

## Effect of transitory glucose deprivation on mitochondrial structure and functions in cultured cerebellar granule neurons

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### ABSTRACT

We found that 60-min glucose deprivation leads to progressive decrease in the mitochondrial membrane potential and increase in  $[Ca^{2+}]_i$  in cultured cerebellar granule neurons. The latter effect was fully reversible, returning to the basal level 60 min after restoration of normal glucose level in the incubation medium, whereas mitochondrial membrane potential remained at  $10.0 \pm 1.8\%$  below the initial value. Electron microscopy indicated that glucose deprivation induced appearance of mitochondria with local lightening of the matrix and destruction of cristae. This mitochondrial conformation was preserved during the restoration phase after glucose level in the cultivation medium returned to the normal level. Neuronal death within a 24-h period after 60-min glucose deprivation was relatively small, being  $14.0 \pm 4.4\%$ .

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Emerging evidence indicates that cellular effects of some compounds and deleterious factors are mediated by mitochondria [1,2,22–24,26]. Quite often, pathological changes observed within these organelles can be expressed by a fragmentation (fission) of the mitochondrial chondriome, which happens, for example, after exposure of cultivated cells to high concentrations of diazepam, different conventional mitochondrial inhibitors, and to ischemia/reoxygenation or UV irradiation as well [2,22,23,26]. Another result of negative effect on mitochondria is rapid change in mitochondrial traffic in the neuronal cell body and its dendrites. The latter was demonstrated after neuronal exposure to toxic glutamate levels [24]. What is more important, under exposure to pathological factors, the main mitochondrial functions can be damaged, particularly those related to oxidation of organic compounds with further utilization of released energy in the form of the membrane potential ( $\Delta\varphi$ ), which when combined with the energy of proton gradient across the inner mitochondrial membrane ( $\Delta pH$ ) produce the proton motive force ( $\Delta\mu H^+$ , reviewed in Ref. [28]). For instance, very rapid drop of the mitochondrial membrane potential occurs under calcium overload of neuronal cells caused by glutamate toxicity [16,13].

Mitochondrial membrane potential homeostasis has been shown to be necessary for normal cell functioning [28], which can be deteriorated by the loss of energy substrates, particularly under such pathological state as hypoglycemia. Exhaustion of glucose resources under hypoglycemia leads to lower glycolytic rate and acetyl CoA flux into the tricarboxylic acid cycle in mitochondria, resulting in a drop of the mitochondrial membrane potential and thus in lower ATP production [15,14,19,20,27], which can produce irreversible pathological changes in the brain [7]. Moderate insulin-induced hypoglycaemia was shown to have no influence on energy rich phosphates in the human brain of healthy subjects [12]. Recently, *in vivo* studies on brain energy metabolism in humans demonstrated a reduced ratio of phosphocreatine (PCr) to ATP in type 1 diabetic patients which experience moderate insulin-induced hypoglycaemia [4]. That demonstrates that energy metabolism under hypoglycemia may be impaired. Based on these arguments, the importance of substrate deprivation on the morpho-functional state of neuronal mitochondria is certainly very high, and it was within our scope in this study by using a model of hypoglycemia with glucose deprivation (GD) of cultivated cerebellar granule neurons (CGNs). The goal of this study was to compare the changes in the mitochondrial membrane potential with the changes in mitochondrial ultrastructure and intracellular calcium ion level under conditions of GD and after restoration of the glucose level. Although mitochondria *in vitro* display a clear-cut correlation between conformational state and energization [3,10,11], much less is known on this relation for mitochondria *in situ*. In

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addition, besides possible differences in mitochondrial structure and function relations existing *in vitro* and *in situ* models there is an apparent “danger” of having an apparent differences between the data received *in situ* and *in vivo*, but it is a general defect of all *in vitro* systems.

Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Primary cerebellar cultures were prepared from the cerebella of 7–8-day-old Wistar rats of uncertain gender. The number of pups used in the study was 12. After sacrificing the pups with ethyl ester they were treated with 70% ethyl alcohol for 3 min. The skin from the skull and exterior part of the neck was removed and both sides of the skull were scarified. Medulla oblongata was crossed on the border with the spinal cord and the brain was removed and transferred to the Petri dish containing 2–3 ml of  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS. The cerebellum was excised by lancet after dissection of the cerebellum pedicula.

