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The lack of extracellular Na^+ exacerbates Ca^{2+}-dependent damage of cultured cerebellar granule cells

Nikolaj K. Isaev[a,*], Elena V. Stelmashook[b], Olga P. Alexandrova[b], Nadezhda A. Andreeva[b], Irina A. Polyakova[c], Ilya V. Victorov[b], Dmitry B. Zorov[a]

[a] *A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia*
[b] *Brain Research Institute, Russian Academy of Medical Sciences, Moscow, Russia*
[c] *Faculty of Biology, Moscow State University, Moscow, Russia*

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Abstract Rhodamine 123 staining, light and electron microscopy were used to evaluate the ultrastructural and functional state of cultured cerebellar granule cells after short treatment with the solution where NaCl was substituted by sucrose (sucrose balance salt medium, SBSM). Cell exposure to SBSM for 20 min resulted in the fact that mitochondria in the neurons lost their ability to sequester rhodamine 123. This effect could be prevented by: (i) non-competitive *N*-methyl-D-aspartate (NMDA) receptor channel blocker, 10^{-5} M MK-801; (ii) a competitive specific antagonist of NMDA glutamate receptors, 0.25×10^{-3} M D,L-2-amino-7-phosphonoheptanoate (APH); (iii) 10^{-3} M cobalt chloride; (iv) removal of Ca^{2+} from the medium. Low Na^+ in the Ca^{2+}-containing medium caused considerable mitochondrial swelling in granule cells. However, the same treatment in the absence of calcium ions in the medium abolished the deleterious effect of SBSM on the neuronal mitochondrial structure and functions. It is suggested that (i) the exposure of cultured cerebellar granule cells to SBSM leads to a release of endogenous glutamate from cells; (ii) Ca^{2+} ions potentially de-energizing neuronal mitochondria enter the neuron preferentially through the NMDA channels rather than through the Na^+/Ca^{2+} exchanger; (iii) mitochondrial swelling in granule cells is highly Ca^{2+}-dependent; (iv) cellular overload with sodium ions can activate mitochondrial Na^+/Ca^{2+} exchanger and thus prevent permeability transition pore opening in mitochondria.

Key words: Neuron; Glutamate; Mitochondria; Na^+/Ca^{2+} exchange; Membrane potential; Ultrastructure

1. Introduction

In the last decade the significance of sodium and calcium ions in a neuronal glutamate toxicity is widely discussed. It has been found that processes which induce the neuronal degeneration after hyperstimulation of glutamate receptors, result in Ca^{2+} and Na^+ influx into the cell through glutamate-activated ion channels [1,2]. This results, in turn, in overloading of neuronal cytosol with these cations [3,4], distortion of cellular energetics [5–8] and activation of Ca-dependent proteolytic and lipolytic enzymes. At present, two components were proposed as factors of glutamate toxicity, namely sodium and calcium components [9,10]. It has been demonstrated that the lower release of Na^+ from the cell, the higher toxic glutamate can be observed [11]. External sodium ions are necessary for Na^+/Ca^{2+} exchange which activity results in a release of Ca^{2+} excess from neurons under glutamate exposure [12–14]. However, Na^+/Ca^{2+} exchange may serve as a root for Ca^{2+} entry [4,15,16].

Mitochondria extensively accumulate Ca^{2+} which caused us to ascribe to mitochondria the role of preventing the cell from Ca^{2+} overload [17]. Glutamate induces in neurons a dramatic rise not only of intracellular Ca^{2+} but also of Na^+, which overload may decrease mitochondrial Ca^{2+}-buffering capacity [18]. Besides this, transmembrane glutamate transport is Na^+-dependent [19] and due to this lowering of external Na^+ may result in a release of intracellular glutamate into the medium [20].

So far, the role of sodium ions for neuronal viability and functioning is not well defined and additional experiments and theories are needed. In the present study the morpho-functional state of cultivated rat granule cells was investigated after short-term abolishing of Na^+ from the medium.

