

Effects of immunosuppressants FK506 and cyclosporin A on the developing rat brain

Zuzanna Setkowicz, Jaroslaw Kadulski

Department of Neuroanatomy, Institute of Zoology, Jagiellonian University,
6 Ingardena St., 30-060 Kraków, Poland

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Corresponding author:

Zuzanna Setkowicz

Department of Neuroanatomy

Institute of Zoology

Jagiellonian University

Ingardena 6

30-060 Kraków, Poland

tel/fax: +48 12 6632462

e-mail: zuzanna.setkowicz@gmail.com; zuzanna70@poczta.onet.pl

Abstract

Tacrolimus (FK506) and cyclosporin A (CsA) are immunosuppressants commonly used in transplantology. Their neuroprotective actions have also frequently been reported. Unfortunately, after prolonged administration, the drugs have numerous negative neurological side-effects which could also be observed in paediatric clinical cases. Since the problem has never been explored experimentally, the present study focuses on FK506 and CsA influence on the developing rat brain. Six- and 30-day-old rats (P6s and P30s, respectively) received two injections of FK506 or CsA, at a 24-hr interval. Control rats were injected with vehicle alone (Cremophor and ethanol mixture). When the rats were 60-day-old, weights and sizes of their brains were recorded. Additionally, quantitative assessment of calretinin-(CR+) and parvalbumin-immunopositive (PV+) inhibitory neurons was performed. In comparison to naive or vehicle-injected controls, FK506 or CsA-treated P6s showed decreases in the brain weight. In P30s, a decrease in the brain weight was observed only following the vehicle injections. In P6s, CsA injections reduced both CR+ and PV+ neuronal populations while FK506 injections reduced only numbers of PV+ neurons. In P30s, injections of the vehicle alone, but not those of FK506 or CsA, led to significant reductions of the CR+ and PV+ neurons. Generally, the results suggest negative long-term effects of FK506 or CsA on the developing brain. Interestingly, the negative effects of the vehicle were much stronger.

Key Words

calretinin- or parvalbumin-containing neurons; Cremophor

Introduction

Every year the number of successful organ transplantations increases. However, final results of the surgical interventions are determined by effectiveness of long-term immunosuppressive therapy. Among immunosuppressive drugs, Tacrolimus (FK506) and cyclosporin A (CsA) are the most frequently used but, unfortunately, negative side-effects of their prolonged application are often observed. They include headaches, altered mental functioning, tremours, cerebellar syndromes, reversible leukoencephalopathy, increased risks of tumour growth^{1,2} or even seizures³. On the other hand, in experimental studies these drugs showed strong neuroprotective actions following cerebral ischemia^{4,5,6,7}, spinal cord ische-

mia⁸ or traumatic brain injury⁹. So far, those experiments were carried out on adult animals or *in vitro*. To the best of our knowledge, no published systematic research report describes effects of CsA or FK506 on the developing central nervous system. Since the drugs are commonly administered to children and adolescents in clinical immunosuppressive therapy, exploration of their effects on the developing brain appears important.

In order to explore the above presented problem, we exposed the rat brain to FK506 or CsA on postnatal days 6 or 30, and investigated what would be the effect of this intervention on the adult, 60-day-old brain. The two age groups represent considerably different stages of the brain development. The first one is char-

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acterised by dynamic glial cell production, neuronal apoptosis¹⁰ being of importance to functional abilities of the brain in adulthood. At the second stage, the two developmentally programmed processes are basically stopped and different cell populations within the brain approach their final quantities. We focused our attention on populations of GABA-ergic interneurons containing calcium-binding proteins (CaBPs) – parvalbumin or calretinin. Because of their ability to bind calcium, these proteins are involved in many developmental processes (cell differentiation, elongation of nervous cell processes, cell motility)¹¹. On the other hand, the same ability allows these proteins to reduce calcium overloads, which could initiate neurodegenerative changes or modify neuronal conductance. Since any developmental pathology of the neuronal population may have severe consequences to the brain function in adulthood, including epileptic disorders, the obtained results may be of importance to the strategy of prolonged immunosuppressive treatments in children and adolescent patients.

Methods

Animals

All animal-use procedures followed 'Principles of Laboratory animal care', NIH publication (Vol 25, No. 28 revised 1996) and were also approved by the Bioethical Commission of the Jagiellonian University.