The cerebella were washed with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS (Dulbecco) and incubated in this solution containing 0.05% trypsin and 0.02% EDTA (15 min, 37 °C). After incubation, the tissue was washed twice in PBS and dissociated by repeated pipetting in medium of the following composition: fetal calf serum (10%), minimum essential Eagle medium (90%), glutamine (2 mM).

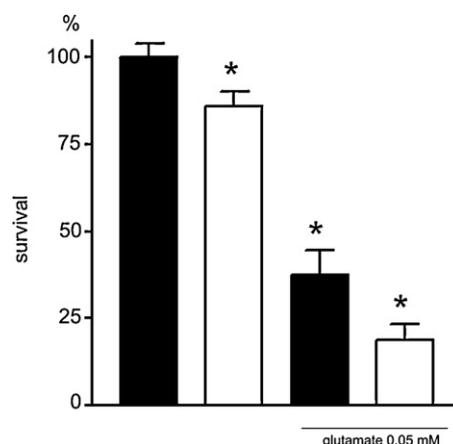
After mild centrifugation (1000 rpm, 1 min) at room temperature, the cells were resuspended in the required volume of the nutrient medium. Cell suspension (0.1 ml/well) was applied to poly-L-lysine-coated (0.5%; 1 h) 96-multiwell plate and placed in a  $\text{CO}_2$ -incubator (5%  $\text{CO}_2$ , 95% air, and 98% relative humidity,  $36.5 \pm 0.5$  °C).

For electron microscopy we used the same volume of cell suspension placed on polylysine-coated coverslips in a 40-mm diameter Petri dish which were fixed using 2.5% glutaraldehyde prepared in phosphate buffer (pH 7.2) followed by postfixation with 1% osmium tetroxide, dehydration in ethanol, and embedding in Epon 812. Ultrathin sections were prepared using a “Leica Ultracut UCT” ultramicrotome and examined at 100 kV in a “JEOL 1011” (JEOL, Japan) electron microscope. 58 cells were examined with comprehensive exploration of their mitochondria (24 of controls, 13 from glucose-deprivation experiment and 21 after restoration).

On the second day *in vitro*, cultures were supplemented with fresh medium containing 25 mM KCl and maintained up to 7–8 days *in vitro*. To prevent proliferation of non-neuronal cells, 1  $\mu\text{M}$  arabinoside monocyotide was added to the cell cultures on the second day *in vitro*.

For GD, the cultured cells were washed twice in balanced salt solution (BSS) containing (in mM): NaCl 154, KCl 25,  $\text{CaCl}_2$  2.3,  $\text{MgCl}_2$  1,  $\text{NaHCO}_3$  3.6,  $\text{Na}_2\text{HPO}_4$  0.35, HEPES 10; at pH 7.3, and were incubated in this medium for 60 min. Control cultures were incubated in BSS containing 1 g/l glucose. After experiment, CGNs were placed into cultured medium and maintained in a  $\text{CO}_2$ -incubator for 24 h up to formation of pyknotic nuclei. Then the cultures were fixed with ethanol–formaldehyde–acetic acid (7:2:1) mixture and stained with trypan blue. The percentage of surviving neurons was estimated by counting the intact nuclei of the CGNs in five fields of view. The viability of untreated control cultures was taken as 100%, and the viability of treated cells were expressed as a percentage referred to control values.

Each value was derived from a minimum of eight cultures from three independent experiments. For analysis of mitochondrial membrane potential changes, cells were loaded with 0.1  $\mu\text{M}$  tetramethylrhodamine ethyl ester (TMRE) (with excitation at 530 nm and emission at >640 nm) for 15 min, and for  $[\text{Ca}]_i$  measurements cells were loaded with 5  $\mu\text{M}$  Fluo-4 AM (excitation at 485 nm, emission at >530 nm) for 30 min at  $36.5 \pm 0.5$  °C followed by triple washing with BSS. TMRE fluorescence in separate mitochondria or in cells was recorded by using inverted fluorescent



**Fig. 1.** Survival of cultivated cerebellar granule neurons measured 24 h after 60-min glucose deprivation. Glutamate added to the cells within the 60-min interval causes extensive neuronal death 24 h after the withdrawal of glutamate and normalization of glucose level. Black bars, under normal glucose level; white bars, GD. \*Statistically significant difference from control values (at normal glucose without glutamate),  $p < 0.05$ ,  $n = 42$ –47, where  $n$  is the number of fields used for calculation of dead cells in three independent experiments.

microscope (Olympus CKX41) equipped with a digital camera. Fluorescence intensity was measured using ImageJ image analysis software (Bethesda, USA). Fluorescence intensity of TMRE was measured in separate mitochondria of live CGNs, while that of Fluo-4 was measured in the bodies of these cells.

