brainstem decreased only with 120 min of exhaustive exercise. At the same time, both depletion of liver glycogen and hypoglycaemia occurred (Fig. 2C–E), which may in turn result in decreased brain glucose levels ( $\sim 50$ – $67\%$  of sedentary control, Table 1). Indeed, brain glucose levels were correlated with blood glucose levels ( $r = \sim 0.71$ – $0.86$ ; data not shown). Also brain glycogen levels during exercise were positively correlated with blood and brain glucose levels (Fig. 3A and B). These results suggest that the decrease in brain glycogen elicited by exercise is due to hypoglycaemia.

We found that brain lactate levels increased with prolonged exercise (Table 1) and brain glycogen levels during exercise were negatively correlated with brain lactate (Fig. 3C). It is therefore likely that exercise-induced hypoglycaemia causes astrocytic glycogenolysis, leading to the production of lactate as fuel for neurons in the brain. This is consistent with findings that an astrocyte–neuron lactate shuttle (Pellerin & Magistretti, 1994; Brooks, 2009) and glycogen shunt (Shulman *et al.* 2001) function during exhaustive exercise. The increase in brain lactate would also be due to increased blood lactate uptake by the brain (Ide *et al.* 2000). However, it is uncertain which is dominant, glycolysis or blood lactate uptake, in the increase in brain lactate during exercise.

During exercise, skeletal muscle glycogen is degraded in an activity-dependent manner, related to exercise intensity and/or duration, and is utilized to supply energy for adenosine triphosphate (ATP) synthesis (Gollnick *et al.* 1974). In contrast, Vissing *et al.* (1996) examined brain glucose uptake as an index of functional neuronal activity and found that glucose uptake was increased in motor and somatosensory cortices, and the cerebellum during 30 min of high-intensity running (28 m  $\text{min}^{-1}$ ,  $\sim 85\%$  of maximum oxygen consumption). Levels of c-Fos protein, as a cell activation marker, in the hypothalamus increase with moderate exercise just above the lactate threshold (LT); LT speed has been found to be around 20 m  $\text{min}^{-1}$

in male rats who underwent 10–14 days of running habituation (Saito & Soya, 2004; Soya *et al.* 2007a). Furthermore, levels of c-Fos protein increase in the brainstem (Ohiwa *et al.* 2006, 2007), and there is also an increase of BDNF mRNA/protein levels in the hippocampus (Soya

*et al.* 2007b). In the present study, a prolonged running test was performed at the LT level ( $20 \text{ m min}^{-1}$ ); thus, neuronal activity would increase in the cortex, hippocampus, hypothalamus, cerebellum and brainstem. Therefore, brain glycogen decreases with prolonged exercise due to



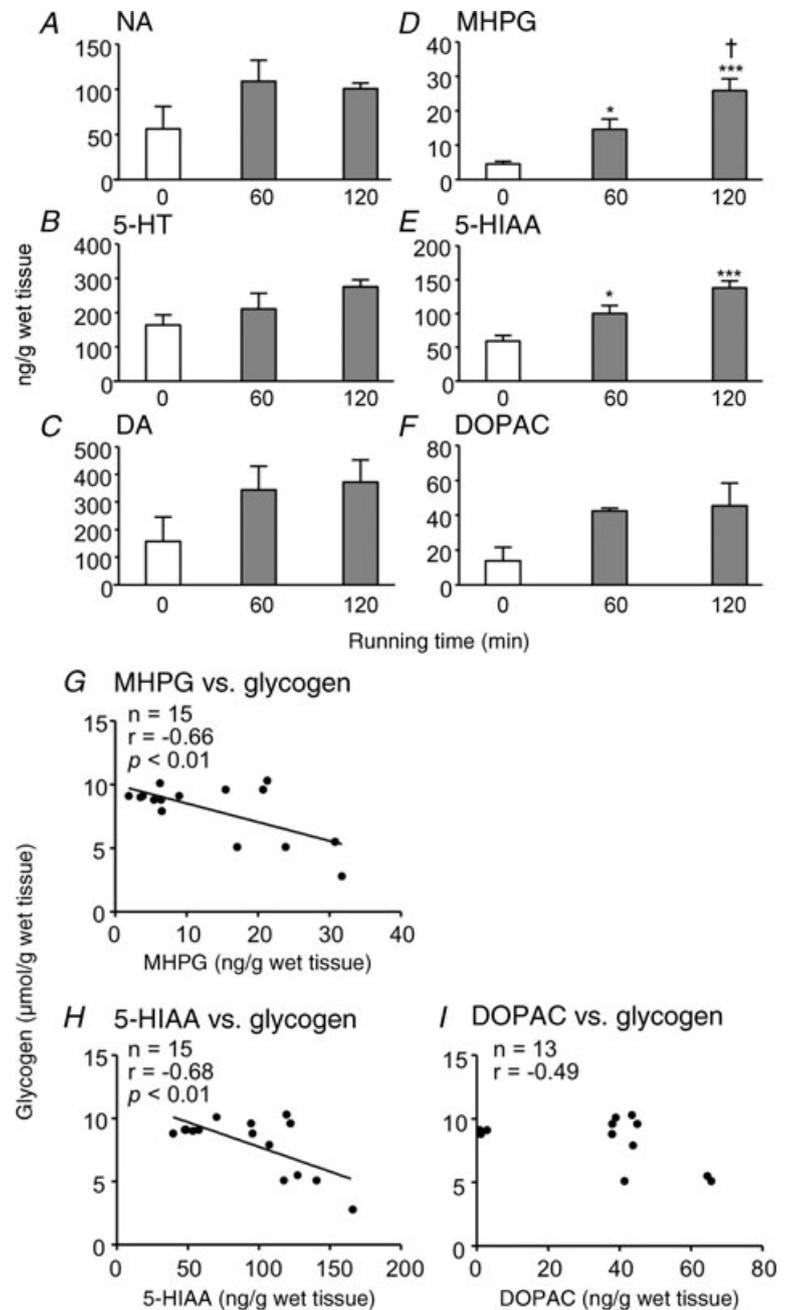
**Figure 3. Correlation between brain glycogen levels and blood glucose, brain glucose and brain lactate**  
Correlation between brain glycogen levels and blood glucose (A), brain glucose (B) and brain lactate (C) (Pearson's product-moment correlation test).