Adult Wistar rats were obtained from an animal colony of the Institute of Paediatrics, Collegium Medicum, Jagiellonian University, Kraków and maintained under conditions of controlled temperature (20 ±2°C) and illumination (12h light/dark cycle). A solid diet (Labofeed) and water were available ad libitum.

Pregnant females were housed in individual cages and allowed to give birth. Within 24 hrs. postpartum, the litters were reduced to 10. The young rats were weaned at 28 days of age.

FK506 or Cyclosporin A administration

Male 6-day- or 30-day-old rats (P6s and P30s, respectively) obtained from the females were injected i.p. with 2 mg/kg of FK506 (Prograf, Fujisawa; n=12 and n=8, respectively for each age group) or with 20 mg/kg of cyclosporin A (Novartis; n=12 and n=7, respectively). The injection was repeated 24 hours later. Each pharmaceutical was dissolved in saline containing 0.25% polyoxyl hydrogenated castor oil (BASF, Germany) and 0.25% ethanol. Control rats were injected with the vehicle alone (n=11 and n=9, respectively). For comparative pur-

poses, naive rats (n=13) were also used. Neuroprotective efficacy of the above-indicated dosage of FK506 and CsA used in the present study has been tested by Uchino et al.¹² and Sullivan et al.¹³.

Tissue fixation and staining procedures

On day 60 of postnatal development, each rat received a lethal dose of pentobarbital and was perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. After removing from the skull, the medulla was dissected, the brain was weighted, post-fixed overnight and embedded in paraffin. Ten mm-thick frontal sections were cut and mounted on slides covered with polylysine (Sigma).

Immunohistochemical procedure

The brain sections on slides were deparaffinised, hydrated, rinsed in Tris buffer (TB) (5 min) on a shaker table and treated with 10% H₂O₂ in TB for 30 min. This was followed by another wash in TB (3 x 5 min) and by digestion in a 0.1% trypsin solution in TB, pH 7.8 for 1 h. Subsequently, the slides were washed in TB (3 x 5 min) and incubated for 48 hrs at 4°C with primary antibodies: monoclonal anti-parvalbumin (Chemicon, MAB1572) or rabbit anti-calretinin (Chemicon, AB5054) dissolved in 0.5% Triton X-100-containing TB, each at concentration 1:1000. Thereafter, the slides were washed in TB (3 x 10 min) and incubated with secondary antibodies: goat anti-mouse or goat anti-rabbit (Sigma), respectively (1:100 in TB, 1 hr at room temp.). Following a further wash in TB (3 x 10 min), the sequence was completed by the addition of the mouse or rabbit PAP (Sigma), respectively (1:200 in TB, 1 hr at room temp.). The slides were then rinsed with TB (3 x 10 min), and the sites of HRP binding were visualised using diaminobenzidine (Sigma) and H₂O₂ as the substrate (5 mg diaminobenzidine in 10 ml of TB containing 5 ml of 30% H₂O₂, 15 min). Finally, the slides were counterstained with cresyl violet, dehydrated and coverslipped using De-PeX (Fluka). Controls of the immunostaining, performed by omitting the primary or primary plus secondary antibody, gave negative results.

Nerve cell counting

Two sections from each brain at the level of the anterior commissure were chosen for microscopic examination using a square frame-containing eyepiece. At a magnification of 1000X, the frame delimited a 100 X 100 mm area. In each section, standard sample zones within dorsal parts of each cerebral hemisphere were delineated (Fig. 1). The zones

were examined square by square and all parvalbumin (PV+) or calretinin-immunopositive (CR+) neurons (Fig. 2) were recorded. Surface areas of the examined zones were also measured using a 2.5x objective on a Nikon Microphot SA microscope mounted with a JVS colour camera connected to an image analysis computer system. Finally, numbers of immunopositive cells per mm² of the examined zone were calculated.

