Gene expression of GLUT3 glucose transporter regulated by glucose *in vivo* in mouse brain and *in vitro* in neuronal cell cultures from rat embryos

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This study was designed to determine whether glucose regulates the gene expression of glucose transporter GLUT3 in neurons. We examined the regulation of GLUT3 mRNA by glucose *in vivo* in mouse brain and *in vitro* by using neuronal cultures from rat embryos. Hypoglycaemia (< 30 mg/dl), produced by 72 h of starvation, increased GLUT3 mRNA in mouse brain by 2-fold. Hybridization studies *in situ* demonstrated that hypoglycaemia-induced increases in GLUT3 mRNA expression were observed selectively in brain regions including the hippocampus, dentate gyrus, cerebral cortex and piriform cortex, but not the cerebellum. Primary neuronal cultures from rat embryos deprived of glucose for 48 h also showed an increase (4-fold over control) in GLUT3

mRNA, indicating that glucose can directly regulate expression of GLUT3 mRNA. In contrast with hypoglycaemia, hyperglycaemia produced by streptozotocin did not alter the expression of GLUT3 mRNA. We also confirmed previous findings that hypoglycaemia increases GLUT1 mRNA expression in brain. The increase in GLUT1 expression was probably limited to the blood–brain barrier *in vivo*, since GLUT1 mRNA could not be detected in neurons of the mouse cerebrum. Thus we conclude that up-regulation of neuronal GLUT3 in response to glucose starvation represents a protective mechanism against energy depletion in neurons.

INTRODUCTION

Under physiological conditions, maintenance of normal cerebral functions depends almost entirely on the availability of glucose for the supply of ATP [1,2]. Since the brain cannot store the significant carbohydrates, a steady glucose supply is required from the blood. Thus glucose transport into the brain is critical for the maintenance of brain metabolism. Although under basal conditions the rate of glucose transport is not the rate-limiting step for glycolysis in the central nervous system [3,4], hypoglycaemia or hyperglycaemia is known to change the glucose transport system in the brain [5,6], suggesting that there should be glucose-regulatable mechanisms associated with the transport of glucose.

On the other hand, glucose transport into the brain is mediated by a facilitative-diffusion-type transport system [2,7–9]. It has been suggested that glucose transport is regulated at two levels in the central nervous system: (1) the blood–brain barrier, which allows the facilitated diffusion of glucose into the brain's extracellular space; and (2) the neuronal and glial cell membranes, where glucose transport into the cells is also mediated by facilitated diffusion. Recently, a number of glucose transporter subtypes have been identified by molecular cloning, consisting of at least five isoforms of glucose transporter, GLUT1–GLUT5 [10]. GLUT1 is predominantly expressed in brain microvascular endothelium [11,12], and plays an important role in glucose transport through the blood–brain barrier into underlying tissue layers. Recently we have isolated the cDNAs encoding mouse and rat GLUT3, which were predominantly expressed in the brain [13,14], in contrast with the human GLUT3, which is expressed in most tissues and tumours [15–18]. Both hybridization *in situ* and immunohistochemical studies clearly demonstrate the expression of rodent GLUT3 mRNA and proteins in neurons [13,14,19]. Thus the cloning of rodent GLUT3 cDNA enabled us to study the glucose-regulatory mechanisms of

neuronal glucose-transporter gene expression. In the present study, we have determined the effects of glucose on gene expression and the localization of GLUT3 mRNA *in vivo* in mouse brain and *in vitro* by using primary cultures of rat brain neuronal cells. The results indicate that the expression of GLUT3 in neurons was regulated by glucose.

EXPERIMENTAL

Materials

[α -³²P]dCTP and a kit for nick translation were from Amersham Corp. [³²P]UTP and 2-deoxy[¹⁴C]glucose were obtained from DuPont NEN. Biotinylated anti-rabbit IgG (goat) and peroxidase-avidin complexes were from Dako Japan Co. RNA-labelling kit was obtained from Boehringer Mannheim Corp. Kits for transcription *in vitro* (MAXIscrip) and RNAase protection assay (RPA II) were obtained from Ambion Inc. Nitrocellulose membranes were from Schleicher and Schuell. Reagents for cell culture were obtained from GIBCO. Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Wako Chemical Industries.

