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Influence of Neuroprotectants, Cyclosporin A and Tacrolimus, on the Development of Hippocampal Formation in the Rat

Z. Setkowicz*, K. Radomska, K. Rogóż, Ł. Uram, and K. Janeczko

Department of Neuroanatomy, Institute of Zoology, Jagiellonian University, 30-060 Kraków, Poland

Tacrolimus (FK506) and cyclosporin A (CsA) are immunosuppressants that have well-known neuroprotective actions. However, the drugs also have numerous negative neurological side effects observed in clinical practice. The problem of the long-term consequences of the immunosuppressive treatment has never been explored experimentally. Therefore, we focused on the FK506 and CsA influence on the developing hippocampal formation as a region of very high vulnerability to different kinds of damages. 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 a vehicle alone (Cremophor and ethanol mixture). When the rats were 60 days old, quantitative assessments of calretinin- (CR+) and parvalbumin-immunopositive (PV+) inhibitory neurons and S-100β protein-immunopositive (S-100β+) astrocytes were performed within the hippocampal formation. In comparison to naive rats, vehicle-, FK506- or CsA-treated P6s showed no changes in CR+ or PV+ neuronal quantity, but a significant increase in the density of S-100β+ astrocytes was noted. Additionally, reduction in the size of the hippocampal formation was recorded following CsA injections performed on P6. In P30s, injections of the vehicle alone and of FK506 but not those of CsA, led to significant reductions of the CR+ neurons. The results suggest similar negative long-term effects of FK506 or CsA on the developing hippocampus. Of particular interest are the negative effects of the vehicle alone.

Keywords: Calretinin- or Parvalbumin-Containing Neurons, Astrocytes, Cremophor.

RESEARCH ARTICLE

1. INTRODUCTION

Cyclosporin A (CsA) was first used in clinical practice in 1978 as an immunosuppressant medication after kidney transplants. The first reports of tacrolimus (FK506) use in post-transplant pediatric patients date to 1991. Apart from the widely described immunosuppressant potency of these drugs, they were observed to have neuroprotective properties, i.e., the ability to increase nervous cell survival after exposure to a neurotoxic agent. This ability was demonstrated in a multitude of experimental models, including transient brain ischemia,[1–4] and brain trauma.[5–7] Those studies were conducted on adult animals. However, when CsA and FK506 actions were tested on the developing rat brain, it was observed that their administration in the first postnatal week could permanently decrease brain weight and size, and diminish cortical parvalbumin—or calretynin-positive neuronal count.[8] These changes were less pronounced if the compounds were given to 1-month-old rats, in which a majority of developmental processes had already been finished.[9] In addition, the disadvantageous effects of the vehicle (a mixture of ethyl alcohol and castor oil), used to dissolve CsA and FK506 because of their lipophilic character, were noticed.[8] These facts suggest that CsA and FK506 can have a serious negative impact on the developing brain. Hippocampus is the brain structure that is very sensitive to damage. Due to its key role in the mechanism of memory formation and learning, normal development of its neurons is of crucial significance. Therefore, our present studies aim to determine how CsA and FK506 influence development of the hippocampal formation if they are used in pharmaceutical formulations commonly used in clinical practice. To answer this question, we would like to examine the fate of two populations of GABAergic neurons, the containing calcium-binding proteins: calretynin and parvalbumin. Since the newest concept of molecular mechanism underlying the neuroprotective action of immunosuppressants assumes that they target not only neurons but also glial cells,[4] we are going to investigate whether similar changes occur also in astrocytic population, containing

*Author to whom correspondence should be addressed.

S100β protein also able to bind free calcium.[10] According to excitotoxicity theory, calcium excess can be a cause of both neuronal and glial cell death.[11]

2. METHODS

2.1. Animals

All animal-use procedures followed the "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 (12 h 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 ten. The young rats were weaned at 28 days of age.

