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Effect of tacrolimus (FK506) on ischemia-induced brain damage and memory dysfunction in rats

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Abstract

The behavioral and neurohistological protective effects of tacrolimus (FK506) were examined in rats subjected to 15-min global forebrain ischemia. Learning and memory performance were evaluated in an aversive, non-food-motivated, eight-arm radial maze. In one experiment, naive rats were rendered ischemic, and 15 days later they were tested for acquisition of a spatial task (postoperative training). In a complementary experiment, rats were trained for 8 days and then subjected to ischemia (preoperative training); 15 days later (on Day 24 of testing) they were retested for retention of cognition. FK506 (1.0 mg/kg) was given intravenously at the beginning of reperfusion, followed by doses applied intraperitoneally 6, 24, 48 and 72 h postischemia. Behavioral performance was expressed by latency to find the goal box, and number of errors. Ischemia did not affect acquisition performance. In contrast, retention of cognition was markedly impaired by ischemia, particularly working memory ($P < .05–.001$). This ischemia-induced, retrograde amnesia was significantly reduced by FK506 compared to vehicle alone on Day 24, as measured by latency and working memory errors ($P < .025$). A neuroprotective effect of FK506 was also seen on working memory, when postischemic performance was compared to that prior to ischemia ($P > .05$, Day 24 vs. Day 8, paired samples), in contrast to the significant, retrograde amnesia found in the ischemic, vehicle-treated group ($P < .01$). FK506 also significantly reduced the extent of hippocampal CA1 cell loss; however, this effect did not correlate with behavior. The present results suggest that the histological, neuroprotective effect of FK506 may be accompanied by a reduction in cognitive impairment, as assessed in a novel, non-food-motivated, eight-arm radial maze after transient, global, cerebral ischemia in rats.

Keywords: Cerebral ischemia; Neurodegeneration; FK506; Neuroprotection; Learning and memory; Radial maze

1. Introduction

The brief interruption of cerebral blood flow, such as occurs during reversible cardiac arrest, causes permanent brain damage and behavioral dysfunction (Zola-Morgan et al., 1986; Petito et al., 1987). Differing degrees of brain injury and neuropsychological sequelae are also expected in humans subjected to cardiopulmonary bypass surgery (Cummings et al., 1984; Sudo et al., 2001). Despite promising results obtained in animal models, no pharmacological strategy has been clinically effective in counteracting the outcome of cerebral ischemia (Gladstone et al., 2002).

In evaluations of neuroprotectors, the use of immunophilin ligands is considered to be a novel and potentially useful strategy. The importance of immunophilins in the development of neuroprotectors emerged from observations that tacrolimus (FK506), a potent immunosuppressant used in organ transplants, provides neuroprotection against glutamate-induced neurotoxicity in vitro (Dawson et al., 1993). Several subsequent studies performed in animal models of focal (Sharkey and Butcher, 1994; Butcher et al., 1997; Arii et al., 2001) or transient, global forebrain ischemia in vivo (Ide et al., 1996; Tokime et al., 1996; Yagita et al., 1996; Drake et al., 1996) revealed its neuroprotective properties. Recently, we extended these findings to the 4-VO occlusion model of transient, forebrain ischemia in rats and suggested that the neuroprotective efficacy of FK506 can be sustained over time (Giordani et al., 2003). Since FK506 easily crosses the blood–brain barrier (Yoshimoto and Siesjo,

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1999) and is available for human use, the above data suggest that clinical trials of FK506 in patients with cerebral ischemia may be promising. However, additional studies using animal models of ischemic brain damage are needed.

It has been widely emphasized that morphometric end points are insufficient to reveal accurately the neuroprotective potential of drugs (Corbett and Nurse, 1998; Hunter et al., 1998). It is thus important that neurohistological analyses be complemented by measurements of behavioral function before assuming that a given drug may be clinically useful. The beneficial effect of FK506 in reducing the functional deficits that follow brain damage has already been demonstrated in focal brain ischemia (Sharkey et al., 1996) and chronic cerebral hypoperfusion subsequent to permanent 2-VO (Tanaka et al., 2001). Chronic treatment with a nonimmunosuppressive analogue of FK506 (GPI 1046) also improves the retention performance of aged rats in the water maze task (Sauer et al., 1999). However, despite various works reporting on the neuroprotective effect of FK506 at the histological level, apparently no study has investigated whether FK506 reduces the cognitive disruption caused by global, transient, forebrain ischemia, including that produced in the rat 4-VO model. If FK506 prevents pyramidal cell death in the hippocampus, this agent should reduce the memory impairment usually associated with global ischemia. In the present study, we investigated whether the neuroprotective effect of FK506 at the histological level might be accompanied by improvement of ischemia-induced learning and memory impairment as tested in an aversive version of the eight-arm radial maze (Paganelli et al., 2004).