Animals

C57BL/6J mice, obtained from Cier Japan, were housed in a room maintained at 23 °C and fed *ad libitum*. Animals used in the experiments were 6 weeks old, with an average weight of 20–30 g. Diabetes was induced by a single injection of 320 mg/kg streptozotocin dissolved in sodium citrate (pH 5.4) into the peritoneum. The control animals were handled, but received no intraperitoneal injection. Hypoglycaemia was caused by starvation for 72 h with drinking water. The animals were killed by decapitation, and blood was collected for glucose and insulin measurements.

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Northern blots

Total cellular RNA was prepared from whole mouse brain and/or neuronal cell cultures by the guanidine isothiocyanate procedure [20]. RNA was analysed by electrophoresis through 1.5% agarose/0.66 M formaldehyde gel, blotted on to a nitrocellulose filter, and hybridized with nick-translated mouse GLUT1, mouse GLUT3, or chicken β -actin cDNAs as previously described [13]. Filters were washed under high-stringency conditions [$0.1 \times$ SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS, 60 °C] and autoradiographed with intensifying screens at -80°C for 7 days. Intensities of the band were determined with a densitometric analyser (model TIAS-2000; ACI Japan Co.).

Immunoblot analysis

The whole brain of the mouse was homogenized, and mixed with Laemmli sampling solution. Western-blot analysis was performed as described previously [14]. Briefly, approx. 50 μg of protein was subjected to SDS/PAGE (10% gel), and electrophoretically transferred to nitrocellulose. After the filters were incubated with 1:500 dilutions of anti-GLUT1 or anti-GLUT3 antibodies [14,21], immunoreactive bands were detected by the alkaline phosphatase reaction.

Hybridization *in situ*

Mouse brains were removed immediately after killing, embedded in O.C.T. compound (Tissue Tek) in liquid nitrogen, and stored at -80°C until sectioned. Then 10 μm sagittal sections were cut at -20°C throughout the brain and collected. Sections were then air-dried, washed with 0.1 M PBS, fixed with 4% paraformaldehyde, and quenched with 2 mg/ml glycine. Sections were digested with 1 $\mu\text{g}/\text{ml}$ protease K and prehybridized with 50% (v/v) deionized formamide/2 $\times$ SSC at 42 °C for 30 min. Digoxigenin-labelled sense and antisense riboprobes were prepared by transcription of plasmids coding for mouse GLUT3 by using T7 or SP6 RNA polymerase as described in the manual of the RNA labelling kit (Boehringer Mannheim). The cDNA insert in this plasmid, cloned into pGEM4Z (Promega Biotec), encodes amino acids 207–394 of mouse GLUT3. Slides were hybridized for 16 h at 42 °C in 100 μl of a solution containing 20 mM Tris/HCl (pH 7.4), 2.5 mM EDTA, 1 $\times$ Denhardt's solution, 300 mM NaCl, 0.5 mg/ml yeast tRNA, 50% formamide and 50–100 ng/ml antisense or sense digoxigenin-labelled riboprobes. After the slides were washed with 50% formamide/2 $\times$ SSC for 30 min at 45 °C and rinsed with 10 mM Tris/HCl (pH 7.4)/500 mM NaCl, they were treated with 20 $\mu\text{g}/\text{ml}$ boiled RNAase A solution in the same buffer for 1 h at room temperature. Slides were then washed twice with 50% formamide/2 $\times$ SSC for 30 min at 45 °C, and washed once with 50% formamide/1 $\times$ SSC for 30 min at room temperature. To detect the signals, slides were washed twice with 50 mM Tris/HCl (pH 8.0)/150 mM NaCl and incubated with 1:500 dilution of anti-digoxigenin Fab fragment conjugated with alkaline phosphatase for 1 h at room temperature. After washing the slides three times with 50 mM Tris/HCl (pH 8.0)/150 mM NaCl, they were rinsed with 100 mM Tris/HCl (pH 9.0)/100 mM NaCl/5 mM MgCl_2 . The colour was developed by incubation with Nitro Blue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in a dark box. The reaction was stopped with TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA); slides were mounted in gelatin/glycine solution, and then observed by light microscopy.

