

Injections of vehicle, but not cyclosporin A or tacrolimus (FK506), afford neuroprotection following injury in the developing rat brain

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Abstract. Susceptibility of the injured rat brain to seizures depends on the developmental stage at which the injury had been inflicted. Our previous study shows that tacrolimus (FK506) and cyclosporin A (CsA) applied following the injury can also decrease or increase the seizure susceptibility in an age-dependent way. To find possible neuronal substrates of the effects, we examined influences of the agents on the injured brain and on its neuronal population. Rat brains were mechanically injured on postnatal days 6 (P6) or 30 (P30). Twenty minutes and 24 hours following the injury, FK506 or CsA were injected in clinically used pharmaceutical formulations (Prograf® or Sandimmun®, respectively). The brains were fixed on postnatal day 60 and processed for histological examinations. To detect if negative effects of the injury could be abolished by the treatments, we examined the brain weight, the size of the injured region, and the nerve cell density, including the density of calretinin- and parvalbumin-immunopositive cells. We have found that long-term effects of treatments with the FK506- and CsA-containing pharmaceutical formulations were never better than those of the vehicle alone (Cremophor and ethanol mixture). Moreover, the treatments could even amplify negative consequences of the injury alone. It could, therefore, be concluded that all the neuroprotective effects observed in the present study resulted exclusively from the influence of the vehicle alone. These effects of the brain injury and of subsequent treatments performed at different developmental stages were considered as possible determinants of further increase or decrease in susceptibility to seizures observed in adulthood.

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INTRODUCTION

Our previous study (Setkowicz and Janeczko 2003) showed that a mechanical brain injury in rats on post-natal days 6 (P6) or 30 (P30) changed in an age-dependent way the brain susceptibility to pilocarpine-induced seizures evoked in adulthood. When the injury was made in the younger 6-day-old group, the course of seizures showed no significant change while a similar injury in 30-day-old animals led to an extremely strong aggravation of the seizures accompanied with very high mortality (Setkowicz and Janeczko 2003). Therefore, in our next study, we attempted to check (Setkowicz et al. 2004) if a neuroprotective treatment following the injury would ameliorate the subsequent susceptibility to seizures in adulthood. For the experiment we selected two agents showing well-known neuroprotective actions: cyclosporin A (CsA) and FK506 (tacrolimus). CsA significantly decreases the infarct area in the experimental ischemia (Miyata et al. 2001) and promotes survival of affected neurons. In the injured brain, FK506 inhibits the neuronal death in the hypoxic cerebral cortex (Sharkey and Butcher 1994), reduces the volume of destroyed tissue following MCAO (Wakita et al. 1998) and traumatic brain injury (Buki et al. 1999, Kaminska et al. 2004, Okonkwo and Povlishock 1999).

In our previous above mentioned experiment (Setkowicz et al. 2004), adult rats treated with CsA following brain injuries on postnatal day 6 showed a significant increase in the intensity of pilocarpine-induced seizures and, consequently, much higher mortality. Rats injured on P30 displayed the epileptiform activity much longer but accompanied by better survivals. Following FK506 administration, rats injured on P6 showed a decreased duration of seizures and significantly increased survival. Thus, the observed changes depended not only on the agent itself but also on the developmental stage at which the brain was injured. It became evident that, in spite of previously defined neuroprotective action of FK506 or CsA (Uchino et al. 2002), the agents did not always abrogate the elevated susceptibility to pilocarpine-induced seizures following the brain injury. Moreover, significant aggravation of the susceptibility could sometimes be observed indicating rather worse functional ability of the brain following the apparently neuroprotective treatments.

To the best of our knowledge, FK506 or CsA have never been tested in the injured but still developing brain. Therefore, it appeared necessary to define what

general effects these agents would produce in this experimental situation. Our present study was particularly focused on neurons containing calcium-binding proteins – calretinin and parvalbumin – belonging to GABA-ergic neuronal system (Pothuizen et al. 2004) which could hypothetically determine the previously observed (Setkowicz et al. 2004) changes in seizure susceptibility. These proteins were characteristic of neurons showing relatively higher resistance to epilepsy-induced brain damages (Sloviter 1989).

METHODS

Animals

All animal-use procedures were approved by the Bioethical Commission of the Jagiellonian University in accordance with international standards.

Adult Wistar rats were obtained from an animal colony of the Institute of Pediatrics, Collegium Medicum, Jagiellonian University, Kraków and maintained under conditions of controlled temperature ($20 \pm 2^\circ\text{C}$) 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 h postpartum, the litters were reduced to ten. The young rats were weaned at 28 days of age.