2. Experimental procedures

2.1. Animals

Adult male Wistar rats weighing 270–300 g were used. The rats were housed in groups of three to four in plastic cages (39 × 33 × 16 cm) at a controlled temperature (22 ± 1 °C) on a 12-h light/dark cycle (lights on at 0700 h) with constant air renewal. Food and water were offered ad libitum. These housing conditions were maintained until the end of the experiments.

2.2. Ischemia

Transient, global forebrain ischemia was induced using the 4-VO method (Pulsinelli and Brierley, 1979) with modifications (Milani et al., 1998). The vertebral arteries were bilaterally electrocoagulated under ether anesthesia plus the local application of 2% xylocaine. The carotid arteries were then loosely snared with a silk thread, and 5 to 6 h later the thread was gently tightened for 15 min. Loss of the righting reflex within 2 min of carotid occlusion, unresponsiveness to gentle touch, mydriasis and tonic extension of the paws were considered indicative of effective ischemia. Core temperature was monitored but not controlled during ischemia and up to 3 1/2 h of reperfusion, using a rectal probe inserted to approximately 6 cm. Sham-operated animals were submitted to the same manipulations, except for vertebral and carotid artery occlusion.

2.3. Drug treatment

FK506 (1.0 mg/kg) was given intravenously at reperfusion, followed by intraperitoneal injections applied 6, 24, 48 and 72 h postischemia. Repeated FK506 application, in contrast to a single-injection regimen, reduces ischemia-induced, CA1 pyramidal cell loss, an effect sustained up to 30 days after ischemia (Giordani et al., 2003). Ischemic control animals received vehicle alone (0.1 ml/100 g body weight). Sham-operated rats received no treatment. Both FK506 (solution, 10 mg/ml ampoule) and vehicle (polyoxyethylenehydrogenated castor oil 60 and anhydrous ethanol) were kindly supplied by Fujisawa Pharmaceutical, Osaka, Japan.

2.4. Apparatus

We used an aversive version of the unconfined, eight-arm radial maze as described elsewhere (Paganelli et al., 2004). In this maze, eight arms (55 × 15 cm) radiate from alternate sides of a 16-sided central, polygonal platform (71 cm in diameter). At the end of each arm, an opening 9 cm in diameter provides access to a darkened wooden box (23 × 11 × 9.5 cm) that can be inserted and removed like a drawer below any opening, serving as a refuge for the rat (the goal box). Of the eight arms, only one contained the true refuge; in the remaining arms, the boxes were open-ended. Rails 2.5 cm in height bordered each arm to prevent the animal from falling. The rotatable maze was elevated 90 cm above the floor on a metal stand. The start box was a dark, open-ended cylinder positioned in the center of the maze. From a separate room, a pulley system allowed the experimenter to raise the start box and release the animal. Several extramaze cues were available in the room. A small ventilator located on the floor generated constant noise in the testing room throughout the experiment. Two spotlights of 200 W each plus a pair of ordinary incandescent lamps (40 W each) were fixed on the ceiling 180 cm above the maze. The video camera was positioned 220 cm away from, and 130 cm above, the maze.

2.5. Behavioral procedures

2.5.1. Postoperative acquisition trial (Experiment 1)

Rats were examined for acquisition performance postoperatively. They were randomly assigned to one of the following treatments: (1) sham operation, (2) ischemia + vehicle and (3) ischemia + FK506. Seventeen days after ischemia, the animals were habituated to the testing appa-

ratus. They were placed individually and directly in the center of the maze, which they were allowed to explore until finding the goal box or a 4-min period had elapsed. If the goal box was not found within 4 min, the rat was placed into the arm containing the correct goal box and gently forced to enter it by the experimenter. The rat remained in the goal box for 4 min, after which it was returned to its home cage. During habituation, the extramaze cues were removed and the spatial position of the goal box was randomly changed between subjects. The habituation procedure was repeated for 3 days. On the 4th day, the extramaze cues were replaced and acquisition training was started. The rat was placed into the start box through the open top and released 30 s later. When the animal found and entered the goal box it was allowed to stay there for 1 min. If the goal box was not found within 4 min, the rat was placed into the correct arm and gently forced to enter the shelter. Acquisition training was carried out using a schedule of three trials per session, one session per day, for 8 days (from Day 20 to Day 27 postischemia). From trial to trial the maze was cleaned of excrement and randomly rotated on its central axis; the goal box was randomly moved to any of the other seven arms, but its spatial position in relation to the extramaze cues was kept unchanged across trials and sessions, and was the same for all rats. Behavioral performance was measured by latency to find the goal box, the number of reference memory errors and the number of working memory errors. Within a given trial, a reference error was counted when the rat visited an arm containing a false goal box for the first time. However, if the rat returned to an arm that had been visited previously during that trial, a working memory error was registered. An arm was considered visited when the rat entered halfway down the arm. The animal was considered to have left an arm when it placed all four paws on the central platform.