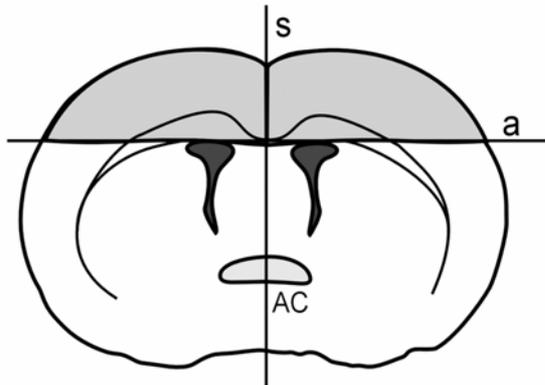
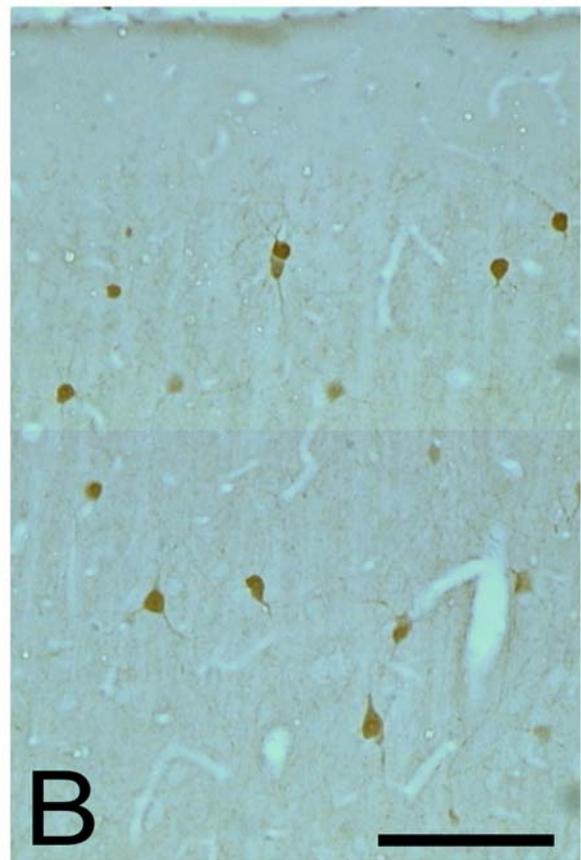
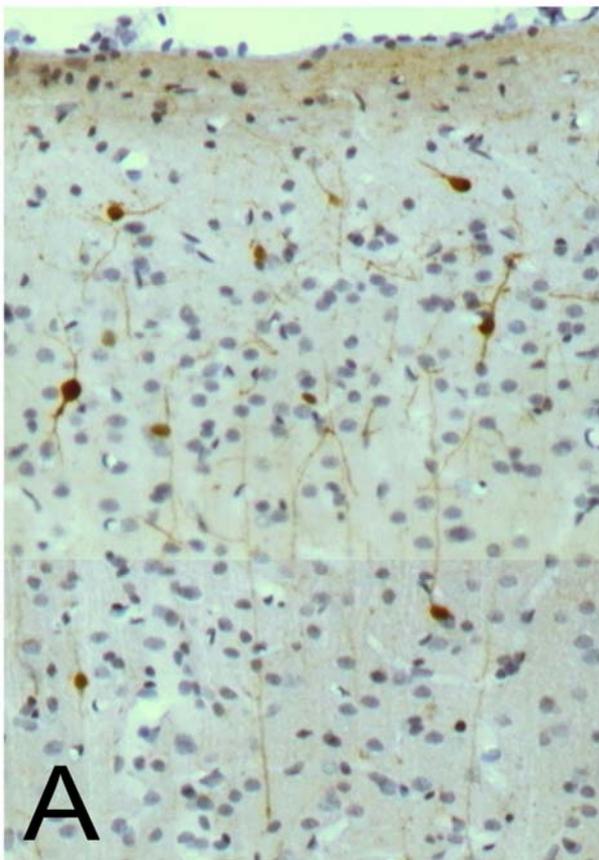


Figure 1. Delineation of microscopically examined standard zones. S, axis of symmetry of the brain section at the level of the anterior commissure (AC); a, line perpendicular to the axis S passing through the top of lateral ventricle. Shaded areas - zones within the dorsal part of the cerebral hemispheres where calretinin or parvalbumin-immunopositive neurons were counted. St, stripes of tissue where Nissl-stained neurons were counted.

To detect possible quantitative changes of the whole neuronal population, an additional counting of Nissl-stained neurons was carried out. For comparative purposes, the counting method had to be similar to the above described non-stereological quantitative assessment applied to PV+ or CR+ neurons. Consequently, the density of Nissl-stained cells was calculated and expressed as the average number of neurons per one mm² of the tissue section area. From technical reasons, the time-consuming counting was limited to stripes of tissue between the lateral ventricle and the surface in each cerebral hemisphere shown (Fig. 1). The width of the stripe was 200 mm i.e. it included two sides of the frame used during the counting. All visible neurons with a clearly defined nucleus and cytoplasm, within the exclusion lines of the frame, were counted under oil immersion using a 100x objective. Microscopic observations were carried out without the knowledge of the previous treatment of the rats.