Brain lesioning

Male 6- and 30-day-old rats (P6s and P30s, respectively) obtained from the females were anaesthetized with pentobarbital (Vetbutal, Biowet, Poland, 30 mg/kg i.p.). Under aseptic conditions, a lesion was made in the left cerebral hemisphere by insertion a rotating dental drill down to the white matter underlying the cerebral cortex, avoiding perforation of the whole thickness of the cerebral hemisphere wall. The drill was inserted at the level of the coronal suture and 2.5 or 3.0 mm lateral to bregma in P6s and P30s, respectively. The depth of drill penetration below the skull surface was limited to 2.0 and 3.0 mm, for P6s and P30s, respectively, by a plastic ring fixed on the drill. The diameter of the drill was 0.9 and 1.2 mm in P6s and P30s, respectively, to produce lesions of size proportional to the size of injured brain. Thereafter, the skin was sutured and animals were returned to their cages. Figure 1 shows location of the lesion in brains

of different age groups. This model of brain lesion was used in our previous studies (Janeczko 1989, 1994, Setkowicz and Janeczko 2003, Setkowicz et al. 2004).

FK506 or cyclosporin A administration

Twenty minutes after the injury, the rats were injected i.p. with 2 mg/kg of FK506 (Prograf®, Fujisawa) or with 20 mg/kg of cyclosporin A (CsA, Sandimmun®, Novartis). The injection was repeated 24 hours after the injury. Each of the pharmaceuticals was dissolved separately in saline containing 0.25% polyoxyl hydrogenated castor oil (Cremophor®, BASF, Germany) and 0.25% ethanol. Therefore, further in the text, when we refer to FK506 or CsA injections, we mean application of these agents as ingredients of clinically used pharmaceutical formulations, i.e. injections of Prograf® or Sandimmun®, respectively. Control rats were subjected to brain lesioning on P6 or P30 without any post-treatment or were injected with the vehicle alone. For comparative purposes, naive rats were also used. Neuroprotective efficacy of the above-indicated dosage of FK506 and CsA used in the present study has been tested by Uchino and coauthors (2002) and Sullivan and others (2000).

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 from the skull, the medulla was dissected, the brain was weighted, post-fixed overnight and embedded in paraffin. Ten μm -thick coronal sections were cut and mounted on slides covered with polylysine (Sigma).

Immunohistochemical procedure

The brain sections on slides were deparaffinized, hydrated, rinsed in Tris buffered saline (TBS, 0.05 M Tris (Sigma T-1378) in 0.9% NaCl, pH 7.6) (5 min) on a shaker table and treated with 1.0% H_2O_2 in TBS for 30 min. This was followed by another wash in TBS (3×5 min) and by digestion in a 0.1% trypsin solution (Sigma, T-4799) in TBS, pH 7.8 for 1 h. Subsequently, the slides were washed in TBS (3×5 min) and incubated 48 h at 4°C with primary antibodies: monoclonal anti-parvalbu-

min (Chemicon, MAB1572) or rabbit anti-calretinin (Chemicon, AB5054) dissolved in 0.5% Triton X-100-containing TBS, each at concentration 1:1000. Thereafter, the slides were washed in TBS (3×10 min) and incubated with secondary antibodies: goat anti-mouse (Sigma, M-8642) or goat anti-rabbit (Sigma, R-2004), respectively (1:100 in TBS, 1 hr at room temp.). Following a further wash in TBS (3×10 min), the sequence was completed by the addition of the mouse or rabbit PAP (Sigma, P-3039 or P-1291, respectively) (1:200 in TBS, 1 h at room temp.). The slides were then rinsed with TBS (3×10 min), and the sites of HRP binding were visualized using 0.02% 3,3'-diaminobenzidine-4HCl (Sigma) and 0.003% H_2O_2 in TBS. 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.

Nerve cell counting

Microscopic observations were carried out by an observer unaware of the previous treatment of the rats.