2.5.2. Postoperative retention trial (Experiment 2)

Naive, intact animals were habituated and trained for acquisition of the spatial task for 8 days, as in Experiment 1. On the day after the last training session, the rats were subjected to sham operation, ischemia + vehicle or ischemia + FK506 as in Experiment 1. The rats recovered from surgery for 15 days (24th experimental day) when they were tested for retention of cognition acquired during the training sessions from Days 1 to 8. The results of this experiment have been reported in part elsewhere (Paganelli et al., 2004).

2.6. Histological analysis

One day after behavioral testing, the animals were deeply anesthetized with ether and perfused transcardiacally with 0.9% saline followed by Bouin's fixative (20 ml/min for 7–10 min). Following decapitation, the head was immersed in crushed ice (1–2 °C) for 1 h. The brain was then carefully removed and fixed in Bouin's fluid for 3 days. Eight to 12 paraffin-embedded, coronal sections (5-μm thickness) were taken from each brain at a level corresponding to approximately 4.52 mm posterior to bregma and stained with celestine blue/acid fuchsin. Three well-stained coronal sections were chosen for bilateral counts of normal-appearing neurons in the dorsal portion of the CA1 subfield. In each hemisphere, the number of intact-appearing pyramidal cells showing a distinct nucleus and nucleolus was counted along a transect of 1.35 mm length (magnification 400×, field diameter = 450 μm, Olympus). The number of pyramidal cells in each rat was expressed as the mean of the three coronal sections. The identity of the groups was not revealed during histological assessment. Experimental procedures followed the principles set down by the Brazilian College of Animal Experimentation (COBEA).

2.7. Data analysis

The acquisition performance was analyzed using a multifactorial analysis of variance for repeated measures (MANOVA) with groups as the between-subjects and sessions as the within-subjects factors (StatGraphics Plus 6.0). In the case of a significant group effect, Duncan's multiple range test was used to distinguish among groups. For each individual, the mean value from three trials per session (day) was used. Retention performance measured 2 weeks after ischemia was analyzed by both Student's *t* test (paired sample comparisons, Day 8 vs. Day 24) and one-way analysis of variance, followed by Duncan's multiple range test (unpaired sample comparisons on Day 24). The degree of ischemia-induced, CA1 pyramidal cell loss was also quantified by one-way analysis of variance, followed by Duncan's test. Correlation between the number of pyramidal cells remaining in the CA1 subfield and behavioral deficit in the ischemic rats was analyzed using Pearson's correlation test. The sham-operated animals were not included in the correlation analysis since their brains were not damaged.

3. Results

Fig. 1 shows acquisition performance for latency (A), number of working memory errors (B), and number of reference memory errors (C) measured after ischemia. The MANOVA did not reveal a significant group effect for any of the behavioral parameters [$F(2,35) = 0.56–1.40$; $P > .05$]. However, a significant session effect for all parameters was detected [$F(7,237) = 2.74–5.47$, $P < .01–.0001$]. There was no significant group vs. session interaction [$F(14,237) = 0.61–1.02$, $P > .05$]. Thus, the rate at which latency and the number of errors decreased over time was similar in the three groups, indicating that animals subjected to ischemia also improve with training.

Fig. 2 illustrates latency to find the hidden goal box (A), and the number of working (B) and reference (C) memory errors registered during the preoperative training days (from