The ANOVA test with Bonferroni or Dunnett post hoc test was used for statistical comparisons. Levels of  $p < 0.05$  were considered statistically significant. The results are given as means  $\pm$  SEM.

Evaluation of the CGN viability 24 h after 60 min GD showed that it diminished by only  $14.0 \pm 4.4\%$ . However, if the incubation medium was supplemented with glutamate (50  $\mu\text{M}$ ), more than 80% of neurons died, which demonstrated high sensitivity of CGNs to this neurotransmitter, confirming their neurochemical maturity (Fig. 1).

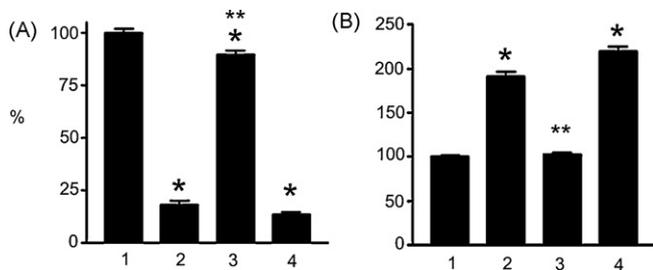
Using TMRE fluorescent probe for the mitochondrial membrane potential, we found that 60 min of GD causes a significant decrease in the membrane potential in cultivated CGNs (to  $18.0 \pm 1.6\%$  in terms of mitochondrial TMRE fluorescence from that of the basal values). After restoring the glucose level, the membrane potential was restored incompletely—even 60 min after transfer of the cells into incubation medium with normal glucose level the mitochondrial TMRE fluorescence still remained at  $90.0 \pm 1.8\%$  of the basal values (Fig. 2A).

We found that intracellular calcium concentration  $[\text{Ca}^{2+}]$  measured with Fluo-4 AM increased to  $91 \pm 5\%$  for 60 min of GD. Induced calcium ion overload was completely reversible and returned to the basal level 60 min after returning to normal glucose content in the incubation medium (Fig. 2B).

The study of mitochondrial ultrastructure showed that mitochondria in CGNs cultivated for 2 h under normal conditions in balanced salt solution containing glucose were not enlarged, having slightly oval or elongated shape. Mitochondrial matrix had higher electron density than that of cytosol (Fig. 3).

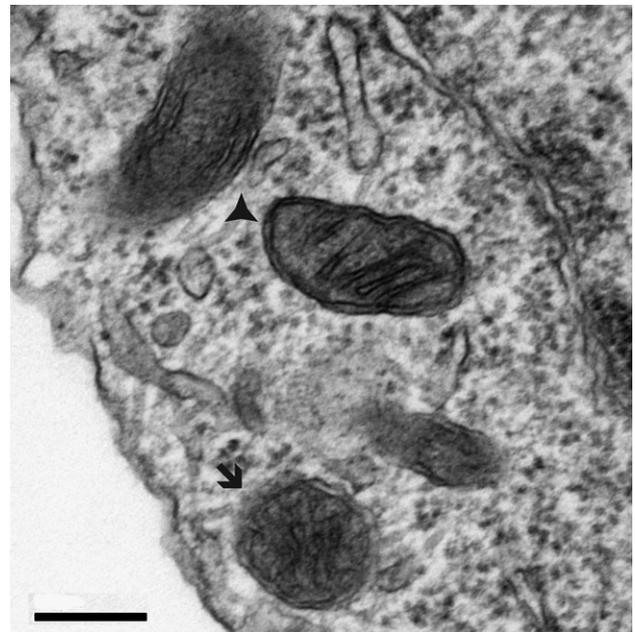
Under control conditions, as well as under GD and during the restoration phase, the ultrastructure of the mitochondrial population was highly variable. In all three cases we could observe mitochondria with orthodox conformation and those having high density of mitochondrial matrix and barely visible cristae as well (Figs. 4 and 5).