2. Materials and methods

Primary cerebellar cultures were prepared from the cerebella of 7–8-day-old Wistar rats using procedures described earlier [12]. On the second day of cultivation the potassium concentration had risen up to 25 mM. In the medium mainly used for cultivation sodium chloride was iso-osmotically substituted for sucrose [10]. Cells were exposed for 20 min to sucrose balanced salt medium (SBSM) or basic salt medium (BSM) of the following content (in mM): sucrose 274 (or NaCl 137), KCl 5, Na_2HPO_4 0.035, $NaHCO_3$ 12, glucose 11, pH 7.6–7.8, $t = 20$°C. Thus in SBSM the total Na^+ content was as low as 12.07 mM. Cells incubated in BSM were chosen as a control. $CoCl_2$ (10^{-3} M) was used to block Ca channels, MK-801 (10^{-5} M) as a non-competitive specific antagonist of *N*-methyl-D-aspartate (NMDA) channels and D,L-2-amino-7-phosphonoheptanoate (APH) (0.25×10^{-3} M) as a competitive specific antagonist of NMDA glutamate receptors. All these drugs were added to SBSM simultaneously with 2.3 mM $CaCl_2$. Mitochondrial energization in a cell was monitored by rhodamine 123 (R123) accumulation after 10 min of incubation (5×10^{-6} g/ml) in BSM or SBSM. Cellular fluorescence was monitored by a Univar fluorescent microscope (Reichert, Austria). For estimation of delayed neuronal death, treated cells were washed twice with BSM and incubated in this medium for 2 h in a CO_2 incubator for the development of delayed neuronal death. After incubation, cells were fixed with an ethanol/formaldehyde/acetic acid (7:2:1) mixture and stained with vanadium hematoxiline. The percentage of damaged neurons was determined by counting the intact and pyknotic nuclei of the granule cells in 9-view fields. In experiments with exogenous glutamate, cells were exposed to glutamate ($100–500\times10^{-6}$ M) for 20 min in BSM. For electron microscopic study cells were fixed by 2.5% glutaraldehyde prepared on phosphate buffer (pH 7.2) with post-fixation by 1% osmium tetroxide, dehydration in

*Corresponding author.
E-mail: isaev@energ.genebee.msu.su

Abbreviations: R123, rhodamine 123; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo(*a,d*)cyclohepten-5,10-imine hydrogen maleate; NMDA, *N*-methyl-D-aspartate; BSM, balanced salt medium; SBSM, sucrose balanced salt medium; APH, D,L-2-amino-7-phosphonoheptanoate

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ethanol, staining by uranyl acetate and embedding in Epon 812. Ultrathin sections were prepared on ultramicrotome LKB-3 and examined at 75 kV in a Hitachi HU-11 electron microscope.

3. Results and discussion

After 20 min incubation of granule cells in BSM or in Ca^{2+}-free SBSM neuronal mitochondria show very active accumulation of R123 which was indicated by an intensive green fluorescence after excitation of the cells with blue light (Fig. 1A,B). Sister cell cultures were incubated during a similar time in SBSM with 2.3 mM Ca^{2+}. Granular cell cytosol had rather intense scattered fluorescence, while mitochondria did not accumulate R123 (Fig. 1C). The latter effects were observed in about 80% of all viewed neurons; the total number of cells studied was above 50. In all cells the R123 fluorescence was sensitive to protonophores; 1 μM FCCP completely abolished fluorescence within cell mitochondria (not shown). Previously we demonstrated that excessive entry of Ca^{2+} into granule cells induced a fast collapse of the mitochondrial membrane