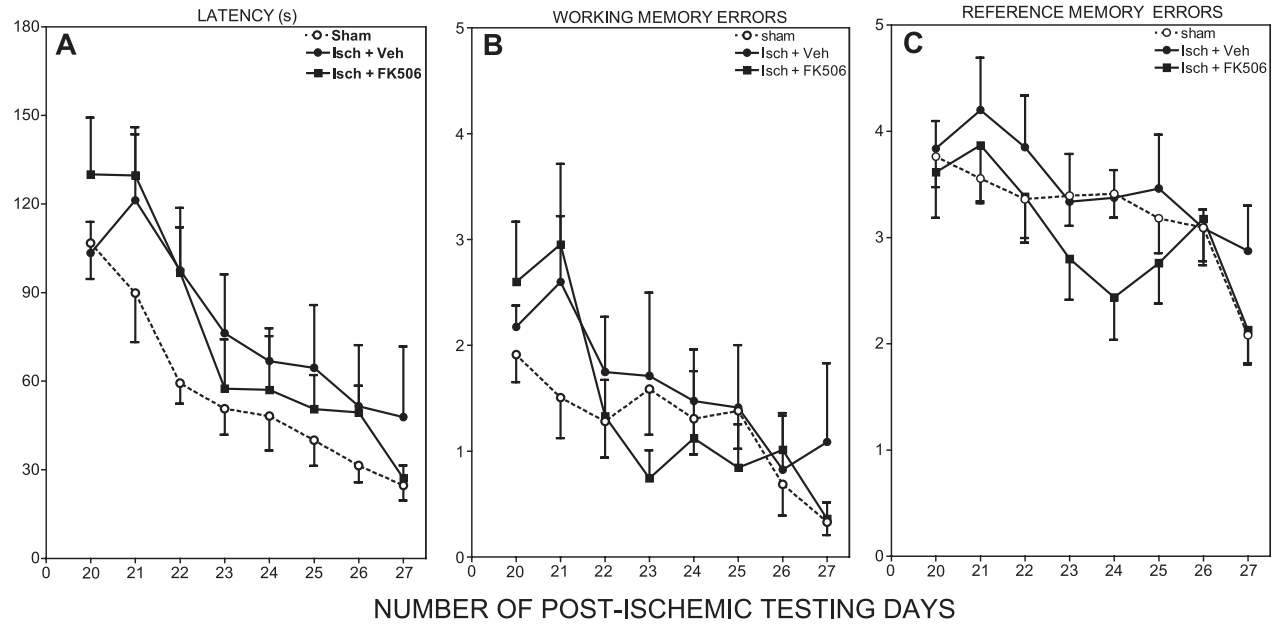

Fig. 1. The effect of FK506 (1.0 mg/kg, 1 injection iv + 4 injections ip) on acquisition performance of rats subjected to 15 min transient forebrain ischemia and tested in the aversive radial maze. FK506 was given 0, 6, 24, 48 or 72 h postischemia. For each individual, the mean of the values obtained from three trials in each session (day) was used to express performance in terms of latency to find the goal box (A), number of working memory errors (B) and number of reference memory errors (C). The analysis of variance revealed no group effect. Values are the mean ± S.E.M. Sample sizes: Sham = 18; Isch + Veh = 8; Isch + FK506 = 13.

Day 1 to Day 8) and postoperative testing day (on Day 24). Before ischemia, the groups assigned to each treatment exhibited similar latencies and number of errors during acquisition training [group effect, $F(2,35) = 0.01 - 0.27$, $P > .05$]. A significant session effect was revealed by the MANOVA [$F(7,245) = 4.08 - 26.71$, $P < .01 - .0001$], indicating that the rats learned the task very well. There was no significant Group × Session interaction for any of the