Figure 2. Neurons immunoreactive for calretinin (A) and parvalbumin (B) in the cerebral cortex. Scale bar shows 100 mm.



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Statistical analysis

Statistical analysis was performed with the STATISTICA work package for Windows (Statsoft, Inc.). Because of non-normal distribution of data, we used the Kruskal-Wallis analysis of variance (differences among all the examined groups) followed by Mann-Whitney U test (differences between groups). The level of statistical significance was set at 0.05.

Results

All the measurements were carried out on the histological material from brains of 60-day-old rats which were injected with CsA, FK506 or vehicle on P6 or P30, i.e. 54 or 30 days earlier. We evaluated statistically relations between the following groups: (1) naive (non-treated rats) or injected with (2) vehicle alone, (3) FK506 or (4) CsA at each of the two developmental stages.

Brain weight

In animals treated with vehicle alone on P6, the brain weight remained unchanged on P60 in comparison to the group of naive, non-treated rats (Fig. 3 A). However, injections of FK506 dissolved in the vehicle made the brain weight significantly lower than that in non-treated controls ($P < 0.0002$) and also than in those treated with the vehicle alone ($P < 0.0001$) or CsA ($P < 0.008$). Following CsA injection on P6, the brain weight was lower than that in vehicle-treated rats ($P < 0.02$) but not when compared to naive rats.

In the group of 30-day-old rats, only the vehicle injections led to a decrease in the brain weight on P60 ($P < 0.04$ vs. naive rats, Fig. 3 B) but no effect of FK506 or CsA was observed. It is noteworthy that neither the average body weight nor the brain-to-body weight ratio were affected in any of the differently treated animal groups.

Changes in neuronal populations

Quantitative assessment of Nissl-stained or CR+ and PV+ neurons detected no significant difference between two hemispheres in any of the examined groups. Therefore, further statistical analysis of differences between experimental and control groups was performed on the basis of average data calculated for both cerebral hemispheres.

None of differently treated groups of 6- or 30-day-old animals showed any significant change in the density of Nissl-stained neurons calculated per one mm^2 of the examined area.

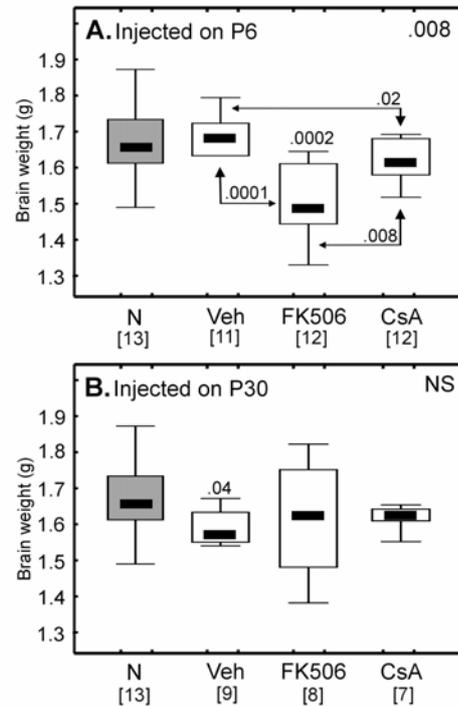


Figure 3. Changes in the brain weight. Rats injected on post-natal days 6 (A) or 30 (B) were examined on P60. Abbreviations for Figs. 3-5: N – naive, control rats; Veh, FK506, CsA – rats treated with vehicle alone or with FK506 or CsA, respectively. Numbers of animals belonging to each group are shown in square brackets. The box and whisker diagram shows the median (small black rectangle in the box), the 25–75% variability range (large box), and maximal and minimal values (whiskers). NS means that the difference was non-significant. Decimal indexes located over a box show statistical significance of difference between a differently-treated group and the group of control rats. Indexes located at double-headed arrows show statistical significance of differences between two examined groups (Mann-Whitney U test). The decimal index in the upper right corner of the diagram shows statistical significance of differences between the examined groups (Kruskal-Wallis test).

a. Calretinin-immunopositive (CR+) neuronal population

In P6s, both vehicle and CsA injections significantly decreased number of CR+ neurons ($P < 0.01$ and $P < 0.004$ respectively, Fig. 4 A) in comparison to the control, naive group. The FK506-injected group showed no significant change.