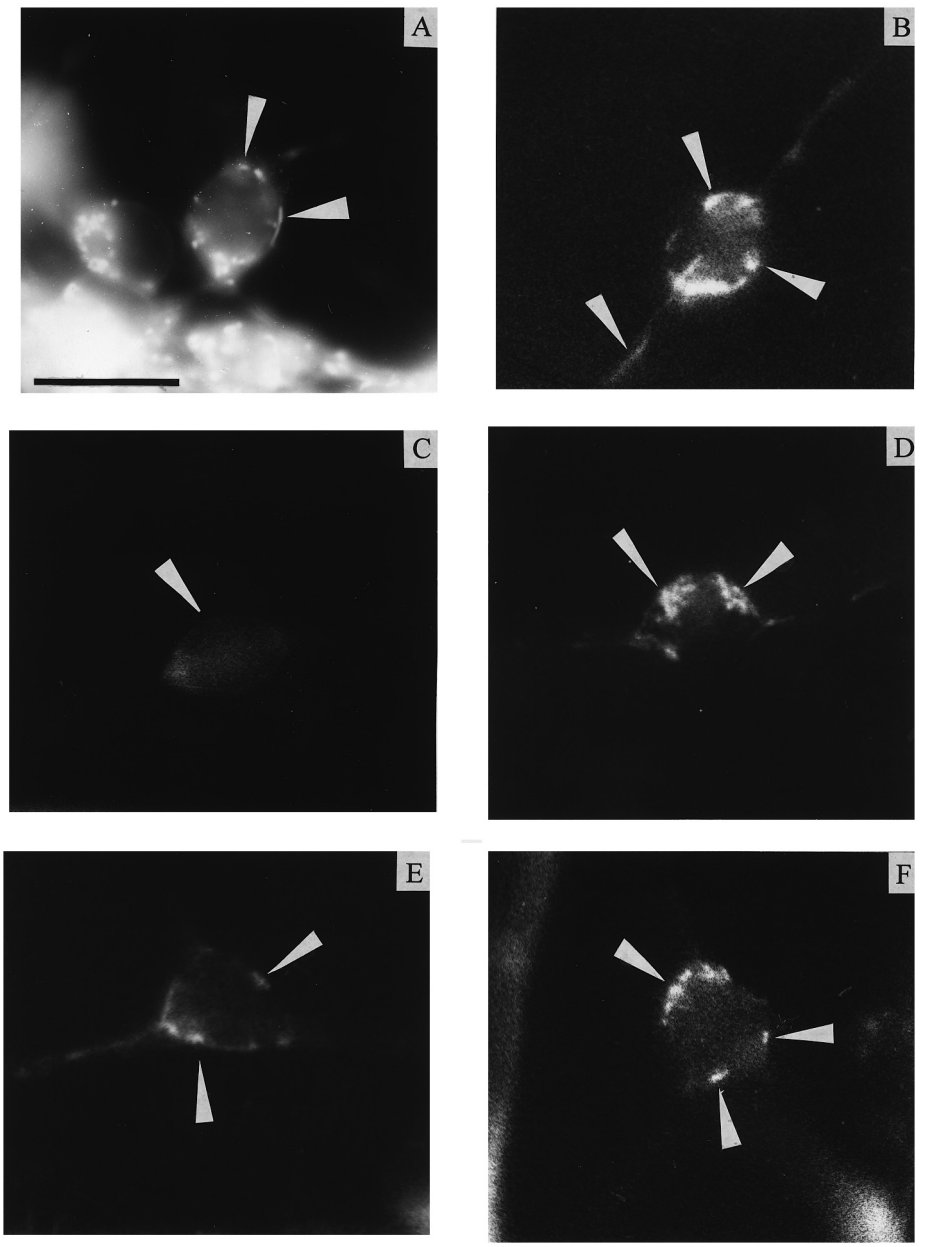

Fig. 1. Living granule cells in cerebellar dissociated culture stained by rhodamine 123. A: Cells were balanced in salt medium for 20 min and then stained. Brightly fluorescent mitochondria can be easily distinguished inside a cell (arrows). Bar = 10^{-5} m. B: Cells were exposed in Na^+- and Ca^{2+}-free sucrose balanced salt solution for 20 min and then stained. Arrows show mitochondria within the cell. C: Granule cell after 20 min exposure in Na^+-free sucrose balanced solution with 2.3 mM Ca^{2+} and then stained. Faintly fluorescent granule cell shown by arrow. Mitochondria are not distinguishable. D: Granule cell after 20 min exposure in Na^+-free sucrose balanced solution with 2.3 mM Ca^{2+} and 1 mM Co^{2+} followed by rhodamine staining. Arrows show fluorescing mitochondria. E: Granule cells after 20 min exposure and in Na^+-free sucrose balanced solution with 2.3 mM Ca^{2+} and 10^{-5} M MK-801 and followed by 10 min treatment with rhodamine 123. Brightly fluorescent mitochondria can be easily distinguished inside granule cells (arrows). F: Granule cells after 20 min exposure and in Na^+-free sucrose balanced solution with 2.3 mM Ca^{2+} and 0.25 mM APH and followed by 10 min treatment with rhodamine 123. Brightly fluorescent mitochondria can be seen within granule cells (arrows).