2.2. 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; $n = 10$ and $n = 9$, respectively, for each age group) or with 20 mg/kg of cyclosporin A (Sandimmune, Novartis; $n = 10$ and $n = 6$, 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 ($n = 9$ and $n = 8$, for each age group, respectively). For comparative purposes, naive rats ($n = 9$) 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. 2002[2] and Sullivan et al. 2000.[12]

2.3. 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 0.1 M phosphate buffer, pH 7.4. After removing the skull, the medulla was dissected, the brain was weighed, post-fixed overnight, and embedded in paraffin. Ten μm-thick frontal sections were cut and mounted on slides covered with polylysine (Sigma).

2.4. Immunohistochemical Procedures

The brain sections on slides were deparafinized, hydrated, rinsed in Tris buffer (TB) (5 min) on a shaker table and

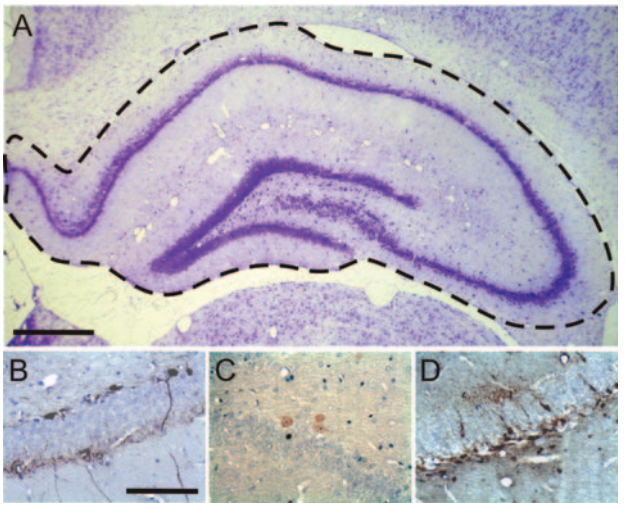

Fig. 1. Delineation of microscopically examined region of the hippocampal formation (broken line). Nissl staining. Scale bar shows 1 mm (A); neurons immunoreactive for calretinin (B) and parvalbumin (C), and S100β-immunopositive astrocytes (D) at the granular layer of the dental gyrus. Scale bar shows 100 μm.

treated with 10% H_2O_2 in TB for 30 min. This was followed by another wash in TB (3×5 min) and by digestion in a 0.1% trypsin solution in TB, pH 7.8 for 1 hr. Subsequently, the slides were washed in TB (3×5 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. To visualize astrocytes, monoclonal anti-S100β protein primary antibody (Sigma, S2532) was used at a concentration of 1:500 according to the same immunostaining protocol. Thereafter, the slides were washed in TB (3×10 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 (3×10 min), the sequence was completed by the addition of the mouse or rabbit PAP (Sigma), respectively (1:200 in TB, 1 hr at room temperature). The slides were then rinsed with TB (3×10 min), and the sites of HRP binding were visualized using diaminobenzidine (Sigma) and H_2O_2 as the substrate (5 mg diaminobenzidine in 10 ml of TB containing 5 ml of 30% H_2O_2, 15 min). Finally, the slides were counterstained with cresyl violet, dehydrated, and coverslipped using DePeX (Fluka). Controls of the immunostaining, performed by omitting the primary or primary plus secondary antibody, gave negative results.

2.5. Immunopositive Nerve Cell Counting

Two sections from each brain at the level Bregma −3.80 according to the atlas by Paxinos and Watson,[13] containing the dorsal part of the hippocampal formation were