Two non-adjacent sections (from each brain at the level of the anterior commissure) were chosen for microscopic examination. In each section a standard zone around the lesion within the dorsal part of the cerebral hemisphere was delineated (Fig. 1). Age-dependent changes occurring in this zone following injury, including astroglial and macrophage/microglia

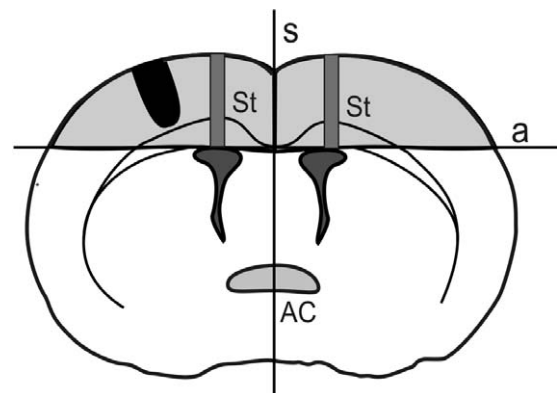


Fig. 1. Delineation of microscopically examined standard zones. (S) axis of symmetry of the brain section at the level of the anterior commissure (AC); (a) line perpendicular to the axis S passing through the top of lateral ventricle. Black area – the lesion site; shadowed areas – zones where calretinin or parvalbumin-immunopositive neurons were counted; (St) stripes of tissue where Nissl-stained neurons were counted.

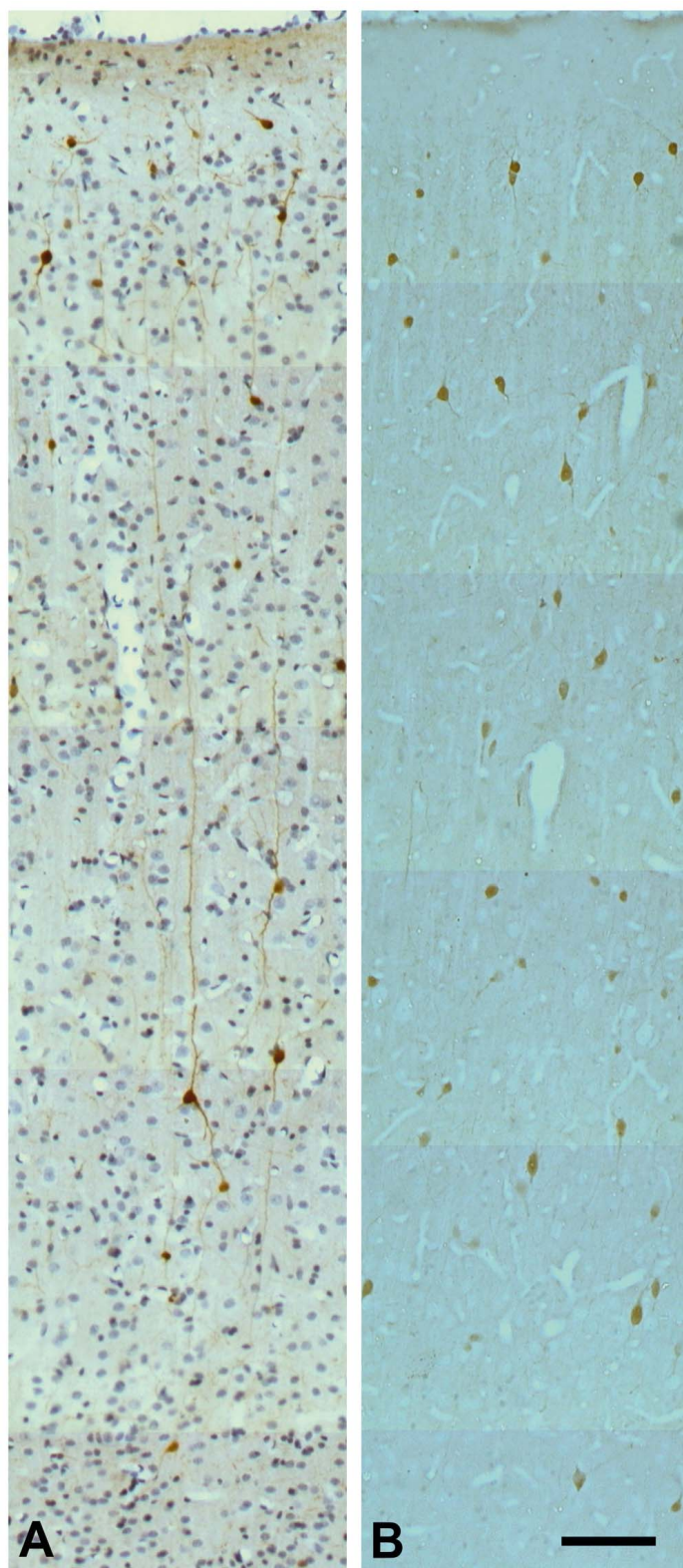


Fig. 2. Neurons immunoreactive for calretinin (A) and parvalbumin (B) in the examined part of the cerebral cortex. Scale bar shows 100 μm .

responses, have already been examined (Janeczko 1989, 1994, Janeczko et al. 1997, Setkowicz and Janeczko 1998) and their influence on the brain susceptibility to seizures has also been analyzed (Setkowicz and Janeczko 2003, Setkowicz et al. 2004).