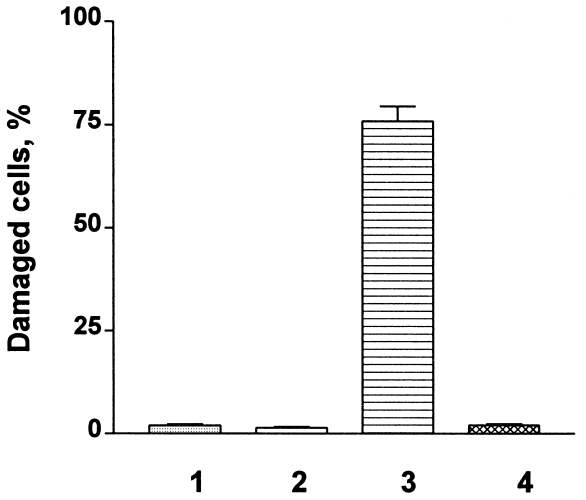

Fig. 2. Cellular viability. Lane 1: control cells incubated for 20 min in BSM; lane 2: cells incubated for 20 min in BSM and 10^{-5} M MK-801; lane 3: cells incubated for 20 min in SBSM; lane 4: cells incubated for 20 min in SBSM with 10^{-5} M MK 801. After this all cells stayed in BSM for 2 h and were then stained as described in Section 2. Lanes 1 and 3, 3 and 4: $P < 0.001$, $n = 54$, Student's t-test.
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potential within this type of neurons [6,7]. To elucidate the Ca^{2+} dependence of mitochondrial membrane potential in granule cells, inhibitors of Ca^{2+} channels were used in the present study. It was found that 10^{-3} M $CoCl_2$ completely abolished the effect of SBSM on the mitochondrial membrane potential (Fig. 1D).

Under iso-osmotic substitution of Na^+, calcium ions can enter the neuron via Na^+/Ca^{2+} exchange or through NMDA channels, activated by internal glutamate. The latter