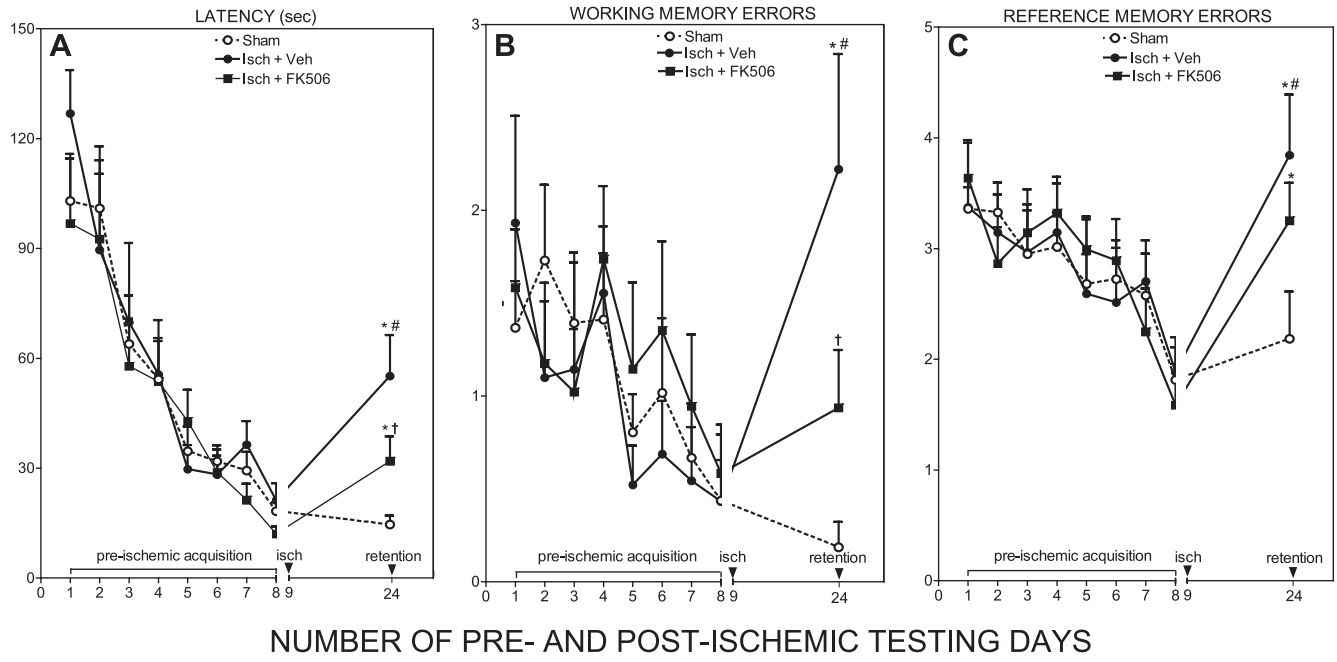

Fig. 2. The effect of FK506 on the ischemia-induced disruption of memory retention in the aversive radial maze. The rats received three trials per day for 8 days before ischemia. On Day 9, ischemia was induced for 15 min, and 15 days later the rats were retested for latency (A), number of working memory errors (B) and number of reference memory errors (C). Values are mean ± S.E.M. Sample sizes: Sham = 16; Isch + Veh = 9; Isch + FK506 = 13. * $P < .05$ (paired t test, Day 24 vs. Day 8); # $P < .001 - .025$ (vehicle vs. sham); † $P < .025$ (vehicle vs. FK506).

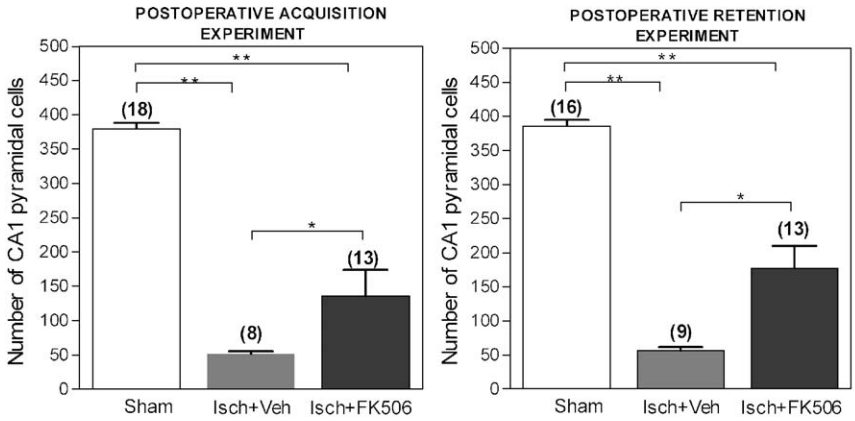

Fig. 3. Effect of FK506 on ischemia-induced, hippocampal pyramidal CA1 cell loss. Cells were counted along a transect 1.35 mm in length. Histological analysis was performed 1 day after the end of behavioral testing. The numbers in parentheses refer to sample size. Values are the mean ± S.E.M. * $P < .001–.025$; * * $P < .0001$.

parameters analyzed [$F(14,245) = 0.61–1.02$, $P > .05$]. When these rats were retested 15 days after ischemia (on Day 24), significant disruption of memory storage was revealed in the vehicle-treated group for all parameters (paired *t* test, Day 24 vs. Day 8, latency; $P = .009$; working errors: $P = .024$; reference errors: $P = .005$). The amnesic effect of ischemia was also evident when a one-way ANOVA was used for between-group comparisons on Day 24 [main effect of group: $F(2,35) = 3.54–9.54$, $P < .05–.001$; Duncan's multiple comparison test, latency: $P < .001$; working errors: $P < .001$; reference errors: $P < .025$, vehicle vs. sham]. This amnesic effect of ischemia was significantly reduced by FK506 as measured by latency ($P < .05$) and working memory errors ($P < .05$); however, the number of reference memory errors was not affected ($P > .05$, Duncan's test, FK506 vs. vehicle on Day 24). Comparing the performance on Day 24 with that on Day 8 (two-sample paired *t* test), FK506 also significantly reduced the ischemia-induced, retrograde amnesia of working memory (paired *t* test, $P > .05$), in contrast to the vehicle-treated group (paired *t* test, $P < .01$, Fig. 2B). In this paired sample analysis, however, FK506 did not reduce the ischemia-induced increase in latency and number of reference errors on Day 24 (paired *t* test, latency: $P = .011$; reference errors: $P = .016$).

Fig. 3 (panels A and B) illustrates the effect of FK506 on ischemia-induced, hippocampal neurodegeneration. Fifteen minutes 4-VO caused 85.3% to 86.4% pyramidal cell loss in the hippocampus ($P < .0001$ sham vs. vehicle). After FK506, the extent of cell death fell to 64.3% and 53.3%, respectively, in the groups tested for postoperative acquisition (panel A) or retention (panel B). Compared with vehicle alone, the neuroprotective effect of FK506 (21 to 33.1% cell preservation) was statistically significant (Duncan's multiple range test, Vehicle vs. FK506: $P < .025–.001$). There was no correlation between the number of intact-appearing pyramidal cells remaining in the CA1 region of the hippocampus and retention of cognition measured on Day 24 (Fig. 4; $r = .005–.3$, $P > .05$).