Consequently, the same zone surrounding the lesion site has been chosen for the present study. Using a square frame-containing eyepiece, the zone was examined square by square to count pericaria of all parvalbumin (PV+) or calretinin-immunopositive (CR+) neurons (Fig. 2). Under oil immersion, at a magnification of 1000 $\times$, the frame delimited a $100 \times 100 \mu\text{m}$ area. All immunopositive neuronal pericaria located within the exclusion lines of the frame, were counted. For comparative purposes, the immunopositive pericaria were counted in a similar zone located in the right, uninjured cerebral hemisphere (Fig. 1). Diameters of the pericaria did not vary among the groups.

The area of each examined zone was also measured using a 2.5 $\times$ objective on a Nikon Microphot SA microscope mounted with a JVS colour camera connected to a computer image analysis system. The area was multiplied by the thickness of the section to obtain the volume of examined tissue. Finally, the density, (number) of immunopositive cells per 1 mm^3 of the tissue volume was calculated.

To detect possible quantitative changes in the whole neuronal population, Nissl-stained neurons were counted on two non-adjacent sections taken at the level of the anterior commissure from each brain. The counting was limited to stripes of tissue located between the lateral ventricle and the surface of each cerebral hemisphere as shown in Fig. 1. The width of the stripe was 200 μm , which is equal to twice width of the frame used during the counting (100 μm). The nucleolus surrounded by the rim of neuronal nucleus was used as the unit of count, i.e. one Nissl-stained neuron. Two nucleoli found within the same nucleus were understood to indicate a single neuron. The percentage of neurons having two nucleoli was very low and similar in each examined group. The diameter of nucleoli did not vary among the groups. The nucleoli located within the exclusion lines of the counting frame were counted under oil immersion and their numbers per the tissue strip were assessed. Finally, the volume of the stripe was determined and the cell number was calculated per a standard volume of 1 mm^3 .

As mentioned above, diameters of all the types of counted object (i.e. CR+ and PV+ pericaria and nerve

cell nucleoli) did not vary between the examined groups. Therefore, they could not influence quantitative relations between the groups including results of statistical tests (Gittins and Harison 2004, Oorschot 1994). Nevertheless, to obtain possibly most actual values of the cell density, we applied the Abercrombie correction factors calculated separately for each group and for each type of the counted objects.

The analysis does not take into account a significant tissue shrinkage following fixation and dehydration procedures (Dorph-Petersen et al. 2001, Tramontin et al. 1998). In fact, however, this study is not focused on the assessment of exact cell density but on differences existing between the examined groups in cell density, which, obviously, is affected by a systematic error (Coggeshall and Lekan 1996, Gittins and Harison 2004).

Brain section measurements

All the measurements were carried out on the histological material from brains of 60-day-old rats which were injured on P6 or P30, i.e. 54 or 30 days earlier, respectively, and subsequently subjected to different treatments.

After the 54- or 30-day-long survival periods (in P6s and P30s, respectively) apoptotic or necrotic neurons disappeared and the lesion cavity could totally be closed with the collapsing but still growing tissue. After the injury made on P6 a locally lowered cortical surface was the only marker of the former lesion site. Control examination of this region immediately after the injury proved that the lesioning technique was effective producing brain injuries of relatively similar sizes in each age-group. A quantitative assessment of long-term changes in the size of the lesion cavity (possible reduction of necrotic changes following neuroprotective treatments) could not be carried out similarly as that following lesions made in adult brains. Therefore, in each examined brain section we measured the surface area of the region of injury delineated within the dorsal part of cerebral hemisphere as it is shown in Fig. 1. For comparative purposes, the surface area of the corresponding zone delineated in the right, non-injured hemisphere was also measured.

Statistical analysis

Statistical analyses were 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.