enhanced glycogenolysis, since astrocytic glycogenolysis is enhanced with neuro-activating stimulation (for example, sleep deprivation and sensory stimulation) (Swanson *et al.* 1992; Cruz & Dienel, 2002; Kong *et al.* 2002).

The levels of MHPG and 5-HIAA, which are metabolites of NA and 5-HT, respectively, increased in the cortex during exercise, especially at 120 min (Fig. 4). This is consistent with NA or 5-HT levels increasing in the cortex with prolonged (~1–2 h) treadmill exercise (Pagliari & Peyrin, 1995; Gomez-Merino *et al.* 2001). Furthermore, as shown in Fig. 4G and H, the decrease in cortical glycogen levels during exercise was negatively correlated with increased cortical MHPG and 5-HIAA levels. Since brain

glycogenolysis is promoted by activation of noradrenergic and/or serotonergic mechanisms (Benington & Heller, 1995; Pachmerhiwala *et al.* 2010), our present results suggest that the reduction of cortex glycogen during exercise is not only due to hypoglycaemia but also to increased NA and 5-HT metabolism. In addition, since neuronal activation would promote NA and 5-HT metabolism, our results suggest that cortical neuronal activity increases with exercise. Thus, brain glycogenolysis could be promoted in order to supply ATP for neuronal activation during exercise.

Hypoglycaemia and increased 5-HT levels in the brain could serve as indicators of central fatigue during



**Figure 4.** Levels of monoamines and their metabolites in the cortex immediately after treadmill running exercise for 0 (pre-exercise), 60 and 120 min, and correlation between monoamine metabolites and glycogen levels in the cortex (Pearson's product-moment correlation test)

Data represent the mean  $\pm$  SEM ( $n = 4-6$  rats). A, (NA); B, 5-hydroxytryptamine (5-HT); C, dopamine (DA); D, methoxyhydroxyphenylglycol (MHPG); E, 5-hydroxyindoleacetic acid (5-HIAA); and F, 3,4-dihydroxyphenylacetic acid (DOPAC), \* $P < 0.05$ ; \*\*\* $P < 0.0001$  compared to pre-exercise, † $P < 0.05$  compared to 60 min (Tucky's *post hoc* test). G, correlation between MHPG and glycogen levels; H, correlation between 5-HIAA and glycogen levels; and I, correlation between DOPAC and glycogen levels.

prolonged exercise (Newsholme *et al.* 1992; Nybo & Secher, 2004). On the other hand, hypoglycaemia and 5-HT are factors of glycogenolysis in the brain (Benington & Heller, 1995). Thus, brain glycogen decreases with prolonged exercise lead us to postulate that hypoglycaemia together with brain activation with NA and/or 5-HT metabolism might be involved in the development of decreases in brain glycogen, and may shed light on studies examining how brain glycogen metabolism is involved in central fatigue during exercise (i.e. understanding how enhancing the effects of brain glycogen storage and availability may delay exercise-induced exhaustion).

Collectively, this study provides evidence indicating that exercise results in a decrease in brain glycogen due to hypoglycaemia and to an increase of NA and 5-HT metabolism in the cortex. Exercise-induced hypoglycaemia and increased 5-HT levels could be indicators of central fatigue; however, our findings suggest that whether these phenomena cause exhaustion depends on the reduction in brain glycogen levels. These findings may aid in elucidating the mechanisms underlying central fatigue.

### Methodological implication

Although brain glycogen has a long history of being difficult to determine, there are still few reports showing its *in vivo* implications. This is because the rapid breakdown of brain glycogen, compared with that of glycogen in peripheral areas, makes it impossible to reliably measure stable brain glycogen levels. However, a breakthrough study by Kong *et al.* (2002) revealed that a higher power (10 kW, 1.2 s) MI, which immediately fixes brains, rendering glycogen-producing and -metabolizing enzymes inactive (with an averaged temperature of  $82 \pm 3^\circ\text{C}$  in their study), can allow the determination of values tenfold higher than without MI. They were able to finely monitor brain glycogen levels, which varied between brain regions, in rats with sleep deprivation and hypoglycaemia compared to respective controls. Similarly to our study, Herzog *et al.* (2008) also recently reported high, and valid, brain glycogen levels in hypoglycaemic rats using high-powered MI (9.5 kW, 1.7 s). High-powered MI is necessary in order to correctly detect original brain glycogen levels and should be taken as the best method for assessing this. Thus, our findings using MI to show prolonged exercise-induced decrease in brain glycogen is compelling.

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### Author contributions

Experiments were performed at the Laboratory of Exercise and Neuroendocrinology at the University of Tsukuba. All the listed authors contributed in the following ways: (1) Conception and design of the experiment; (2) Collection, analysis and/or interpretation of data; (3) Drafting the article or revising it critically for important intellectual content. All authors approved the final version.

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