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Tacrolimus (FK506) reduces hippocampal damage but fails to prevent learning and memory deficits after transient, global cerebral ischemia in rats

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Abstract

Transient, global cerebral ischemia (TGCI) leads to hippocampal damage and disruption of spatial learning and memory. The immunosuppressant, tacrolimus (FK506), prevents TGCI-induced hippocampal neurodegeneration, but its effectiveness in promoting the recovery of learning and memory performance after TGCI has been little investigated. Here, we use a confined version of the aversive, non-food rewarded radial maze to evaluate further the effects of FK506 on TGCI-induced learning and memory deficits. In the first experiment, rats were rendered ischemic (15 min 4-VO) and 20 days later were tested for acquisition of the radial maze task over 15 consecutive days (post-operative training). In the second experiment, naive rats were trained for 10 days and subjected to TGCI (pre-operative training); retention of task performance was assessed on days 31, 35 and 39 post-ischemia. Acquisition and retention performances were expressed as a) latency to find a goal box, b) number of reference memory errors, and c) number of working memory errors. Data are presented both across daily training sessions (15 days, 3-day blocks) and as a total value (summed over the 15 days). Histological examination was performed on the day after behavioral testing. In both experiments, FK506 (1.0 mg/kg) was given i.v. at the beginning of reperfusion, followed by doses applied intraperitoneally (i.p.) 6, 24, 48 and 72 h post-ischemia. TGCI markedly disrupted both acquisition and retention performance ($p<0.0001$–0.05). Treatment with FK506 did not prevent the TGCI-induced acquisition and retention deficits, independently of whether performances were quantified 'daily' or as a 'total' value. In contrast, FK506 reduced hippocampal damage significantly compared to the vehicle alone ($p<0.001$–0.05). We conclude that the present study did not confirm our earlier behavioral data, and suggest that FK506 is not effective in treating the behavioral outcomes of TGCI, despite its efficacy in reducing CA1, hippocampal damage. However, further studies including other behavioral tasks and more extensive neurohistological analysis, are needed to better elucidate the effectiveness of FK506 in promoting functional recovery in models of transient, global cerebral ischemia.

Keywords: Global cerebral ischemia; Neurodegeneration; FK506; Neuroprotection; Learning and memory; Aversive radial maze

1. Introduction

Transient, global cerebral ischemia (TGCI) mainly results from unexpected, reversible cardiac arrest. The hippocampus is the brain structure most vulnerable to the neurodegenerative effects of such ischemia in humans (Cummings et al., 1984; Zola-Morgan et al., 1986; Petito et al., 1987) and animals (Pulsinelli and Brierley, 1979; Pulsinelli and Buchan, 1988). Behavioral and cognitive disturbances, particularly within the learning and memory domains, are the most visible symptoms of TGCI. The post-ischemic amnesic syndrome described in humans has been characterized as a combination of cognitive, executive, sensorial and motor impairments occurring after cardiopulmonary arrest (Earnest et al., 1980; Volpe and Hirst, 1983; Longstreth et al., 1983; Cummings et al., 1984; Zola-Morgan et al., 1986; Lim et al., 2004). Some patients become severely disabled, suffering a

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wide range of memory deficits and executive dysfunctions (Peskine et al., 2004). In rodent models of TGCI, disruption of spatial learning and memory abilities are the parameters most often described. Thus, in evaluating the pre-clinical potential of drugs to treat ischemic brain damage, the use of long-term functional/behavioral end-points is an important requirement (STAIR, 1999).

Tacrolimus (FK506) is a fungal-derived macrolide exhibiting potent immunosuppressant effects, and used to treat organ transplantation. This effect is based on its specific interaction with the immunophilin FKBP12 (FK506-binding protein) followed by inhibition of the Ca^{+2}/calmodulin dependent phosphatase calcineurin, and consequent inhibition of T-cells proliferation (Hamilton and Steiner, 1997). The importance of immunophilins as potential targets for the development of neuroprotectors emerged from observations that FK506 reduces glutamate-induced neurotoxicity *in vitro* (Dawson et al., 1993), as well as the size of infarct in models of stroke (Sharkey and Butcher, 1994). Other studies demonstrated further that FK506 reduces hippocampal pyramidal cell death after TGCI in the gerbil (Ide et al., 1996; Tokime et al., 1996; Yagita et al., 1996) and rat 2-VO models (Drake et al., 1996). We have found that FK506 prevents ischemia-induced neurodegeneration at different septo-temporal levels of the hippocampus in the rat 4-VO model and, most importantly, that this effect is sustained up to 30 days after reperfusion (Giordani et al., 2003). Long-lasting neuroprotection by FK506 also has been found in the gerbil model (Furuichi et al., 2003a). Recent evidence suggest that suppression of proinflamatory, cytokine-mediated responses in astrocyte and microglia (Kaminska et al., 2004), as well as inhibition of ischemia-induced necrotic and apoptotic processes (Furuichi et al., 2004) are important mechanisms underlying the neuroprotetctive effects of FK506.