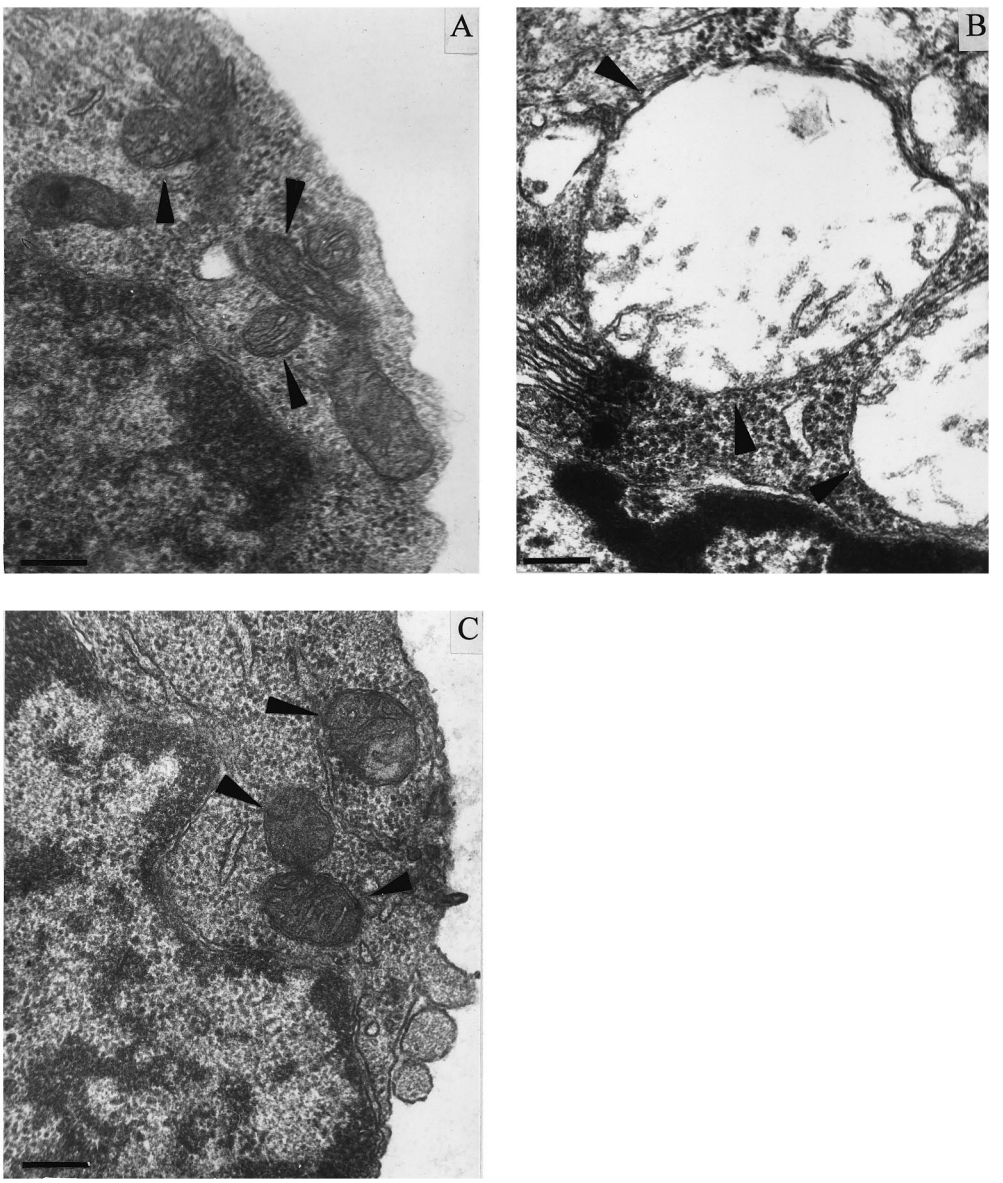

Fig. 3. Transmission electron micrograph of cultured cerebellar granule cells. A: Control cultures were exposed to balanced salt solution for 20 min. Mitochondria (arrows) appear undamaged. Bar = 0.2 10^{-6} m. B: Cells after 20 min exposure in Na^+-free sucrose balanced solution with 2.3 mM Ca^{2+}. Note highly swollen mitochondria (arrows). There is diffuse clumping of heterochromatin. Bar = 0.2 10^{-6} m. C: Cells after 20 min exposure in Na^+- and Ca^{2+}-free sucrose balanced salt solution. Mitochondria (arrows) are swollen. Bar = 0.2 10^{-6} m.

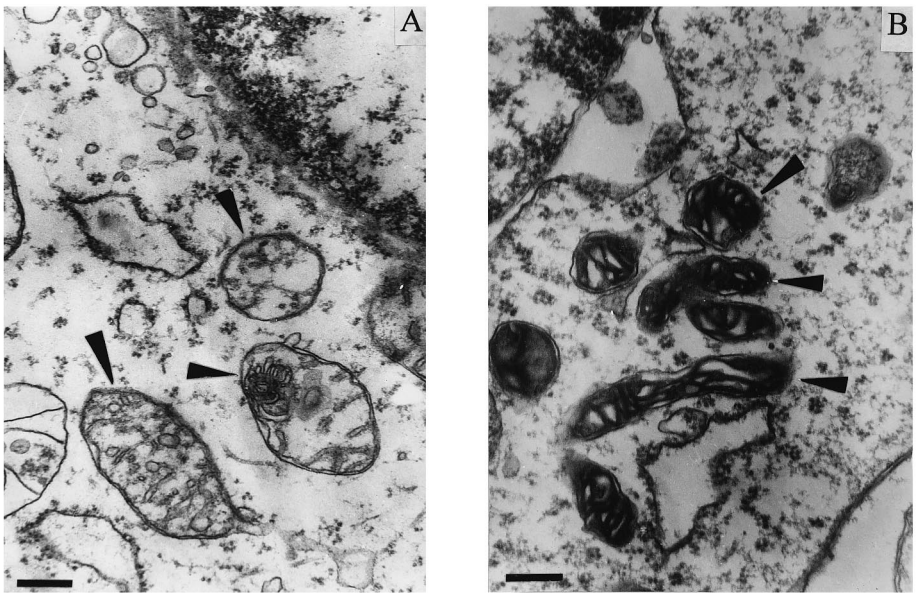

Fig. 4. Transmission electron micrograph of cultured cerebellar granule cells after 20 min exposure to 10^{-4} M glutamate in BSM. A: Swollen mitochondria (arrows). B: Condensed mitochondria (arrows). Bar = 0.2 10^{-6} m.