Fig. 5 shows that FK506 did not affect core temperature compared to rats receiving vehicle alone, at least over the

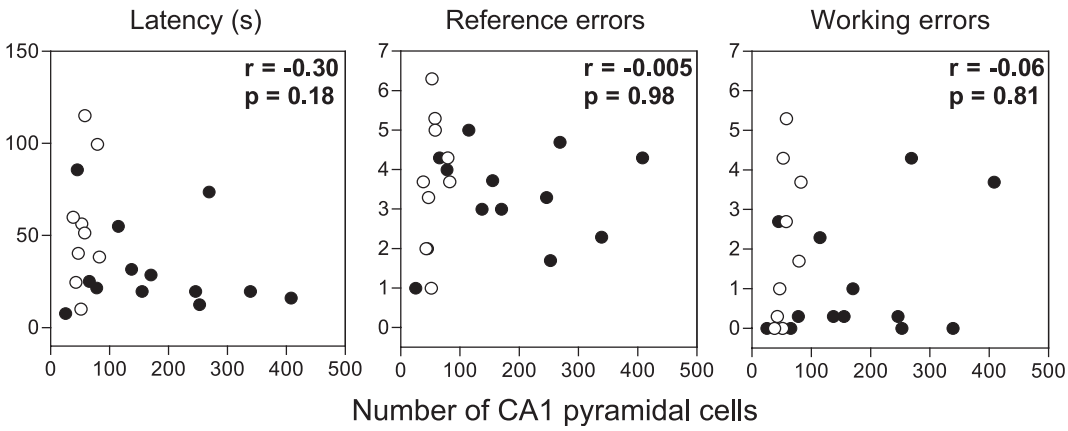

Fig. 4. The latency, number of reference memory errors and number of operational memory errors registered during the retention test (Day 24), plotted against the CA1 cell counts for each animal (Isch + Veh: $n = 9$, open circles; Isch + FK506: $n = 13$, filled circles). Pearson's correlation coefficient *r* and respective *P* value are given at the top right of each scatterplot.

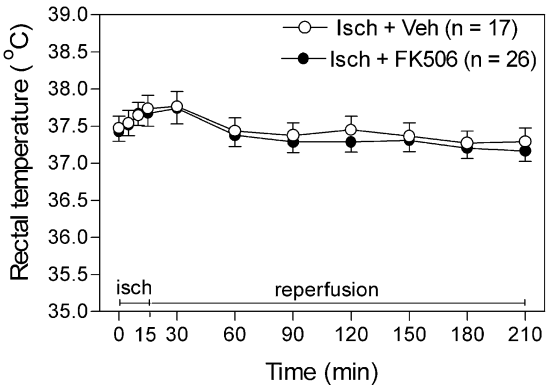

Fig. 5. Rectal temperature of rats subjected to 15-min, transient, forebrain ischemia and then treated with vehicle or FK506. The data obtained from the animals used in both the acquisition and retention experiments have been pooled. MANOVA, group effect: $P>.05$.

period during which temperature was monitored (MANOVA, $P>.05$ for main effect of group and session).

4. Discussion

FK506 is known to reduce neuronal brain damage in animal models of global (see Introduction) or focal, transient, cerebral ischemia (Sharkey et al., 1996, 1997). In an animal model of chronic, cerebral hypoperfusion, FK506 reduces both striatal neuronal degeneration as well as white matter rarefaction (Tanaka et al., 2001). Some studies also show that FK506 ameliorates the functional impairments caused by hypoxic (Tanaka et al., 2001) or focal, ischemic (Sharkey et al., 1996) brain damage. However, apparently there are no data available concerning the effect of FK506 on functional preservation/recovery after transient, global, forebrain ischemia. The present study not only corroborates previous morphometric findings, but extends them from the histological to the behavioral level, demonstrating for the first time that FK506 also reduces the ischemia-induced, memory dysfunction seen after global, transient, forebrain ischemia in the 4-VO model.

The Stroke Therapy Academic Industry Roundtable (STAIR, 1999) emphasizes the importance of functional measurements as one of the main steps in the preclinical evaluation of neuroprotective drug efficacy prior to beginning clinical trials. The effect of FK506 observed here does not appear to alter body temperature. Although core temperature was only monitored up to 3 1/2 h postreperfusion, it is unlikely that the subsequent injections of FK506 would induce hypothermia. In the gerbil, repeated daily injections of FK506 (1.0 mg/kg) for 4 days provided neuroprotection without hypothermia during the subsequent 24 h (Ide et al., 1996; Yagita et al., 1996). In rats, FK506 prevents ischemia-induced brain damage without reducing core temperature when given daily for 4 (Drake et al., 1996) or 14 days (Wakita et al., 1998). The mechanism by which FK506 prevents ischemic brain damage is not well understood and may depend on de novo protein synthesis rather than on the inhibition of calcineurin (Klettner et al., 2001; Morioka et al., 2001).