While the aforementioned data indicate that FK506 might be useful to treat ischemia-induced hippocampal cell death, its efficacy in alleviating the effects of TGCI on learning and memory has not been investigated. Therefore, in a previous study we used a modified, non-food rewarded, 8-arm radial maze task to evaluate whether FK506 might facilitate recovery from TGCI-induced learning and memory deficits (Benetoli et al., 2004). In that study, TGCI affected the capacity of rats to remember the task acquired before ischemia, an effect that characterises a retention deficit, that is, amnesia. This amnesic effect of ischemia was reduced by FK506. The same could not be concluded, however, about the effect of FK506 on TGCI-induced acquisition deficit, as expressed by an impairment of the rat's ability to learn the goal box location, when it is trained after ischemia. In that study, TGCI failed to disrupt acquisition performance in the vehicle-treated group, despite extensive hippocampal cell death, thus making interpretation on the effect of FK506 difficult. This issue was discussed in terms of possible influences concerning the nature and/or disposition of the visuo-spatial, extra-maze cues provided.

The non-food rewarded radial maze has been termed the aversive radial maze (AvRM) (Paganelli et al., 2004), since it functions on the basis of rat's natural behavior of avoiding aversive, open, illuminated areas, and its preference for a darkened, enclosed shelter. In Benetoli's study, the AvRM was used as an unconfined maze, in which the central arena and the radial arms constituted a single compartment. This layout allows the animal to use egocentric, non-spatial guidance strategies to solve the task. One of the most efficient tactics that many subjects employ to find the reward in the unconfined radial maze entails a series of uninterrupted, sequential entries into arms adjacent to that previously visited, until encountering the rewarded arm (Dale, 1986; Hodges, 1996). This behavioral response was quantified in our original, unconfined AvRM study (Paganelli et al., 2004) and corroborates observations that under such conditions the subjects may use sensory (kinesthetic) cues rather than spatial navigating capabilities (Roullet and Lassalle, 1995). Since not all rats adopt such a strategy, this may constitute an important source of variability. Thus, we have transformed the unconfined AvRM into a confined version (Fig. 1), to eliminate the sequential entry choices.

Since we could not interpret some of our previous findings, the present study reevaluates whether FK506 might facilitate the recovery of spatial learning and memory performances after TGCI in rats. We used the AvRM in its confined version, which may represent a more reliable task to measure spatially-guided behaviors, since this configuration abolishes the use of the 'automatic', sequential entries into adjacent arms. Further, retention performance was assessed several times, in contrast to the single trial session used in our previous study, and the behavioral data analysis was extended to include different presentations and statistics.