The advantage of using in our study dissociated cultures from cerebellum is the morphological and neurochemical homogeneity



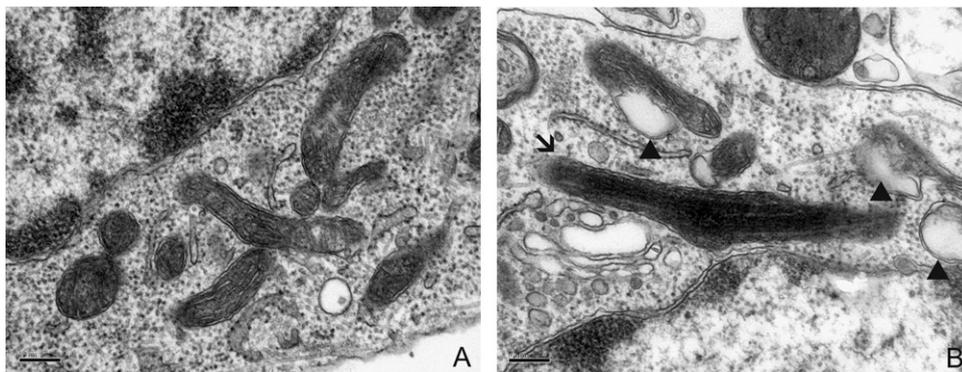
**Fig. 2.** Glucose deprivation of cerebellar cultivated granule neurons causes a reversible decrease in the mitochondrial membrane potential in these cells (A) and increase in cytosolic level of calcium ions (B). (1) cell cultures were incubated for 2 h in balanced salt solution (BSS) containing glucose; (2) cell cultures were incubated for 1 h in a BSS and further for 1 h in the same solution but without glucose; (3) cell cultures were incubated for 1 h in the absence of glucose and further for 1 h with glucose; (4) cell cultures were incubated for 2 h in a BSS without glucose. \* $p < 0.05$  as compared with 1, \*\* $p < 0.05$ , as compared with 2 and 3. For (A),  $n = 252$ – $272$ , where  $n$  is the number in three independent experiments, for (B)  $n = 319$ – $330$ , the number of neurons in three independent experiments.

(meaning that the cells are similar by their type of expressed neuromediator and the sensitivity to this mediator) of neurons mainly represented by granule neurons. It makes these cells a very convenient model for studying the mechanisms of the progression of different pathological processes in neurons including those related to the distortion of the energy metabolism [17]. Earlier using this model, we were able to demonstrate that 60-min GD causes an increase in  $[Ca^{2+}]_i$  and decrease in the membrane potential with blockers of ionotropic receptors being unable to prevent these changes. In this study we demonstrate that although neurons were highly sensitive to the toxic effect of glutamate, GD caused small

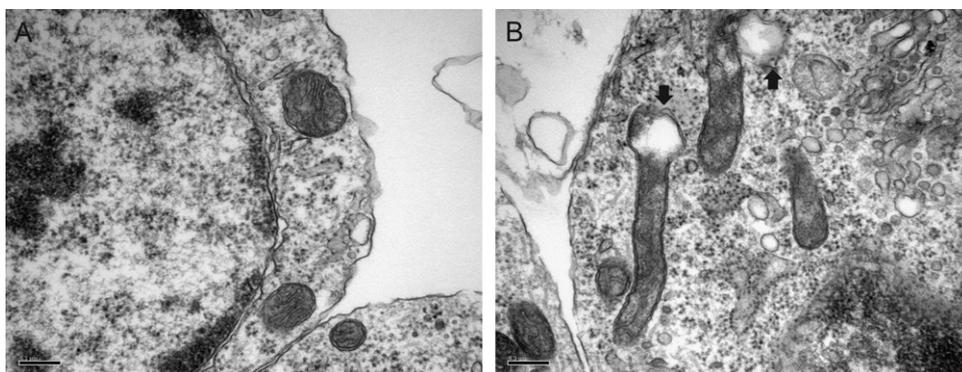


**Fig. 3.** Cultivated granule neurons incubated for 2 h in BSS containing glucose. The cell contains not only normal, orthodox mitochondria with intact ultrastructure (shown by arrow) but also mitochondria with barely recognizable cristae (shown by arrowhead).

but statistically significant increase in neuronal death. Although GD was accompanied by a marked decrease in the mitochondrial membrane potential and increase in intracellular calcium ion level, these changes were very reversible. Earlier the reversibility of hypo-



**Fig. 4.** Cultivated granule neurons incubated for 1 h in BSS containing glucose and further for 1 h under the same solution but without glucose. The cell contains not only normal, orthodox mitochondria with intact ultrastructure (A) but also swollen mitochondria (shown by arrowheads) as well as mitochondria with barely recognizable cristae (shown by arrow). Scale  $0.2 \mu\text{m}$ .