We idealized the aversive radial maze (Paganelli et al., 2004) on the basis of our previous work with the circular platform task (CPT) (Milani et al., 1998). Acquisition in the CPT or in the aversive eight-arm radial maze is based on the rat's natural behavior of avoiding open, illuminated areas, and its preference for a darkened and enclosed shelter. The CPT is assumed to be conceptually similar to the water maze task, with the advantage that it does not require immersion of the animals in (cold) water, or food deprivation as required in the appetitive eight-arm radial maze, T-maze and DNMS tasks (Barnes, 1979, 1988). Using the aversive eight-arm radial maze, the rats performed reference memory tasks by remembering that only one of the eight arms contained the shelter (reward), whose relative spatial location was unchanged over the daily sessions (long-term memory). Working memory required the rat to remember any of the nonrewarded arms that had been visited previously within a given trial (short/intermediate-term memory).

In the present study, however, interpretation of the effect of FK506 on postoperative acquisition performance by ischemic rats was hindered since ischemia did not affect learning acquisition compared to sham-operated rats (Fig. 1). This contrasts with our previous findings in which the aversive radial maze task clearly distinguished ischemic from control subjects. In that study, the acquisition impairment measured after ischemia was expressed as increased latency and number of working memory errors throughout the entire training period (Paganelli et al., 2004). The most important parametric difference between the two studies concerns the extramaze cues, the number, nature and spatial location of which were changed randomly and inadvertently in the present work. This may have affected the sensitivity of the behavioral task between the two studies. Minor mnemonic impairments may not be detected by a task in which demands are insufficient because the task is too easy (a "ceiling effect"); in a task that is excessively demanding, control group performance may be so poor that further impairment is difficult to detect (a "floor effect") (Olton and Markowska, 1994). Under such conditions, both the intact and damaged individuals will perform the task similarly. Although ceiling and floor effects are not apparent in the present data, the failure to detect an effect of ischemia on acquisition in the present study, in contrast to our previous data, may be due to different patterns of the extramaze cues between the two studies. This seems a reasonable explanation since the radial maze task is highly dependent on extramaze cues. Thus, the lack of standardization of the extramaze cues may also partially explain the discrepancy in results between different studies examining the effects of cerebral ischemia on learning and memory, as measured

in the eight-arm radial maze task. Disruption of both reference and working memory has been reported (Kiyota et al., 1991; Gionet et al., 1991), as has impairment of working memory performance alone (Davis et al., 1986, 1987). In addition, neither reference nor working memory disruption was found in ischemic rats tested in the appetitive eight-arm radial maze (Nunn and Hodges, 1994). Distinct patterns of extramaze cues may constitute an important factor leading to discrepant results not only among different laboratories, but also within the same research group, as found in the present study.

In contrast to the acquisition data, 15 min of 4-VO significantly reduced the retention of cognition acquired during the preischemic training period (Fig. 2). This amnesic effect of ischemia was more pronounced on working memory, which supports the hypothesis that the radial maze is particularly well suited to detect this kind of cognitive disturbance, and may rely on the disruption of memory consolidation and/or memory retrieval mechanisms (Hodges, 1996). An amnesic syndrome has been described in humans after cardiopulmonary arrest (Volpe and Hirst, 1983; Cummings et al., 1984). In the present study, FK506 significantly reduced the effect of ischemia on retention of cognition, an effect that seems more evident on working memory. This selective effect agrees with the observation that working memory is more sensitive to drug effects, suggesting that manipulation of recent, transient information is more readily affected by pharmacological changes than is retrieval from long-term memory (Hodges, 1996). The present data agree with other studies showing that FK506 reduces the functional deficits associated with acute, cerebral ischemia (Sharkey et al., 1996) or chronic cerebral hypoperfusion (Tanaka et al., 2001). Our data, therefore, further support the hypothesis that FK506 may be useful to treat both the structural and functional outcomes of cerebral ischemia.

Together with histological data, the demonstration that a drug can protect against ischemia-induced behavioral disturbances is an important finding since a functional level of analysis renders the animal model used more pertinent to the clinical setting. Histomorphological protection may not imply recovery or preservation of function after brain damage (Green et al., 1992, Colbourne and Corbett, 1995). For example, while phenobarbital provides considerable protection against ischemia-induced, hippocampal CA1 cell death, the release of acetylcholine by the cell's presynaptic terminals remains impaired (Ishimaru et al., 1995). This subcellular, functional disturbance may be sufficient to determine broad behavioral impairments, despite neuronal rescue by the drug. The opposite effect also has been reported, i.e., behavioral improvement without histomorphological protection after ischemic (Grotta et al., 1990) or traumatic (Sinson et al., 1995) brain injury. Dysfunction of complex behaviors and recovery may reflect alterations at the subcellular, synaptic or electrophysiological level, or even of widespread morphological changes that cannot be quantified by a simple cell count in a restricted region of a given structure (Aronowski et al., 1996). Thus, the observation that FK506 can concomitantly preserve against both structural and functional disturbances renders its clinical relevance more pertinent. In this respect, the present data further support the notion that FK506 may be of therapeutic use in the context of cerebral ischemia.