Immunohistochemistry

Immunohistochemical studies were performed as previously described [14,21]. The dissected mouse brains were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. The paraffin-embedded tissues were cut into 5 μm sections, and incubated at 4 °C overnight with 1:1000-diluted solution of anti-GLUT1 antibody. Immunohistochemical staining was done by the avidin–biotin peroxide method of Hsu et al. [22].

RNAase protection assay

Labelled antisense RNA probe for rat GLUT3, which was isolated from rat brain [14], was synthesized by using [^{32}P]UTP (800 Ci/mmol) and the RNA polymerase T7 MAXIscript kit (Ambion Inc.). This probe protects a product of 510 nucleotides. A 10 μg portion of total RNA from rat neuronal cell cultures was annealed to the labelled probe (1×10^6 c.p.m.) for 18 h at 42 °C in 20 μl of a hybridization buffer consisting of 40 mM Pipes (pH 6.4), 80% (v/v) deionized formamide, 400 mM sodium acetate and 1 mM EDTA. After hybridization, 200 μl of a digestion buffer consisting of 10 mM Tris/HCl (pH 7.4), 300 mM NaCl, 5 mM EDTA and a 1:100 dilution of RNAase A/RNAase T1 mixture (Ambion Inc.) was added, and the reactions were carried out at 37 °C for 30 min. The reactions were stopped by addition of 300 μl of RNAase inactivation/precipitation mixture (Ambion Inc.), and then precipitated at -20°C for at least 15 min. The reaction products were then size-fractionated on a 6% polyacrylamide gel containing 7 M urea and were revealed by autoradiography. The relative amounts of protected RNA were quantified by laser scanning densitometry of the autoradiograms.

Primary rat brain neuronal-cell cultures

Primary cultures of rat brain neuronal cells were prepared essentially as described previously [23]. Timed 17-day-pregnant Wistar rats were anaesthetized with diethyl ether, and the fetuses were removed by laparotomy. The meninges were removed from each fetus, and cerebral hemispheres were minced with iris scissors. The brain tissue pieces were digested in 0.25% trypsin (Difco) and 0.01% DNAase I (Sigma) at 37 °C. Dissociated cells were collected by centrifugation at 100 g for 10 min, followed by resuspension and filtration. The cells were plated at 5×10^5 cells/ cm^2 on polyethyleneimine (Sigma)-coated tissue-culture dishes in a medium containing 5% (v/v) heat-inactivated horse serum, 5% (v/v) pre-colostrum newborn-calf serum (Mitsubishi Chemical Industry) and 90% DF medium [24], a 1:1 (v/v) mixture of Dulbecco's modified Eagle's and Ham's F12 media (both Gibco) supplemented with 15 mM Hepes/NaOH buffer (pH 7.2), 30 nM selenium (Wako), 1.9 mg/ml NaHCO_3 , 50 units/ml penicillin G and 100 $\mu\text{g}/\text{ml}$ streptomycin sulphate. The cells were cultured in the above medium for 13 days. The medium was changed at 2–3-day intervals, and the medium was supplemented with 1 μM cytosine arabinoside (Sigma) at day 2 in culture and then at every other medium change. Under these conditions, over 98% of the cells were neurons, which were positive against anti-neurofilament monoclonal antibody [23]. The experiments were performed after the cells had been maintained in culture for 13 days.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. and analysed by ANOVA. Significant differences between control and treated states are indicated by P values.

RESULTS AND DISCUSSION

There are several reports in which the glucose regulation of the gene expression of glucose transporter in the brain or neuronal cells was studied [25–29]. However, those studies, done before the cloning of a neuronal glucose transporter (rodent GLUT3) [13,14], were restricted to GLUT1 gene or protein regulation,

Table 1 Blood glucose levels

Results are means $\pm$ S.E.M. ($n = 5-7$); * $P < 0.01$ (compared with control)

Fasting period...	Blood glucose (mg/dl)		
	0 h	24 h	72 h
Control	110 $\pm$ 15	108 $\pm$ 7	112 $\pm$ 18
Treated	105 $\pm$ 10	56 $\pm$ 9*	27 $\pm$ 8*