In P30s, injections of the vehicle alone decreased the number of CR+ neurons (Fig. 4 B) in comparison to naive controls ($P < 0.006$). FK506 and CsA injections had no statistically significant effect.

b. Parvalbumin-immunopositive (PV+) neuronal population

Treatments of P6s with the vehicle alone, FK506 or CsA significantly decreased numbers of PV+ cells ($P < 0.0004$, $P < 0.02$, $P < 0.0007$, respectively, in relation to naive rats, Fig. 5 A). Following the

same treatments performed in P30s, only injections with the vehicle alone resulted in reduction of the PV+ neuron quantity ($P < 0.007$ vs. naive controls, Fig. 5 B).

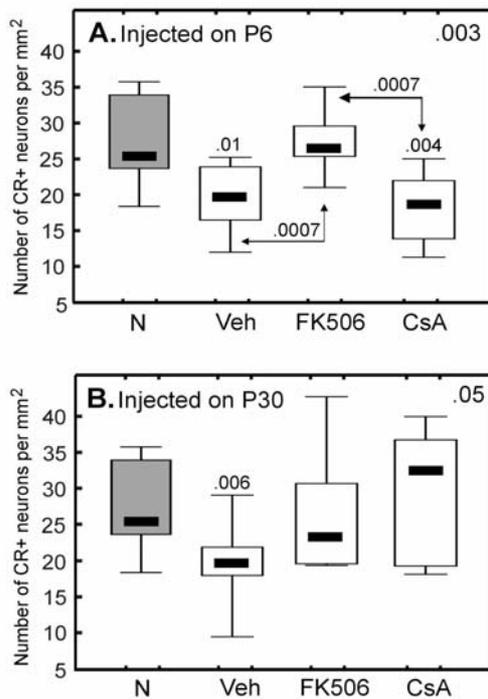


Figure 4. Changes in the number of calretinin-immunopositive neurons per 1 mm² of the brain section area within the dorsal part of the cerebral hemisphere at the level of the anterior commissure shown in Fig. 1. For further explanations see Fig. 3.

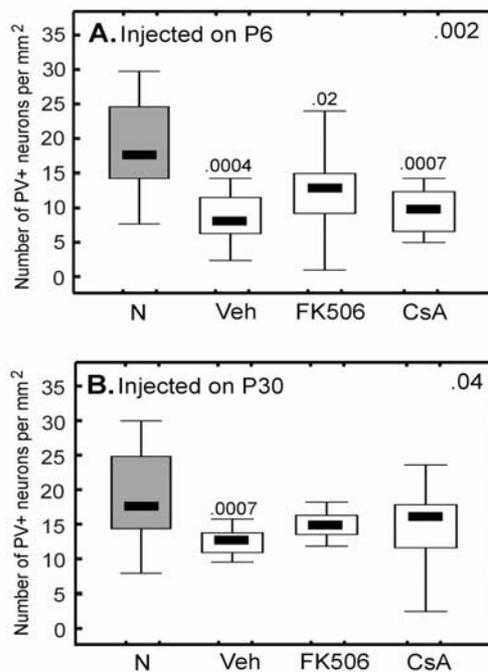


Figure 5. Changes in the number of parvalbumin-immunopositive neurons per 1 mm² of the brain section area within the dorsal part of the cerebral hemisphere at the level of the anterior commissure shown in Fig. 1. For further explanations see Fig. 3.

Discussion

The aim the present study was to answer the question if immunosuppressive agents FK506 and CsA could affect the developing brain. Until now, no systematic study has shown an evidence of permanent developmental changes in the brain evoked by the treatment with FK506 or CsA. The situation appears somewhat surprising since both substances have been routinely used for many years in post-transplantational therapy in children. In such clinical trials, several neurological side-effects have been observed^{1, 2, 14, 15}. The problem becomes more important in several years long therapy after the organ transplantation in pediatric patients who require significantly higher doses of the drugs because of significantly higher metabolic rate and rapid clearance^{16, 17}. Thus, on the basis of clinical experience, in the present experiment negative influences of the two drugs on the developing brain could rather be expected. On the other hand, high neuroprotective efficiency of the used dosage has already been recognised in many previous experiments which, however, have also been performed only on injured adult brains^{12, 13}.