Regarding the pharmacotherapy of brain damage, another important question concerns the therapeutic window of action, i.e., the time elapsed between the onset of ischemia and the beginning of treatment, during which the drug would be effective. In the present study, FK506 delivery was started immediately after beginning reperfusion. This, however, does not invalidate its possible clinical usefulness as a neuroprotector, since hypoxic/ischemic brain damage can be anticipated during surgical or diagnostic procedures that may reduce or interrupt cerebral blood flow (Sudo et al., 2001). The temporal window of neuroprotective efficacy for intravenous FK506 has been evaluated by others. Butcher et al. (1997) showed that FK506 was still effective in providing neuroprotection when applied up to 2 to 3 h after focal ischemia induced by intracerebral microinjection of the potent vasoconstrictor peptide endothelin-1 in rats. A similar therapeutic window also was found after permanent, surgical MCA occlusion in rats. The temporal window for FK506 is shorter (1 h), however, in models of transient, focal ischemia (Arii et al., 2001; Furuichi et al., 2003) or transient, global forebrain ischemia (Furuichi et al., 2003). In the latter study, the neuroprotective effect of FK506 after focal ischemia was sustained for at least 2 weeks, which agrees with previous findings showing that the neuroprotective effect of FK506 persists up to 45 (Ide et al., 1996) or 30 (Giordani et al., 2003) days after transient, global forebrain ischemia in the gerbil and rat, respectively. These findings, together with the present behavioral data, attend three major recommendations of STAIR (1999) for evaluation of preclinical efficacy of neuroprotective drugs, i.e., sustained neuroprotection, temporal window and recovery or preservation of function.

Finally, there was no correlation between any of the behavioral parameters analyzed and the extent of pyramidal cell loss in the CA1 sector of the hippocampus in the groups treated with vehicle or FK506 (Fig. 4). Attempts to establish a quantitative correlation between hippocampal pyramidal cell loss and ischemia-induced behavioral deficits have led to contradictory results: a positive correlation has been found in some cases (Rod et al., 1990; Kiyota et al., 1991; Milani et al., 1998) but not in others (Green et al., 1992; Nunn and Hodges 1994). The close correlation found in some studies may derive from the inclusion of non-ischemic control subjects in the statistical analyses, which leads to bias in favor of a false-positive correlation (Nunn and Hodges, 1994). Also, the number of preserved, intact-appearing pyramidal cells alone, may not be indicative of behavioral changes, and other intra- and/or extrahippocam-

pal effects may determine cognitive disruption by ischemia. In our previous study, however, no correlation was found between the behavioral deficit measured in the circular platform task and the extent of pyramidal cell loss in the subicullum, CA2, CA3 and CA4 sectors of the hippocampus (Milani et al., 1998). The relationship between hippocampal cell loss and cognitive deficits caused by ischemia was reviewed by Bachevalier and Meunier (1999), who concluded that a combination of at least three factors may operate, i.e., the role of intra- and extrahippocampal damage, the nature of the task employed, and the particular memory process taxed. Thus, we cannot exclude the possibility that other brain structures besides the hippocampus may have been lesioned by ischemia. This is an important consideration since in the present study, both ischemia and FK506 profoundly affected the retention of working memory, a mnemonic process involved in the temporary maintenance and manipulation of information, which seems to be crucially dependent on the functional integrity of the prefrontal cortex (for a review, see Funahashi and Kubota, 1994). However, apparently there are no studies of the relationship between ischemia-induced, cognitive deficits and prefrontal, cortical neurodegeneration. Future studies should address this question. Dealing with the issue of the relationship between structure and function, it is also important to emphasize that it is not clear to what extent methodological limitations such as histological assessment and/or random behavioral factors may influence the results of a correlation analysis (Whishaw et al., 1994). In the present study, we cannot exclude the possible influence of technical artifacts, such as a limited cell-count area, and variability among histological preparations. Thus, caution is necessary when interpreting correlation data.

In conclusion, the present study shows that FK506 reduces the ischemia-induced, cognitive impairments seen after transient, global cerebral ischemia as measured in the aversive radial maze. This behavioral effect was concomitant with, but did not correlate with the effect of FK506 in reducing the hippocampal neurodegeneration induced by ischemia. Since this is the first study to report on the protective effect of FK506 against the cognitive outcome of transient, global, forebrain ischemia in the rat, and because the aversive (non-food-motivated) eight-arm, radial maze has been introduced only recently, additional studies are needed to better characterize the effect of FK506 in improving functional recovery after transient, forebrain ischemia. Further evaluation of the behavioral effect of FK506 after ischemic brain damage is now being carried out in a confined version of the aversive radial maze.

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