Figure 1 Effect of hypoglycaemia after 72 h of fasting on the levels of GLUT1 and GLUT3 mRNAs in mouse brain

Mice were fasted with free access to water only for 72 h (Hypo) or were allowed free access to standard mouse chow (Con). RNA from the whole brain was prepared as described in the Experimental section. A representative autoradiograph is shown of RNA-blot analysis of four separate experiments demonstrating a 2.7 kb GLUT1 or a 4.0 kb GLUT3 transcript in 15–20 μ g of total RNA from the control (Con) and fasted hypoglycaemic (Hypo) mice.

which is mostly expressed in the blood–brain barrier [11,12]. The present study is the first to demonstrate the glucose regulation of rodent GLUT3 predominantly expressed in neurons. First, we have determined the effect of hypoglycaemia on the gene expression of GLUT3 in the mouse brain.

Hypoglycaemia was produced by 72 h starvation of the mouse. In most mice, blood glucose was less than 30 mg/dl after 72 h of starvation (Table 1) and serum insulin level was too low to detect by radioimmunoassay at that time (results not shown). The levels of GLUT1 and GLUT3 mRNAs were elevated to approx. 2 times control caused by 72 h fasting hypoglycaemia (Figure 1 and Table 2). Since in the control mouse brain GLUT3 mRNA was mostly expressed in the regions of hippocampus, dentate gyrus, cerebral cortex and Purkinje cells, as previously shown

[13], it would be of interest whether the increase in GLUT3 mRNA response to hypoglycaemia is due either to increased regional expression or to more widespread expression of this gene throughout the brain. Therefore we performed hybridization *in situ*, using the mouse GLUT3 cRNA probe. Figure 2 shows the hybridization *in situ* of mouse GLUT3 mRNA in the mouse cerebrum from control and 72 h-fasted hypoglycaemic mice. The expression of GLUT3 mRNA in the hypoglycaemic mice was markedly increased in the regions of hippocampus, dentate gyrus, cerebral cortex and piriform cortex. Hybridization signals in other regions were not as strong. Thus increases in GLUT3 mRNA expression induced by hypoglycaemia were observed in specific brain regions. It is well known that nerve cells in the hippocampus, striatum, piriform and neocortical regions are susceptible to ischaemic injury and stress [30,31]. Under those circumstances, the heat-shock protein designed 'hsp70' was particularly evident in the hippocampus, and may prevent further cell damage [32]. GLUT3 is probably also a stress-induced protein, which may protect the damaged nerve cell from energy depletion. This indicates that glucose starvation effectively activates the transcriptional rate of the GLUT3 gene in vulnerable neurons.

The effect of hypoglycaemia on the distribution and expression of GLUT3 mRNA in the mouse cerebellum was also studied. GLUT3 mRNA was detected only in Purkinje cells in both control and hypoglycaemic mice (Figure 3). There was no marked difference in hybridization intensities between the control and hypoglycaemic mice, indicating the lack of glucose-regulatory systems in the cerebellum. At present a reason for this lack of change is unknown, though the constant expression of GLUT3 mRNA in the cerebellum may be associated with the physiological functions of this part of the brain.

It is well known that hypoglycaemia increases not only glucose transport activity, but also levels of glucose-transporter (GLUT1) gene expression in brain tissues [27–29,33]. However, the definite expression of GLUT1 in neurons of cerebrum has not been demonstrated *in vivo* so far. As shown in Figure 4, GLUT1 protein was only detectable in the blood–brain barrier by immunohistochemistry in the cerebrum of both control and hypoglycaemic mice. Hybridization of GLUT1 mRNA *in situ* did not show any expression of GLUT1 in neurons of the cerebrum under the hypoglycaemia (results not shown), suggesting that the increase in GLUT1 gene expression in the brain was probably limited to the blood–brain barrier.