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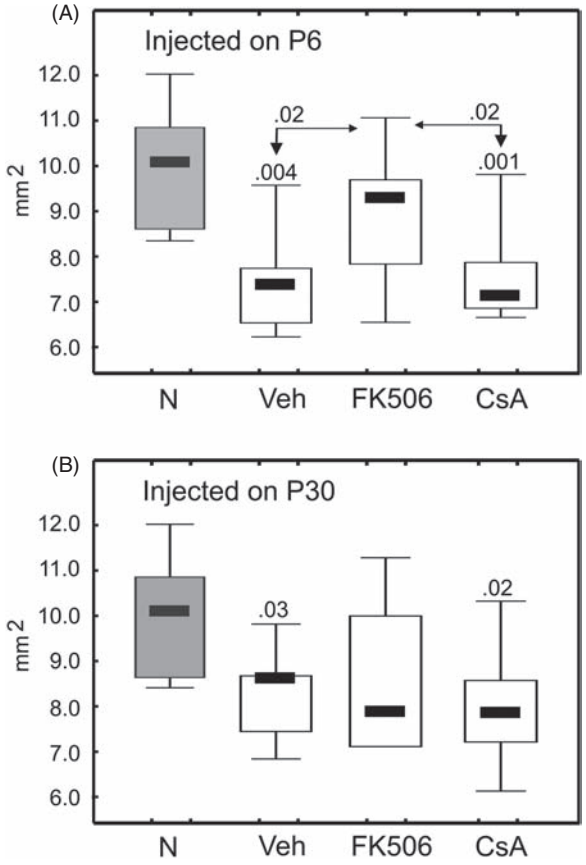

Fig. 2. Changes in the total surface area of the hippocampal region at the level shown in Figure 1(A). Rats treated on postnatal days 6 (A) or 30 (B). Abbreviations for Figures 2–4: N—control, untreated rats; Veh, FK506, CsA—rats treated with vehicle alone or with FK506 or CsA, respectively. The box 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). Decimal indexes located over a box show the statistical significance of the 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).

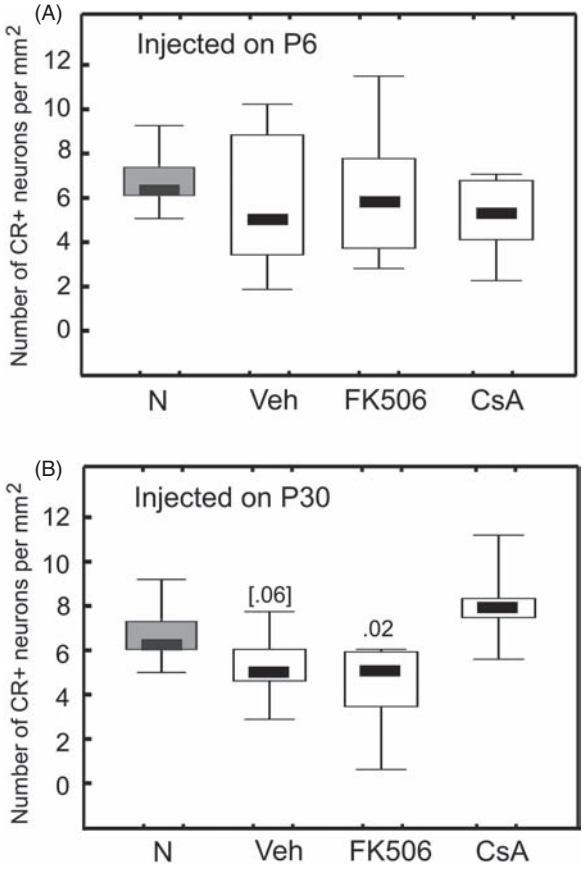

Fig. 3. Changes in the number of calretinin-immunopositive neurons per 1 mm^3 calculated for the total surface area of the examined hippocampal region shown in Figure 1. For further explanations see Figure 2.

chosen for microscopic examination using a square frame-containing eyepiece. At a magnification of 1000×, the frame delimited a 100×100 μm area. In each section, regions of hippocampal formation in both cerebral hemispheres were examined square by square (Fig. 1(A)) under oil immersion using a 100× objective and all parvalbumin (PV+) and calretinin-immunopositive (CR+) neurons, and S100β protein-positive astrocytes (Figs. 1(B)–(D), respectively) were recorded. Surface areas of the examined hippocampal regions were also measured using a 2.5× objective on a Nikon Microphot SA microscope mounted with a JVS color camera connected to an image-analysis computer system. Finally, numbers of immunopositive cells per mm^2 of the examined region of the hippocampal formation were calculated.