To detect absolute ranges at which the injury alone and FK506 or CsA, or their vehicle, influenced the brains, we tested statistically values of all the above described parameters in five groups including the following numbers of rats injured on postnatal days 6 or 30,

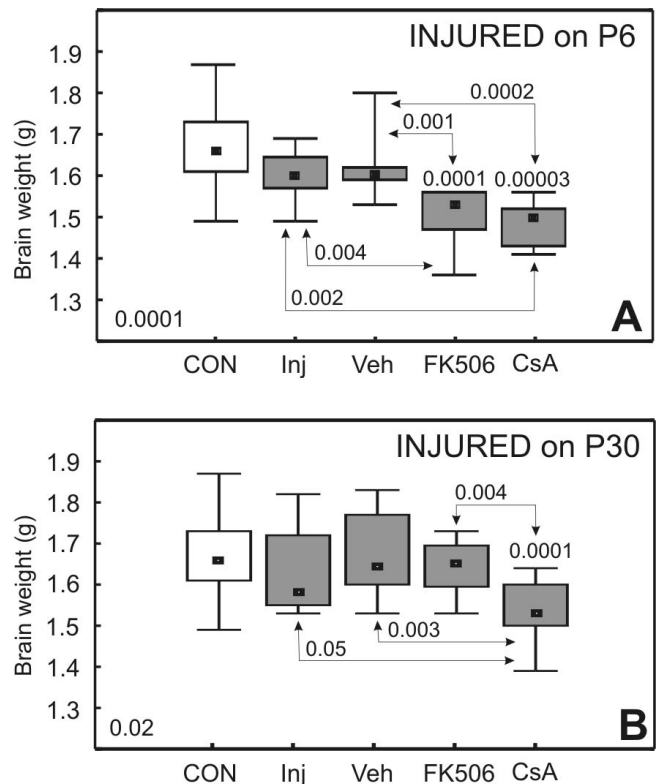


Fig. 3. Changes in the brain weight. Rats injured on postnatal days 6 (A) or 30 (B). Abbreviations for Figs 3–5; (CON) control rats; (Inj) injured but untreated rats; (Veh), (FK506), (CsA) rats treated with vehicle alone or with FK506 or CsA, respectively. The box and whisker diagram shows the median (small black rectangle in the box), the 25–75% variability range (large box), and maximal and minimal values (whiskers). (N.S.) difference 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 lower left corner of the diagram shows statistical significance of differences between the examined groups (Kruskal-Wallis test).

respectively: (1) control non-injured ($n=13$), (2) with the brain injury alone without any additional treatment ($n=8$ and 10), (3) with the brain injury followed by injections of the vehicle alone ($n=10$ and 10) or by injections of (4) FK506 ($n=9$ and 8) or (5) CsA ($n=10$ and 15) dissolved in the vehicle.

RESULTS

Changes in the brain weight

In rats injured on P6, we did not observe statistically significant changes in the brain weight evoked by the mechanical injury alone (Fig. 3A) However, brains from animals injured on P6 and injected with FK506 or CsA had brain weights lower not only than those in naive rats ($P<0.0001$ and $P<0.00003$, respectively) but also in comparison to the remaining two groups with brain injury alone ($P<0.004$ and $P<0.0002$, respectively) or injured and treated

with the vehicle ($P<0.001$ and $P<0.0002$, respectively).

Like in rats injured on P6, in animals injured on P30, neither the injury alone nor a subsequent vehicle or FK506 injection changed the brain weight in comparison to naive controls (Fig. 3B). A considerable decrease, however, was observed exclusively in injured and CsA-injected rats in relation to all the remaining groups (P values between 0.05 and 0.0001).

It is noteworthy that neither the average body weight nor the brain-to-body weight ratio were affected in any of differently treated animal groups.

Changes of the size of injured region

None of all the quantitative assessments of effects induced by the brain injury or by subsequent treatments revealed statistically significant differences between injured and non-injured cerebral hemispheres.

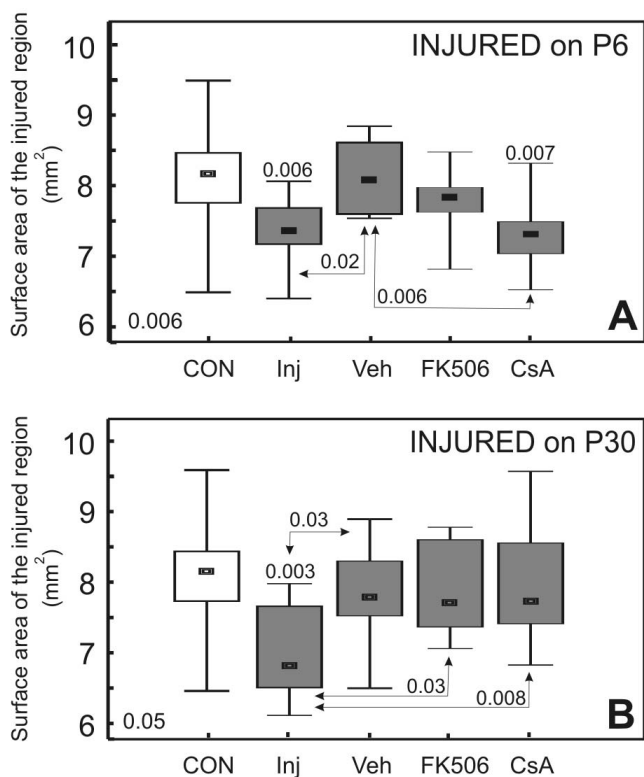


Fig. 4. Changes in the total surface area of the injured region at the level of the anterior commissure, shown schematically in Fig. 1. Brains were injured on postnatal days 6 (A) or 30 (B) and were examined on P60. For further explanations see Fig. 3.