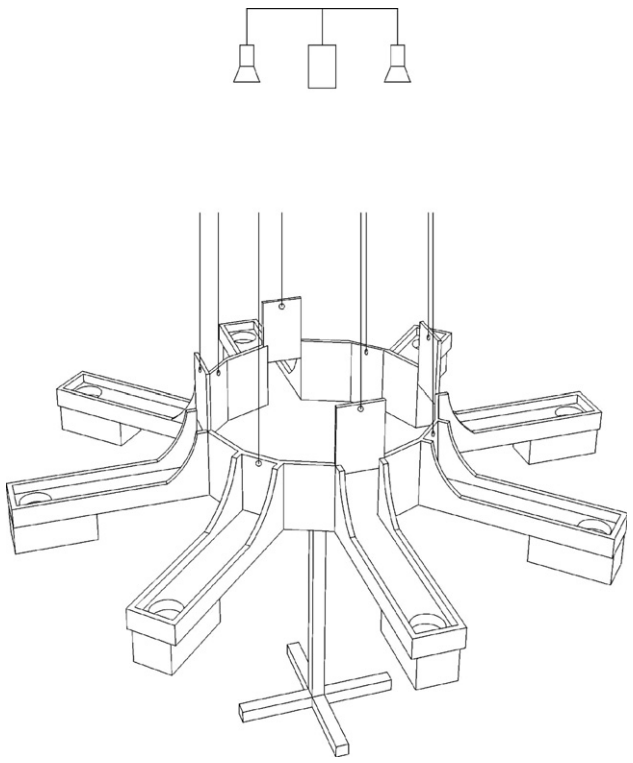

Fig. 1. Schematic representation of the confined version of the AvRM. Each arm has a box just beneath the opening at the distal extremity; however, only one is the true goal box (close-ended box). In the remaining seven arms, the boxes are open-ended, *i.e.*, they have walls like the true goal box, but lack the bottom. The central area is separated from the arms by transparent, acrylic guillotine doors, operated from a separate room by a pulley system. These sides located between the arms are also walled. The maze is well illuminated by two spotlights located above the central area.

2. Experimental procedures

2.1. Animals

A total of 149 young-adult, male Wistar rats (inbred strain, 3–4 months of age, 270–300 g body weight) was acquired from the local vivarium of the State University of Maringá, and used for the 'Post-operative Acquisition Trial' (Experiment 1) and 'Post-operative Retention Trial' (Experiment 2, see details below). Of these, 45 rats were sham-operated and 104 were subjected to TGCI (see 'Drug treatment' for group assignment, and 'Results' for exclusion/mortality rates). The rats were housed at a controlled temperature (22±1 °C) on a 12 h alternating light/dark cycle (lights on at 0700 h). Food and water were provided *ad libitum* until the day of surgery. The experimental procedures performed adhere to the ethical principles set down by the Brazilian College of Animal Experimentation (COBEA), and approved by the Ethics Committee on Animal Experimentation of the State University of Maringá, Paraná, Brazil (Protocol No. 029/2004, Institutional Research Project).

2.2. Ischemia

TGCI was induced by employing the 4-vessel occlusion (4-VO) technique (Pulsinelli and Brierley, 1979; Pulsinelli and Buchan, 1988) with modifications. Under ether anesthesia, each animal was fixed in a stereotaxic frame, and the vertebral arteries were bilaterally electrocoagulated at the level of the first cervical vertebrae. To avoid the risk of defective occlusion, the vertebral arteries were firstly severed using the tip of a unipolar electrode which was gently rotated within the alar foramen until bleeding was produced being immediately stanched by electrocoagulation. An incision into the ventral neck then exposed the common carotid arteries, which were loosely snared with a silk thread. Five to 6 h later, the thread was carefully 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. Rectal temperature was monitored with a digital thermometer (Minipa, APPA MT-520, São Paulo, São Paulo, Brazil) using a rectal probe inserted to approximately 6 cm depth. During surgery, core temperature was kept around 37.5 °C by a heating blanket. Throughout occlusion and during the first hour of reperfusion, the rats were maintained in a warming box at 30 °C (Seif el Nasr et al., 1992), and rectal temperature was monitored up to 3.5 h after reperfusion. Sham-operated animals were subjected to the same surgical intervention, except that the vertebral and carotid arteries were left intact. Occlusion of the vertebral arteries alone does not affect brain perfusion (Pulsinelli and Brierley, 1979).

2.3. Drug treatment

Animals were assigned to the following groups in both experiments: Experiment 1 (acquisition trial): Sham-operated (n=24), ischemia+vehicle (n=30) and ischemia+FK506 (n=40); Experiment 2 (retention trial): Sham (n=21), ischemia+vehicle (n=17) and ischemia+FK506 (n=17). The drug or vehicle treatment regimens followed that used in our previous study. FK506 (1.0 mg/kg) was given intravenously (i.v.) at the beginning of reperfusion, followed by intraperitoneal (i.p.) injections applied 6-, 24-, 48- and 72 h post-ischemia. 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 5% anhydrous ethanol) were kindly supplied by the Fujisawa Pharmaceutical Co., Osaka, Japan.