Microscopic observations were carried out without the knowledge of the previous treatment of the rats.

2.6. Statistical Analysis

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

3. RESULTS

3.1. Surface Area of the Hippocampal Region

In animals administered the vehicle on the 6th postnatal day, the surface area of the hippocampal region (SAHR) measured when they were 67 days old was statistically significantly decreased in comparison with the naive (untreated) group (Fig. 2(A), $P < 0.004$). The administration of FK506 did not cause any changes in the SAHR. CsA administered on the 6th postnatal day, like the vehicle, significantly diminished ($P < 0.001$) the SAHR determined in adult animals. SAHR in FK506-treated animals was similar to that of the naive rats, and thus significantly

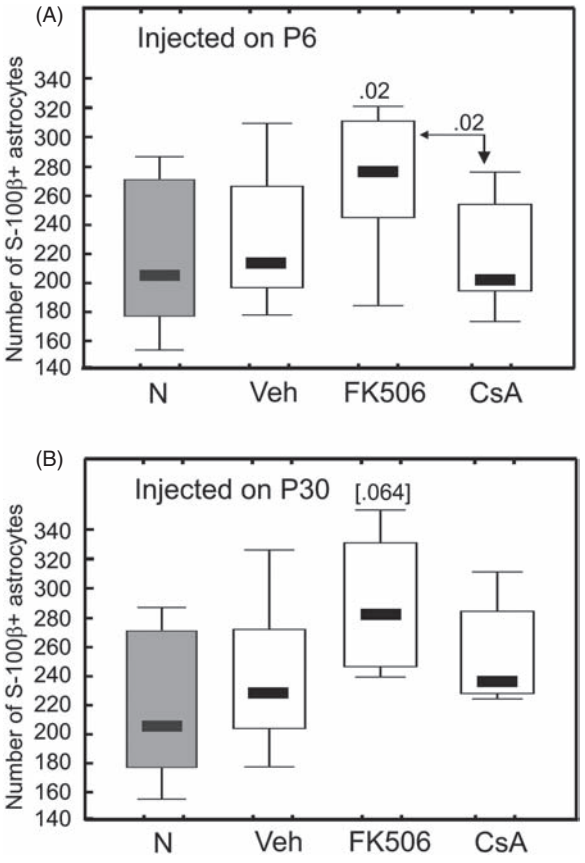

Fig. 4. Changes in the number of S100β-immunopositive astrocytes per 1 mm^3 calculated for the total surface area of the examined hippocampal region shown in Figure 1. For further explanations see Figure 2.

greater than in the groups having received the vehicle or CsA ($P < 0.02$ for each group).

As in the former group, rats treated with the vehicle on the 30th postnatal day showed significantly decreased SAHR versus untreated control animals (Fig. 2(B), $P < 0.03$). FK506 did not cause any changes, while the treatment of 1-month-old animals with the CsA significantly lowered the SAHR ($P < 0.02$) in comparison with the control group. In both age groups, FK506 in the pharmaceutical formulation seemed to abolish the negative effects of the vehicle, but the CsA did not show this effect and the SAHR was decreased, similar to after the vehicle alone.

3.2. Density of Immunopositive Neurons

In P6s, neither the administration of vehicle alone nor FK506, nor CsA induced any alterations in the CR+ neuronal density (Fig. 3(A)). In vehicle-treated P30s, the CR+ density showed a decreasing tendency versus the control group, but the difference was of only borderline statistical significance (Fig. 3(B), $P < 0.055$). FK506 and the vehicle administered to 30-day-old animals caused a significant decrease in CR+ cell density compared to the control group ($P < 0.02$), but the effect of vehicle was not statistically significant. CsA treatment did not produce any significant change.