We have also examined the protein levels of GLUT1 and GLUT3 in hypoglycaemia by immunoblotting. As previously shown [13,14,21], bands of GLUT1 and GLUT3 proteins migrated at 50–60 kDa on SDS/PAGE (Figure 5). There was no difference in level of either protein between the control and hypoglycaemic mice, in spite of the elevation of both GLUT mRNAs in hypoglycaemia (see Figure 1). This may be due to the

Table 2 Effect of 72 h fasting on GLUT1 and GLUT3 mRNA

The relative levels of GLUT1 and GLUT3 mRNAs were determined by laser scanning densitometry of the autoradiograms shown in Figure 1. GLUT mRNA levels were normalized by β -actin concentrations. All parameters are expressed as a percentage of measurements in untreated (control) mice. Results are means $\pm$ S.E.M. ($n = 4$); * $P < 0.01$ (versus control).

	mRNA level (GLUT/actin, as % of control)
GLUT1	183 $\pm$ 15*
GLUT3	179 $\pm$ 20*

Figure 2 Hybridization *in situ* of GLUT3 mRNA in mouse cerebrum

The brain was removed and embedded in O.C.T. compound. Sections of mouse cerebrum were hybridized to digoxigenin-labelled RNA probes for GLUT3 mRNA. (a) and (b) show a sagittal section of the region of hippocampus (Hpc), cerebral cortex (Cx) and piriform cortex (Pir) in the control mouse. (c) and (d) show the same region in the brain of a mouse after 72 h of fasting. The hybridization of a sense-strand probe is shown in (e) and (f). Signals of hybridization were detected by using alkaline phosphatase reaction with NBT and BCIP. Each determination was made at least three times, and results of a representative experiment are shown. Other abbreviations: DG, dentate gyrus; St, striatum. The scale bar represents 0.5 mm.

slow change in GLUT protein relative to its mRNA, as previously described by Sivitz et al. [34].

The results demonstrated here seem to indicate that glucose deprivation enhances the transcriptional rate of GLUT3 mRNA in neurons. However, counter-regulatory hormones induced by hypoglycaemia may play a major role in the increase in GLUT3 mRNA *in vivo*. Indeed, growth hormone increased the levels of glucose transporter mRNAs in other systems [35,36]. Therefore we performed experiments using primary cultures of neuronal

cells prepared from rat embryos to determine whether glucose has direct effects on the gene expression of GLUT3 in neurons.

GLUT3 mRNA was expressed in neuronal cell cultures. Reverse transcriptase-PCR and Northern-blot analysis using poly(A)⁺ RNA (10 µg) clearly showed the presence of GLUT3 mRNA in neuronal cell cultures (results not shown). However, GLUT3 mRNA could barely be detected by Northern-blot analysis using total RNA (20 µg). In this study, the changes in GLUT3 mRNA levels in neuronal cell cultures were determined

Figure 3 Hybridization *in situ* of GLUT3 mRNA in mouse cerebellum from control (a) and 72 h-fasted hypoglycaemic (b) mice

Sections of mouse cerebellum were hybridized with GLUT3 cRNA probe as described in Figure 2. Each determination was made at least three times, and results of a representative experiment are shown. Abbreviations: G, granule cell layer; M, molecular cell layer of the cerebellum; PC, Purkinje cell; W, white matter of the cerebellum. The scale bars represent 0.5 mm.

Figure 4 Immunohistochemical analysis of GLUT1 in cerebral-cortical sections from control (upper panel) and 72 h-fasted hypoglycaemic (lower panel) mice

Paraffin sections treated with a 1:1000 dilution of the anti-GLUT1 antibody are shown, demonstrating the general microvascular distribution. The scale bar represents 0.5 mm.

by RNAase protection assay using a 510 bp DNA fragment of rat GLUT3 cDNA, allowing several orders of magnitude more sensitivity than Northern-blot analysis. Neuronal cell cultures maintained in glucose-free medium for 48 h had a 4-fold increase ($318 \pm 36\%$) in the level of GLUT3 mRNA compared with cells maintained in the presence of 11 mM glucose (Figure 6). Taken together with the results of experiments *in vivo* and *in vitro*, glucose starvation directly up-regulated the gene expression of GLUT3 in neurons.

Contrary to the situation *in vivo*, where GLUT1 is not expressed in neurons, rat neuronal cells in culture expressed GLUT1 mRNA. The expression of GLUT1 in neuronal cell cultures may result from culture conditions. Indeed, the GLUT1 isoform of the glucose transporter is well known to be expressed in culture cells in general [37–39]. In agreement with previous studies [40,41], Northern-blot analysis showed that GLUT1 mRNA increased about 3–4-fold ($345 \pm 55\%$) in the neuronal cell cultures after 48 h deprivation of glucose, compared with cells cultured in 11 mM glucose (Figure 7).