2.4. Apparatus

The aversive, 8-arm, radial maze (AvRM) was used in its confined version as depicted in Fig. 1. Eight arms (55×15 cm) radiate outwards from alternate sides of a central polygonal platform (71 cm across, sixteen sides). At the end of each arm, an opening 9 cm in diameter provides access to a darkened (black inside), 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 8 arms, however, only one contains the true refuge, *i.e.*, a close-ended box, that can be shifted from one arm to another between trials. In the remaining seven arms, the boxes are open-ended, *i.e.*, they have walls like the true goal box, but lack the bottom. When visiting a false goal box, the rat inserts its head into the open-ended box, detects the absence of the bottom, and returns to the central arena. Transparent, acrylic rails, 2.5 cm high, border each arm to prevent the animal from falling. The central arena is separated from the arms by transparent, acrylic guillotine doors (19 cm in height). This rotatable maze is elevated 90 cm above the floor on a metal stand. From a separate room, a pulley system connected to each individual guillotine door allows the experimenter to confine the animal in the central arena before release to explore the arms. Several extra-maze cues (posters on the walls, a closed door, a window and removable, tridimensional objects) 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, 40 W incandescent lamps were fixed to the ceiling, 180 cm above the maze. The video camera was positioned 220 cm away from, and 130 cm above, the maze. For descriptive data analysis, the 8 arms were numbered according to their location in relation to the extra-maze cues such that the sequence and frequency of visits to each different location could be recorded.

2.5. Behavioral procedures

2.5.1. Post-operative acquisition trial (experiment 1)

The rats were randomly assigned to one of the following treatments: sham-operation, ischemia+vehicle, or ischemia+FK506, and examined post-operatively for acquisition performance. Twenty days after ischemia, the animals were habituated to the testing apparatus. They were placed individually and directly in the center of the maze, which they were allowed to explore until finding the goal box, or until 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 extra-maze cues were removed from the testing room, except for the video camera, the pair of lamps, the door and the window. The spatial position of the goal box was randomly changed between subjects. The habituation procedure was repeated for 3 days, *i.e.*, on days 20, 21 and 22 post-ischemia. On day 23, the extra-maze cues were replaced, and acquisition training was started. The rats were trained using three trials/session, one session per day. For training, each rat was placed into the center of the arena, all arms being closed, and the video camera was turned on. Thirty seconds later, the arms were opened simultaneously, and the animal was allowed to explore the entire maze. When the rat entered half way down a non-rewarded arm (containing a false goal box), the guillotine doors of the remaining arms were lowered simultaneously. On the rat's return to the central area, the newly-visited arm was closed immediately, and the animal was again confined in the arena for a further 30 second period. When the rat found and entered half way down the rewarded arm (containing the true goal box), the guillotine door of that arm was lowered, forcing the animal to enter the correct goal box, where it was left for 1 min. If the rat did not find the correct arm within 4 min, it was placed into it and gently introduced into the shelter. When the rat inserted only its head into an incorrect opening and remained there for more than 1 min, it was replaced at the center of the maze and the trial re-started. If an animal persisted with this behavior for more than 4 consecutive sessions (days), it was excluded from the experiment. Between trials, 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, although its spatial position in relation to the extra-maze 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 which had been visited previously during that trial, a working memory error was registered. In rats, working memory has been defined as a short term memory for an object, stimulus, or location that is used within a testing session, but not typically between sessions. It is typically a delay-dependent representation of stimuli that are used to guide behavior within a single trial. In contrast, reference memory would typically be acquired with repeated training trials. Since it would persist from days to months, it would be useful to guide behavior between sessions (Dudchenko, 2004). Accordingly, the present AvRM model requires the rat to learn a reference memory task by remembering the shelter location over several training days; in contrast, the working memory task requires the rat to remember and avoid previously visited, false shelter, within a given trial. An arm was considered visited when the rat entered halfway down its length. The animal was considered to have left an arm when it placed all four paws on the central platform.

2.5.2. Post-operative retention trial (experiment 2)

In a separate experiment, naive, intact animals were habituated and trained for acquisition of the spatial task for 10 days, as described above. On the day after the last training session (day 11), the rats were subjected either to the sham-operation, ischemia+vehicle, or ischemia+FK506, as for experiment 1, and allowed to recover from surgery for 20 days. On days 31, 35 and 39 post-ischemia, they were tested for retention of cognition acquired during the pre-ischemia training.