In the present study, both FK506 and CsA were used in formulas supplied by their manufacturers for clinical purposes, i.e. dissolved in the mixture of Cremophor and ethanol. Several cases of negative influences of FK506 and CsA on the developing brain were observed such as a decrease of its weight or reduction of CR+ or PV+ neuronal populations. It is noteworthy, however, to mention that, from the theoretical point of view, a possibility of positive effects in the same respects could also be considered. Both negative and positive changes would point to structural modifications of unrecognized consequences, including functional impairment of the brain. In the present study, however, only negative changes were revealed or their total absence.

The obtained results can somewhat surprisingly be concluded that highly significant reduction in the quantity of CR+ and PV+ neuronal populations could be evoked by the vehicle alone, i.e. by the mixture in which FK506 or CsA were dissolved and what was previously reported as biologically inactive⁷. Moreover, the vehicle alone, when administered on postnatal day 30 but not on day 6, significantly reduced the brain weight in 60-day-old rats. This fact appears to be preliminary evidence of severe generalised changes which need more detailed investigations since the present study show only a very small, selected aspect of the whole phenomenon.

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In the present experimental situation an apparently paradoxical problem arose: if and at what degree the presence of FK506 or CsA minimised or amplified negative effects of the vehicle itself. It has, however, been proven that FK506 dissolved in the vehicle and administered to six-day-old rats abrogated negative effect of the vehicle administered alone (difference of high statistical significance). A similar effect did not take place when CsA was dissolved in the vehicle. In the same age group, reduction of PV+ neurons following injections of FK506 or CsA in the vehicle was similar to that evoked by the vehicle alone.

The presence of FK506 or CsA in the vehicle before injections in 30-day-old rats abrogated strong negative effects on the quantity of CR+ and PV+ neurons detected following injections of the vehicle alone.

Thus, the results clearly proved that the vehicle itself became the cause of strongest negative long-term effects. There are many published reports on neurotoxic properties of its component - Cremophor. Windenbank et al. [18] observed that *in vitro* Cremophor inhibited the neurite growth of DRG neurons and decreased activity of C proteinase regulating phosphorylation of many proteins engaged in cell proliferation and differentiation¹⁹. Since Cremophor can disturb mitochondrial respiratory processes²⁰, the decrease in the quantity of CR+ and PV+ neurons observed in our study might be the result of its toxic ability especially when mixed with ethanol. In fact, during early developmental periods ethanol induces neuronal degeneration²¹. In the rat, during the first postnatal week, CaBPs-containing neurons undergo considerable developmental changes²². However, according to the report by Granato²³ exposure of neonatal rats during this period can even increase the population of CR+ without any effect on the quantity of PV+ neurons. Nevertheless, the overall population of CaBPs-immunopositive neurons remained stable.

We were aware that the toxic action of the vehicle itself could result not only in quantitative reduction of CR+ and PV+ neuronal populations but also in lower contents of calretinin and/or parvalbumin in affected neurons. The latter, being also of importance to functional features of the brain, could lead to decreases in numbers of neurons detected immunocytochemically in the present study.

Calcium binding proteins (CaBPs): parvalbumin and calretinin are present in GABA-ergic neurons²⁴, where they are involved in the regulation of Ca⁺⁺ level determining their normal

functional abilities. Cellular Ca⁺⁺ overload can frequently initiate neurodegenerative changes leading to cell death²⁵. The main role of CaBPs consists in binding of Ca⁺⁺, which is engaged in the cell division processes, axon elongation and cell motility¹¹.

The treatment with CsA or FK506 could selectively reduce the CaBPs-containing neuronal population without any effect on the total quantity of neurons as it was observed in our experiment. Finally, that might result in changes in susceptibility to seizures in animals which after injury were injected with neuroprotectants^{26, 27}.

The present report demonstrates that a relatively small dose of FK506 or CsA can interrupt the development of inhibitory CaBPs-containing interneurons. Moreover, the presented facts point to adverse effects of the commonly used vehicle on the developing nervous system. It cannot be excluded that the vehicle determines, at least in part, negative side-effects of the neuroprotectants frequently observed in clinical practice.

It appears, therefore, that further experimental and clinical verification of the results presented here could be of importance for planning an optimal post-transplantation therapy in paediatric patients.

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