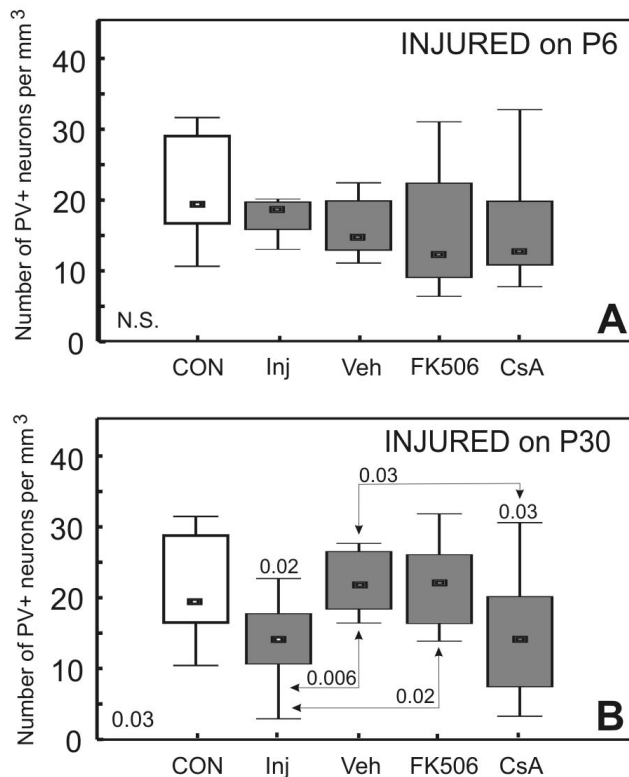


Fig. 5. Changes in the number of parvalbumin-immunopositive neurons per 1 mm^3 calculated for the total surface area of the injured region at the level of the anterior commissure shown in Fig. 1. Brains were injured on postnatal days 6 (A) or 30 (B) and were examined on P60. For further explanations see Fig. 3.

Measurements carried out on coronal brain sections in each group of differently treated rats, injured on P6 or P30, detected no significant difference between surface areas of the zone representing the injured region in the left cerebral hemisphere and a similar zone in the right, non-injured cerebral hemisphere.

Areas of the examined zones underwent significant changes depending on the different animal treatment using FK506 or CsA dissolved in the vehicle, or the vehicle alone.

Sections taken from brains of rats injured on P6 but subsequently untreated showed a significant decrease of the injured region area ($P < 0.006$, Fig. 4 A). When vehicle alone was applied following the injury, the injury-induced decrease of the surface area disappeared. Application of FK-506 did not cause additional effect, while the CsA abolished the effect of vehicle administration ($P < 0.006$) and the surface area remained significantly decreased ($P < 0.007$) similarly as in the injured but untreated rats.

The mechanical injury performed in 30-day-old rats led also to significant reduction of the injured region when examined on P60 ($P < 0.003$; Fig. 4B). This reduction was abolished by vehicle and FK506 or CsA had no additional effect.

Calretinin-immunopositive (CR+) neurons

Neither the brain injury inflicted to 6- or 30-day-old rats nor any subsequent treatment changed the density of CR+ neurons found in the examined parts of each cerebral hemisphere.

Parvalbumin-immunopositive (PV+) neurons

The brain injury on P6 did not modify the density of PV+ neurons in adulthood. Treatments after the injury with FK506, CsA or the vehicle alone had also no additional effects (Fig. 5A).

Following the injury performed on P30, the density of PV+ neurons significantly decreased in non-treated rats ($P < 0.02$, Fig. 5B). In response to vehicle injections, the density of PV+ neurons became significantly higher ($P < 0.006$) and reached the level of control, non-injured brains. Application of FK-506 had no additional effect, while the CsA abolished the effect of vehicle administration ($P < 0.03$) and the surface area remained significantly decreased similarly to that in injured but untreated rats.