2.6. Histology

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 twelve, 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 coronal sections were chosen for bilateral counts of normal-appearing neurons in the dorsal portion of the hippocampal CA1 subfield. In each hemisphere, the number of intact-appearing pyramidal cells showing a distinct nucleus and nucleolus was counted along a transect 1.35 mm in length (magnification 400×, field diameter=450 μm, Olympus). The number of pyramidal cells for each rat was expressed as the mean of the three coronal sections. The identity of the groups was not revealed during histological assessment.

2.7. Statistical analysis

Owing to heterogeneity of variance within the 'Sessions' and 'Trials', the behavioral data were transformed logarithmically for statistical analysis. The data for acquisition and retention performance measured during individual 'Sessions' (days) were analyzed using a Multifactorial Analysis of Variance for Repeated Measures (MANOVA) with Groups as the 'between' and Sessions as the 'within' subjects factors (Statistica 6.0). In the case of a significant Groups effect, the Unequal N HSD Multiple Range Test was used to distinguish between different means. The Kruskal–Wallis Analysis of Variance, followed by Dunn's *post-hoc* test where appropriate, was used to compare the groups when the parameters 'total latency' and 'total number of errors' (summed across 15 acquisition trials or 3 retention trials, respectively) were evaluated. In experiment 2 (retention trial), Wilcoxon's signed rank test was used to compare the performances obtained before and after ischemia (matched samples comparison), and the level of significance "α" was corrected ($\alpha^* = 0.017$) where appropriate, according the Bonferroni's procedure. The histological findings were evaluated by One-way ANOVA, followed by the Tukey's Multiple Comparison Test. Statistical significance was defined as a P value ≤ 0.05.

3. Results

Of the 104 rats assigned to 4-VO, 38 subjects (36.5%) died (17 rats from Experiment 1, and 21 rats from Experiment 2). The main causes of death include: excess of anesthesia, accidental

rupture of the carotid artery, cardiorespiratory arrest and/or convulsion during ischemia, spontaneous death within the first 24–48 h of reperfusion, and a few individuals were sacrificed for technical or ethical reasons. Three rats were excluded for persistently failing to explore the arms over 4 consecutive days (2 FK506-treated rats from Experiment 1, and 1 sham-operated rat from Experiment 2).

Fig. 2 shows the disruptive effect of TGCI on acquisition performance, and the effect of FK506. Examining performances across training Sessions (*upper panels*), the repeated measures ANOVA revealed a significant 'Group' effect on 'latency' ($F_{2,\ 68}$= 8.91, p<0,001), 'number of reference memory errors' ($F_{2,\ 68}$=3.74, p<0,05) and 'number of working memory errors' ($F_{2,\ 68}$=5.19, p<0,01). The HSD multiple range test showed that acquisition