can be released from the cell due to distortion of the Na^+ dependent transport. For identification of the pathway for Ca^{2+} entry into the neuron, NMDA channels were blocked by MK-801, a specific antagonist of NMDA receptors which inhibits glutamate-activated NMDA channels. This drug completely abolished the effect of Ca^{2+} on the mitochondrial membrane potential. Fig. 1E demonstrates the preservation of the mitochondrial membrane potential in a granule cell when SBSM medium was used together with MK-801. For testing the possibility that NMDA channels are activated by a released internal glutamate, granule cells were incubated in a calcium-containing medium in the presence of APH, a competitive specific antagonist of NMDA glutamate receptors. With this drug mitochondria intensively accumulated R123 (Fig. 1F). This set of data gives evidence that Ca^{2+}-dependent mitochondrial depolarization when cells lack sodium ions, may be due to the effect of the intracellular glutamate released into the medium which can promote neuronal death. The latter suggestion we aimed to prove in our further experiments. For this purpose, cells cultivated in Ca^{2+}-containing SBSM (experiment) or BSM (control) for 20 min were transferred to BSM for 2 h. The percentage of dead cells in control cultures was $2.0 \pm 0.3\%$, while in the experiment $76 \pm 4\%$ neurons died. Non-competitive specific antagonists of NMDA channels completely prevented granule cell death (Fig. 2). We concluded that in granule cells when incubated in a medium without Na^+, calcium ions (which induce the collapse of the mitochondrial membrane potential) enter the neuron preferentially through NMDA channels activated by endogenous glutamate but not through Na^+/Ca^{2+} exchange. This conclusion has been supported by a previous set of data demonstrating that (i) glutamate-induced granule cell death rate is higher when Na^+/Ca^{2+} exchange is blocked [12]; (ii) transmembrane transport of glutamate is Na^+ dependent [19]; and (iii) lowering of the external Na^+ results in a release of internal glutamate in hippocampal slices [20].

At the present time, it is known that neuronal mitochondria intensively accumulate calcium ions under glutamate toxicity [14,21] and that glutamate-induced increase of $[Na^+]_i$ lowers mitochondrial Ca^{2+} buffering capacity, presumably via activation of mitochondrial Na^+/Ca^{2+} exchange [18]. The substitution of external Na^+ for sucrose must prevent both neuronal Na^+ overload when glutamate receptors are activated and activation of mitochondrial Na^+/Ca^{2+} exchange. Under these conditions neuronal mitochondria are more loaded with Ca^{2+} which must affect their ultrastructure.

The electron microscopic (EM) study demonstrated that the incubation of granule cells with Ca^{2+}-containing SBSM results in a severe swelling of their mitochondria. Mitochondria of these neurons look roundish and their cristae are well defined, nuclear chromatin is clumped (Fig. 3B). Cellular cytosol did not show any signs of swelling. Note that the same magnification was used to present these data, thus the high amplitude mitochondrial swelling observed in Fig. 3B has been emphasized. Such a swelling was observed in a majority of cells (more than 80% of cells responded to absence of Na but presence of Ca^{2+} in this manner). No visible damage of a cell membrane was detected after exposure to Na-free medium. In granule cells incubated in SBSM without Ca^{2+}, mitochondrial ultrastructure insignificantly differs from that of control (Fig. 3A,C). In granule cells treated during the same time with added glutamate ($100–500 \times 10^{-6}$ M) with normal Na^+ in the medium, mitochondrial swelling was not only less apparent than with endogenous glutamate released into Na^+-free medium (Fig. 4A), but numerous condensed mitochondria were also detected (Fig. 4B). Cytosol swelling in the latter case was apparent. From the whole population of cultured cells less than 20% were not affected by added glutamate, if mitochondrial ultrastructural changes were considered. The ratio of cells with moderate mitochondrial swelling to those with condensed mitochondria was variable with a slight preference for the first ones, sometimes reaching 1:1. The plausible explanation of different cell response to glutamate may consist of a difference in the number of necrotic or apoptotic cells as was described by Ancarkrona et al. [22].

Based on the results listed above and those presented be-

fore, one can conclude that sodium and calcium ions may be considered as partners working within a neuronal cell providing fine tuning of at least some intracellular Ca^{2+}-dependent processes. In the absence of sodium ions in an extracellular medium, the deleterious effect of Ca^{2+} is much more severe, finally leading to cell death. The effect is apparently mediated by glutamate released from intracellular sources. So far, we were not able to identify the source of glutamate release since the cell culture we work with contains, beside granule cells, some glial cells which can contribute to the effect we described. Apart from the source, glutamate activates NMDA channels thus permitting Ca^{2+} to use them as a root to enter the cell. Within a cell, balance between sodium and calcium ions is a factor which determines the probability of cell damage. In this aspect sodium ions can be regarded as a measure against the activation of Ca^{2+}-induced mitochondrial permeability transitions.

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