PV+ cell density was not significantly altered in either age group.

3.3. Density of S100β Protein+ Astrocytes

In P6s, the FK506 administration significantly increased S100β+ astrocytic density (Fig. 4(A), $P < 0.02$) compared to the control group but not versus the vehicle-alone-treated group. After the administration of the vehicle alone or CsA, the astrocytic density did not differ in relation to control.

In P30s having received the vehicle and FK506 or CsA, we observed no significant changes in S100β+ astrocytic density (Fig. 4(B)). However, as in P6s, in P30s FK506 increased the density of these cells, but the significance of the data approximated only the significance threshold (Fig. 4(B), $P < 0.064$). Thus, variability profiles were similar in both groups.

4. DISCUSSION

Our present studies aimed to determine whether systemic administration of CsA or FK506 dissolved in a standard vehicle and vehicle treatment alone, on the 6th or 30th postnatal day cause permanent changes, i.e., changes observable in adulthood. We focused particularly on the observation of the hippocampal formation, neurons of which are especially sensitive to damage. Studies conducted on an experimental model of brain ischemia demonstrated a neuroprotective action of CsA and FK506;[1–4] however, these studies were performed on adult animals. In this context, our studies of undamaged but developing brain gave unexpected results. They question unequivocally neuroprotective activity of CsA and FK506, since each of these compounds showed a negative effect of some kind on the development of the hippocampal formation. However, it should be remembered that both CsA and FK506 have been always administered as components of the pharmaceutical formulation, dissolved in the vehicle, which proved not to be a biologically neutral substance.

The study showed that the administration of CsA and the vehicle alone in both P6 and P30 animals significantly decreased the SAHR in comparison with the control group. Our studies did not involve stereometric measurements; we only assumed that changes in SAHR are an indicator of changes in hippocampal volume/size. The negative effect of CsA in 6-day-old animals can be considered to be a result of its toxic effect on the developing brain, despite that the dose used by us (20 mg/kg) had neuroprotective effect in adult rats.[3] The significant hippocampal shrinkage after CsA administration in 30-day-old rats is more

difficult to explain, because of the impeded penetration of CsA through the blood-brain barrier due to its maturity at this age.[14] Thus, there has to be another way how CsA gets into the brain, maybe through the periventricular organs, which lack this barrier.[14]

This study revealed the negative effect of FK506 on the density of CR+ neurons but only after its administration in P30 groups. This effect was not seen in PV+ neurons, which also were not influenced by CsA. This seems to suggest that the reactivity of these two neuronal types was distinct in respect to this property. Neurons containing calcium-binding proteins, including calretinin-positive neurons, belong to a larger population of GABAergic neurons.[10] Therefore, the reduction of their density can significantly alter excitability of the nervous tissue, including also its vulnerability to epileptic attacks[15, 16]

Functional properties of the nervous tissue can also be modified by elevation of astrocytic density and thus their relations with neurons. In this study, FHJ506 administration significantly increased density of the hippocampal S100β+ astrocytes in P6s and a tendency to such increase in P30s. According to our unpublished results, FK506 augmented the brain-damage-induced proliferation of astrocytes. CsA did not show this property. However, in undamaged developing brain, FK506 could inhibit developmental apoptosis of astrocytes[17] and consequently increase their density. At the present stage of studies, it is difficult to judge whether this is a beneficial phenomenon.

Results at present and our recent study[8] question the data from the above-cited reports of different research groups and manufacturers of cyclosporine A (Sandimmune) or FK506 (Prograf), who presented the vehicle as a neutral substance. If this vehicle shows negative biological effects, it can aggravate unwanted effects of the drug, which is dissolved in it.

Long-term application of drugs in these pharmaceutical formulations, particularly after transplantations in pediatric patients can have serious negative consequences.[18–20] It cannot be resolved to what extent CsA or FK506 contributed to these effects and what was the contribution of vehicle. The effects could have summed up. It is also possible that the vehicle impaired positive neuroprotective action of CsA or FK506 in the adult brain.