Nissl-stained neurons

No significant difference could be detected between two hemispheres in respect to the total number or the density of Nissl-stained neurons in any of the examined groups. Moreover, none of the differently treated rat groups injured on P6 or P30 showed any statistically significant change in the neuronal population.

Because of the lack of statistically significant interhemispheric differences it can be concluded that all the above described morphological and quantitative neuronal changes occurring in the left cerebral hemisphere injured on P6 or P30 including those following subsequent treatments were closely reflected by parallel changes in the respective contralateral region.

DISCUSSION

In our previous study (Setkowicz and Janeczko 2003), we reported that injuries of the rat brain at different developmental stages (postnatal days 6 or 30) led to different changes in susceptibility to pilocarpine-induced seizures when observed in adulthood. Further behavioral study (Setkowicz et al. 2004), proved that treatments with FK506 or CsA could change the susceptibility to seizures depending on the age at which the rats were injured. In that study, each of the neuroprotective agents was applied as the active ingredient of pharmaceutical formulation of Prograf® or Sandimmun®, respectively. The vehicle itself used in the formulations was understood to be a biologically neutral substance (Zawadzka and Kaminska 2005). Generally, in our previous study (Setkowicz et al. 2004), in FK506-treated rats the intensity and duration of seizures were reduced. Conversely, postlesional treatment with CsA aggravated the course of seizures evoked in adulthood. It seems reasonable to stress here that the reported aggravation of the course of seizures could not be a direct effect of CsA seizuregenic action (Setkowicz and Ciarach 2006) since CsA was administered just after the brain injury (performed on P6 or P30) but susceptibility to pilocarpine-induced seizures was tested when the injured brain became 60-day-old.

To obtain preliminary information on possible neural determinants of the observed behavioral phenomena, we defined long-term general effects of the brain injuries themselves. Thereafter, we attempted to

answer the question how the effects could be modified by FK506 or CsA administration after the injuries. The drugs were administered within a therapeutic window ensuring their effectiveness (Uchino et al. 2002). The developing brain-blood barrier was not critical for age-dependent changes in availability of the drugs in the brain since it was disrupted by the injury.

Generally, the present study shows the first evidence that treatments with clinically used pharmaceutical formulations of FK506 (Prograf®) or CsA (Sandimmun®) had significant, long-term effects on the injured and developing rat brain. Unexpectedly, it became also evident that effects of the formulations (i.e. mixtures of the neuroprotective agents with the vehicle) were never better than those of the vehicle alone but, surprisingly, they could be even worse (significant decreases in the brain weight). It could, therefore, be concluded that all the positive effects observed in the present study resulted exclusively from the influence of the vehicle alone. Since FK506 or CsA were used as dissolved in the vehicle, further discussion on their effects always refers to the whole clinically used pharmaceutical formulations in which they were applied.

The vehicle used in the pharmaceutical formulations consists of ethanol and Cremophor (modified castor oil) dissolved in physiological saline. In rats injected with the vehicle alone after the brain injury on P6, the brain weight was similar to controls but injections of FK506 or CsA dissolved in the same vehicle significantly decreased the brain weight. Moreover, in consequence of the vehicle injections, the injured region, which was much smaller in the rats receiving the brain injury alone on P6 or P30, preserved its original size. Finally, the vehicle injections led to respective quantitative preservation of PV+ neurons reduced in untreated brains injured on P30. Thus, surprisingly, the vehicle itself had effects which could rather be expected after CsA or FK506 administration or even better. Similar effects have not been observed when Cremophor alone was administered to the ischemic brain (Sharkey and Butcher 1994, Toung et al. 1999). However, those studies were performed on adult rats and the infarct area was measured without counting surviving neurons.

Since, the vehicle used in the present study contained also 0.25% ethanol, possible effects, even neuroprotective, of the small dose could not be excluded. It was observed in the traumatic brain injury model, where application of ethanol before injury lowered

neurodegeneration within the hippocampal CA1 and CA3 regions (Tureci et al. 2004). Small dose of ethanol inhibited also NMDA-induced excitotoxic damage in the injured nervous tissue (Tureci et al. 2004). Following ethanol inhalation applied to neonatal rats, the population of PV+ cortical interneurons remained unaffected as well as the whole population of neurons containing calcium-binding proteins while CR+ neurons were almost doubled (Granato 2006).

Our observations show clearly for the first time that this defined mixture of Cremophor and ethanol administered to the injured but still developing brain had neuroprotective effects. However, when this mixture served as vehicle for FK506 or CsA, the effects were much weaker, did not occur or were even negative. Unfortunately, it was impossible to test this supposition and assess pure effects of these agents since they are water insoluble.