Finally, we determined the effects of hyperglycaemia on the gene expression of GLUT1 and GLUT3 glucose transporters in the mouse brain. Hyperglycaemia was produced by a single

Figure 5 Effect of 72 h fasting on GLUT1 and GLUT3 proteins

After mice were fasted as described in the legend of Figure 1 (Hypo; Con, control), proteins from the whole brain were prepared. Western-blot analysis is shown for whole brain homogenates (50 μ g/lane) treated with a 1:500 dilution of the anti-GLUT1 or -GLUT3 antibodies. The mobilities of prestained molecular-mass markers (in kDa) are shown to the left.

intraperitoneal injection of streptozotocin and was maintained for 3 weeks. Blood glucose was > 260 mg/dl and immunoreactive insulin was decreased to one-third of the control (results not shown). Figure 8 shows GLUT1 or GLUT3 mRNA levels in the mouse brain from 2-week- and 3-week-diabetic mice. Since the amount of RNA blotted on to filters was not exactly the same, the levels of GLUT mRNAs were normalized by the β -actin mRNA concentrations. As shown in Table 3, the levels of

Figure 6 Regulation of GLUT3 mRNA expression by glucose in neuronal cells

The RNAase protection assay was used to quantify the amount of GLUT3 mRNA in primary neuronal-cell cultures. After neuronal cells were incubated with either 0 or 11 mM glucose for 48 h, 10 µg of total RNA was isolated, and was annealed to a rat GLUT3-specific probe of unique size. The protected fragments (510 bp) were size-fractionated on a 6%-polyacrylamide gel containing 7 M urea after the RNAase protection reaction was completed. The products were detected by autoradiography. The autoradiogram is representative of three separate experiments.

Figure 7 Effect of glucose on the levels of GLUT1 and β-actin mRNAs in primary rat brain neuronal cells

Total cellular RNA (20 µg per lane), isolated from neuronal cells maintained in the absence or presence of glucose for 48 h, was probed with nick-translated [α - 32 P]dCTP-labelled mouse GLUT1 and chicken β-actin cDNAs as described in the Experimental section. This was a representative of experiments performed independently three times.

GLUT1 or GLUT3 mRNAs in the mouse brain were not changed by diabetes. Hybridization *in situ* of mouse GLUT3 mRNA also showed no difference between the untreated and 2-week-diabetic mice (results not shown). Although there are no reports on the effect of hyperglycaemia on the levels of GLUT3 mRNA or protein in the brain, there is dispute whether diabetes-induced hyperglycaemia increases, decreases, or does not affect GLUT1 expression or glucose transport activity in the brain [3,26,30,42–46]. Possibly, the mild hyperglycaemia (260–300 mg/dl) in the present study resulted in the lack of changes in the levels of GLUT1 and GLUT3 mRNA expressions. Our results support the notion that diabetes is not accompanied by changes in glucose transporter expression in the brain.

In conclusion, glucose starvation up-regulated the gene expression of GLUT3 glucose transporter in the brain. The results

Figure 8 Effect of streptozotocin (STZ)-induced diabetes on GLUT1 and GLUT3 mRNA levels in mouse brain

Mice were rendered diabetic with STZ for 3 weeks, and control (Con) mice were used without injection. RNA was extracted and Northern blots were performed and probed as described in the legend of Figure 1. The Northern blot is representative of four separate experiments for each time point.

Table 3 Effect of streptozotocin (STZ)-induced diabetes on GLUT1 and GLUT3 mRNA in mouse brain

The content of GLUT mRNA was normalized by β-actin concentration. All parameters are expressed as a percentage of measurements in untreated (control) mice. Results are means ± S.E.M. ($n = 4$).

Time after STZ injection ...	mRNA level (GLUT/actin, as % of control)	
	2 weeks	3 weeks
GLUT1	96 ± 7	102 ± 5
GLUT3	90 ± 12	108 ± 8

indicated that neurons may have a protective mechanism against energy depletion.

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