**Fig. 5.** Cultivated granule neurons incubated for 1 h in balanced salt medium not containing glucose and further for 1 h with the same solution but containing glucose. The cell contains not only normal, orthodox mitochondria with intact ultrastructure (A) but also mitochondria with local light matrix areas (shown by arrows (B)). Scale  $0.2 \mu\text{m}$ .

glycemic responses of mitochondria was studied mostly in brain hypoglycemia models *in vivo* [8,17], what does not permit to follow a correlation between the mitochondrial membrane potential and ultrastructure and cytosolic calcium ion content. In our study, we showed that under both GD and the period of restoration of the access to glucose, neuronal mitochondrial ultrastructure in the population was highly variable. Under three conditions explored, we could observe both normal, orthodox mitochondria and those in a condensed state with barely recognizable cristae. Mitochondrial heterogeneity in the cell was indicated in earlier studies; it was shown that these organelles are different in the level of their enzymatic activity under normal conditions, while under pathological conditions these differences possibly can also be expressed in variations of morphological changes of these organelles [5]. Even in the system *in vitro* in mitochondrial suspension the significant variability in the relative values of the membrane potential on the level of single mitochondria in the population was revealed [21].

In our study, in some mitochondria population in neurons *in situ* we observed local lightening of the matrix (as we call it “local mitochondrial swellings”) which was accompanied with the disappearance of cristae. While in control neurons its percentage was found not to exceed 5%, after GD they were represented by 12% of the whole mitochondrial population. Of course, these data are based on the analysis of 2-dimensional mitochondrial images without counting the number of locally swollen areas per single mitochondrion, which can be done only after analysis of 3-dimensional mitochondrial architecture.

Apparently, locally swollen mitochondria are incapable of restoring their ultrastructure, while the mitochondrial potential changes caused by depletion of energy substrates can be partially reversible being apparently without close correlation with mitochondrial ultrastructure. That means that these mitochondria having a local damage are unable to restore their membrane potential to full scale possibly due to a limited protons/ions leak through the inner membrane in damaged areas. Apparently these mitochondria in the neuron restore neither their functional activity (the membrane potential) nor the ultrastructure. Possibly, these mitochondria with locally changed ultrastructure are responsible for the neuronal death we observed under GD. In spite of these changes, the majority of neuronal cells survived, which supports the conclusion made by Dubinsky and Rothman [6] that high level of intracellular calcium after some pathological intervention in no case predicts fatal consequences for the cell when oxidative metabolism is disturbed. It also looks possible that on the very first phase of GD the decrease in the mitochondrial membrane potential may serve a protective role by preventing critical accumulation of calcium ions in mitochondria [9] and lowering the production of reactive oxygen species [25], while when reached some critical level they can cause mitochondrial damage followed by destruction and elimination of the organelle and the host cell. Different vulnerability of cells and their mitochondria to pathological stimuli may be a result of functional as well as structural heterogeneity of mitochondria within a single cell and among the cells. It is obvious that mitochondrial structural organization has limited ability to repair a damage reaching the state which was coined the “point of no return” [18] when irreversible destructive processes are activated. From one side, limited mitochondrial structural plasticity after the damage occurred, and significant resistance of other mitochondria in the population to pathological stimuli from another, seems to determine the relative fate of a cell in the cell population. Relative functional mitochondrial plasticity (as for the mitochondrial membrane potential and intracellular calcium level maintenance) under pathological intervention indirectly points to some vital mechanisms the cell and mitochondrion activate to keep these parameters constant. However, since the biggest massive of data on the correlation of mitochondrial structure and functions has been received

under *in vitro* and *in situ* conditions the extrapolation to the *in vivo* conditions must be done with high precaution.

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