The data obtained in the present study appear to disagree with those which could be expected following application of any drug defined as neuroprotective. However, *in vivo* positive influences of FK506 or CsA have been previously observed only in adult brains (Sharkey and Butcher 1994, Wakita et al. 1998, Zawadzka and Kaminska 2005) but never in those injured at so early developmental stages. Therefore, in the present study age-dependent cytotoxic effects of the agents cannot be excluded. However, *in vitro*, FK506 and CsA could be neuroprotective for embryonic neurons (Avramut et al. 2001, Castilho et al. 2000). The above presented facts indicate that prolonged immunosuppressive treatments with FK506 or CsA in children as well as in adults may evoke pathological changes in the brain (Bartynski et al. 2001, 2004, Bechstein 2000, David-Neto et al. 2000). It might be reflected by headaches, altered mental functioning, tremors, cerebellar syndromes, reversible leukoencephalopathy, increased risk of tumour growth (Gijtenbeek et al. 1999, Wijdicks 2001) or even seizures (Gaggero et al. 2006). Such negative influences might underlie the previously observed increase in susceptibility to seizures and shorter survivals in CsA-treated rats injured on P6 (Setkowicz et al. 2004).

Susceptibility to seizures can often be a consequence of dysregulation of neuronal inhibitory mechanisms. Therefore, in the present experiment we tried to detect how FK506 or CsA could affect further development of PV+ or CR+ interneurons in the injured

brain. Their mostly non-overlapping populations of different origin constitute the majority of GABA-ergic inhibitory interneurons (Andressen et al. 1993, Xu et al. 2003) and could play important roles in the control of pyramidal cell excitation (DeFelipe 1997) and regulation of epileptiform activity in the brain (Mihaly et al. 1997).

In the present study, the animals injured on P6 and treated with FK506 or CsA displayed no significant changes in density of CR⁺ or PV⁺ cells. It appears to suggest that the mechanical injury performed at this age, contrary to the injury by injection of trimethyltin (TMT) (Geloso et al. 1997, 1998), did not selectively affect neurons containing these Ca⁺⁺-binding proteins. In this age group, no significant injury-induced change in the density of Nissl-stained neurons was also found. Moreover, in respect to this neurons, no difference was detected between the left, injured and the right, non-injured cerebral hemispheres.

The injury alone in 30-day-old animals did not affect the population of CR⁺ neurons but evoked a considerable quantitative deficit of PV⁺ neurons. The deficit could functionally impair inhibitory neuronal functions in the cerebral hemisphere being also a possible determinant of its increased susceptibility to seizure-genic stimuli like pilocarpine (Setkowicz and Janeczko 2003). According our present results, a mixture of FK506 and the vehicle (Prograf®) prevented the population of PV⁺ neurons from quantitative reduction, even if FK506 had no its own additional influence on the phenomenon. On the contrary, a similar mixture of CsA (Sandimmun®) did not abolish the injury-induced reduction. Therefore, previously observed alleviation or aggravation of susceptibility to seizures in the injured brains (Setkowicz et al. 2004) might be correlated with changes resulting from the two different treatments.

We were aware that the observed significant decreases in numbers of immunocytochemically detected PV⁺ neurons might result not only from virtual quantitative reduction their populations but also from a lower neuronal content of parvalbumin in differently treated brains. However, the latter, if occurred, could also modify functional features of the nervous tissue.

The general and very interesting feature of all the observed reactive changes is their parallel occurrence in investigated regions of both injured and non-injured

cerebral hemispheres. Conversely, in the same experimental condition following the brain injury on P6 or P30, the astrocyte response clearly dominates in the injured region, when compared to the corresponding contralateral areas (Janeczko et al. 1997, Setkowicz and Janeczko 1998, Setkowicz et al. 1999). Permanent results of the glial response (Markiewicz and Lukomska 2006), including age-dependent formation of the glial scar (Janeczko 1994), have already been considered as determinants of epileptogenesis.

The above described age-dependent differences in effects of FK506 or CsA on neurons might have numerous molecular determinants. Both agents block calcineurin (involved in several forms of cell death) but bind to different proteins (Castilho et al. 2000) which may undergo their own development modifying potentials of CsA or FK506 actions in subsequent post-natal periods.

CONCLUSION

The present study shows, for the first time the positive changes evoked by administration of the vehicle alone. Their functional importance remains to be tested using the model of pilocarpine-induced seizures which has already been applied to examine effects of FK506 or CsA on the injured developing brain (Setkowicz et al. 2004). Further studies of long-term effects of the drugs on the developing but non-injured brain may better explain phenomena reported in the present study.

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