The vehicle used in the pharmaceutical formulation of CsA or FK506 is a mixture of ethyl alcohol and castor oil. The observed changes can be attributed to the toxic effect of ethanol on the developing nervous system.[21] However, it is not an unequivocal conclusion, since Zharkovsky et al.[22] and Aberg et al.[23] observed that a slight amount of ethanol could stimulate hippocampal neurogenesis, thus having a positive effect. Our studies did not resolve this question; maybe the second component of the vehicle, namely castor oil, was responsible for the negative changes. So far, no comprehensive studies have been performed to determine its effect on the developing brain.

It can be concluded that both CsA and FK506, and their vehicle administered intraperitoneally in P6s or P30s may influence development of the hippocampus. Further studies should uncover functional significance of these changes.

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References and Notes

1. E. Aberg, C. P. Hofstetter, L. Olson, and S. Brené, *Int. J. Neuropsychopharmacol.* 8, 557 **(2005)**.
2. B. C. Albensi, P. G. Sullivan, M. B. Thompson, S. W. Scheff, and M. P. Mattson, *Exp. Neurol.* 162, 385 **(2000)**.
3. W. O. Bechstein, *Transpl. Int.* 13, 313 **(2000)**.
4. M. L. Buck, *Pediatr. Pharmacother.* 9, 1 **(2003)**.
5. S. P. Butcher, D. C. Henshall, Y. Teramura, K. Iwasaki, and J. Sharkey, *J. Neurosci.* 17, 6939 **(1997)**.
6. J. DeFelipe, *J. Chem. Neuroanat.* 14, 1 **(1997)**.
7. R. G. Giffard and R. A. Swanson, *Glia* 50, 299 **(2005)**.
8. C. Ikonomidou, P. Bittigau, and M. J. Ishimaru, *Science* 287, 1056 **(2000)**.
9. B. Kaminska, K. Gaweda-Walerych, and M. Zawadzka, *J. Cell Mol. Med.* 8, 45 **(2004)**.
10. D. O. Okonkwo and J. T. Povlishock, *J. Cereb. Blood Flow Metab.* 19, 443 **(1999)**.
11. G. Paxinos and C. Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press **(1986)**.
12. C. Ponticelli, *Ann. N. Y. Acad. Sci.* 1051, 551 **(2005)**.
13. R. Quinn, *Nutrition* 21, 775 **(2005)**.
14. Z. Setkowicz, M. Ciarach, R. Guzik, and K. Janeczko, *Epilepsy Res.* 61, 63 **(2004)**.
15. Z. Setkowicz and K. Janeczko, *Epilepsy Res.* 53, 216 **(2003)**.
16. Z. Setkowicz and J. Kadulski, *Int. J. of Neuroprotection and Neuroregeneration* 3, 239 **(2007)**.
17. R. H. Singleton, J. R. Stone, D. O. Okonkwo, A. J. Pellicane, and J. T. Povlishock, *J Neurotrauma* 18, 607 **(2001)**.
18. P. G. Sullivan, A. G. Rabchevsky, R. R. Hicks, T. R. Gibson, A. Fletcher-Turner, and S. W. Scheff, *Neuroscience* 101, 289 **(2000)**.
19. H. Uchino, R. Minamikawa-Tachino, and T. Kristian, *Neurobiol. Dis.* 10, 219 **(2002)**.
20. T. Yoshimoto and B. K. Siesjö, *Brain Res.* 839, 283 **(1999)**.
21. M. Zawadzka and B. Kaminska, *Glia* 49, 36 **(2005)**.
22. K. Zeller, J. Vogel, and W. Kuschinsky, *Brain Res. Dev. Brain Res.* 26, 200 **(1996)**.
23. T. Zharkovsky, A. Kaasik, K. Jaako, and A. Zharkovsky, *Brain Research* 978, 115 **(2003)**.

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