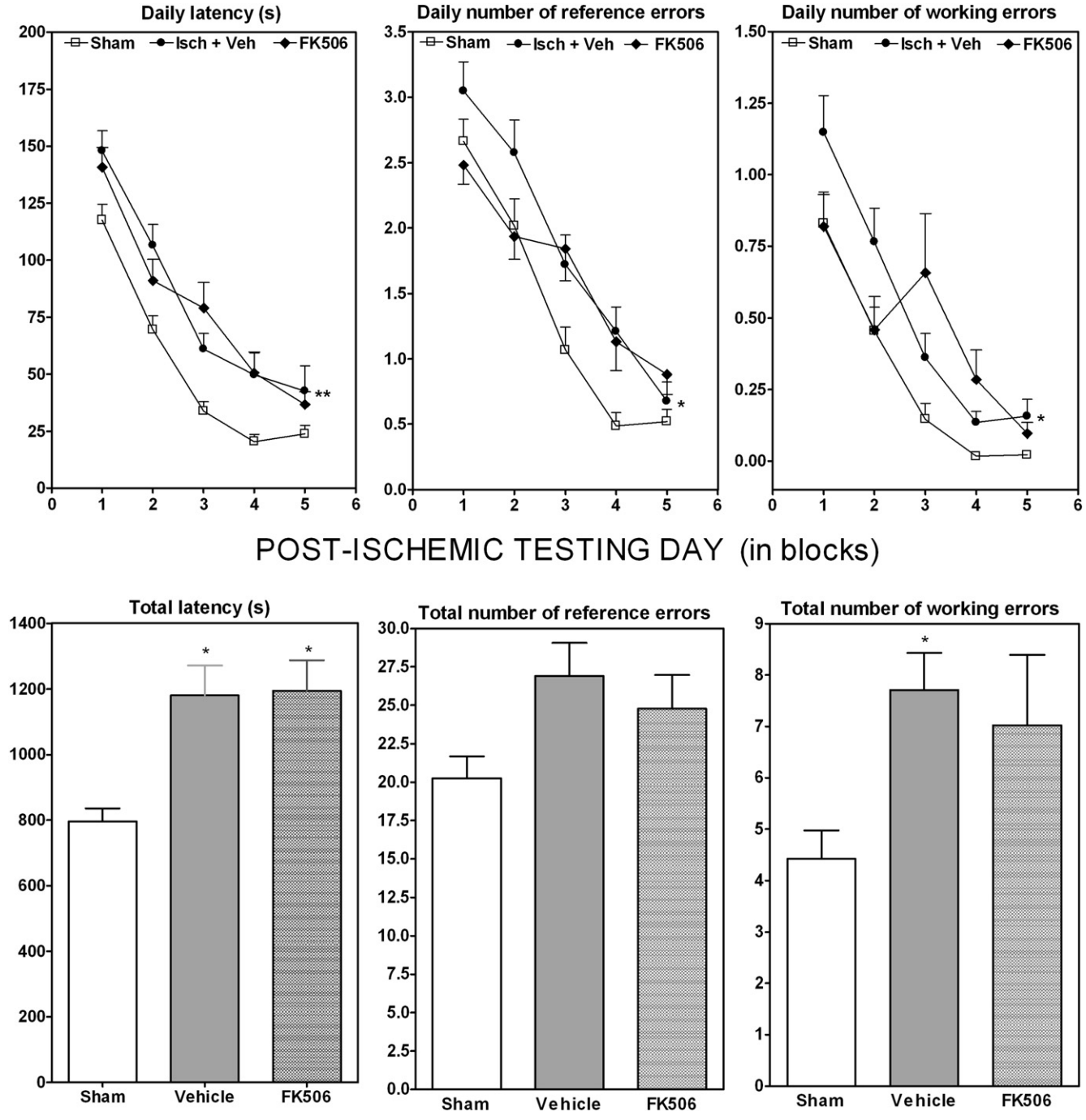

Fig. 2. The effect of FK506 (1.0 mg/kg, 1 injection i.v.+4 injections i.p.) on acquisition performance in rats subjected to TGCI (15 min) and tested in the confined AvRM. FK506 was given 0, 6, 24, 48 or 72 h post-ischemia. For each subject, the mean value obtained from three trials/day expresses performance in terms of 'latency' (left), 'number of reference memory errors' (middle), and 'number of working memory errors' (right panel). Acquisition performance was registered from day 23 to day 37 post-ischemia, and plotted as trial blocks (3 days/block, *upper panels*). The 'total latency' and 'total number of errors' (mean±SEM) are represented in the *lower panels*. Ischemia disrupted acquisition performance (p*<0.05; **p<0.01 sham *vs* vehicle), an effect not prevented by FK506 (p>0.5, FK 506 *vs* vehicle). Data are the group mean±SEM. Sample sizes: Sham=24; Vehicle=21; FK 506=26.

performance was significantly reduced in the vehicle-treated group for all three parameters ($p<0.01$–0.05, Vehicle *vs* Sham). This impairment was also evident when performance was measured as 'total latency' (K–W=12.08, $p<0.01$) and 'total working errors' (K–W=7.13, $p<0.05$) summed from day 1 to day 15 of training (*lower panels*). The number of 'total reference errors' also tended to increase after TGCI (K–W=5.67, $p=0.059$). This ischemia-induced acquisition impairment was not prevented by FK506, when the parameters 'latency' and 'number of errors' were analysed across time (*upper panels*) or as total values (*lower panels*) ($p>0.05$, FK 506 *vs* vehicle). A highly significant 'Session' effect was evident for all three parameters ($F_{4,\ 272}=96.72$–134.70, $p<0.0001$), indicating that the ischemic groups also learned the task, although more slowly than the sham-operated rats. A Group *vs* Session interaction effect appeared for the parameters 'reference memory errors' and 'working memory errors' ($F_{8,\ 296}=2.93$–3.71, $p<0.01$).

The effect of FK506 on the ischemia-induced retention deficit is shown in Fig. 3. Animals were trained for 10 days before

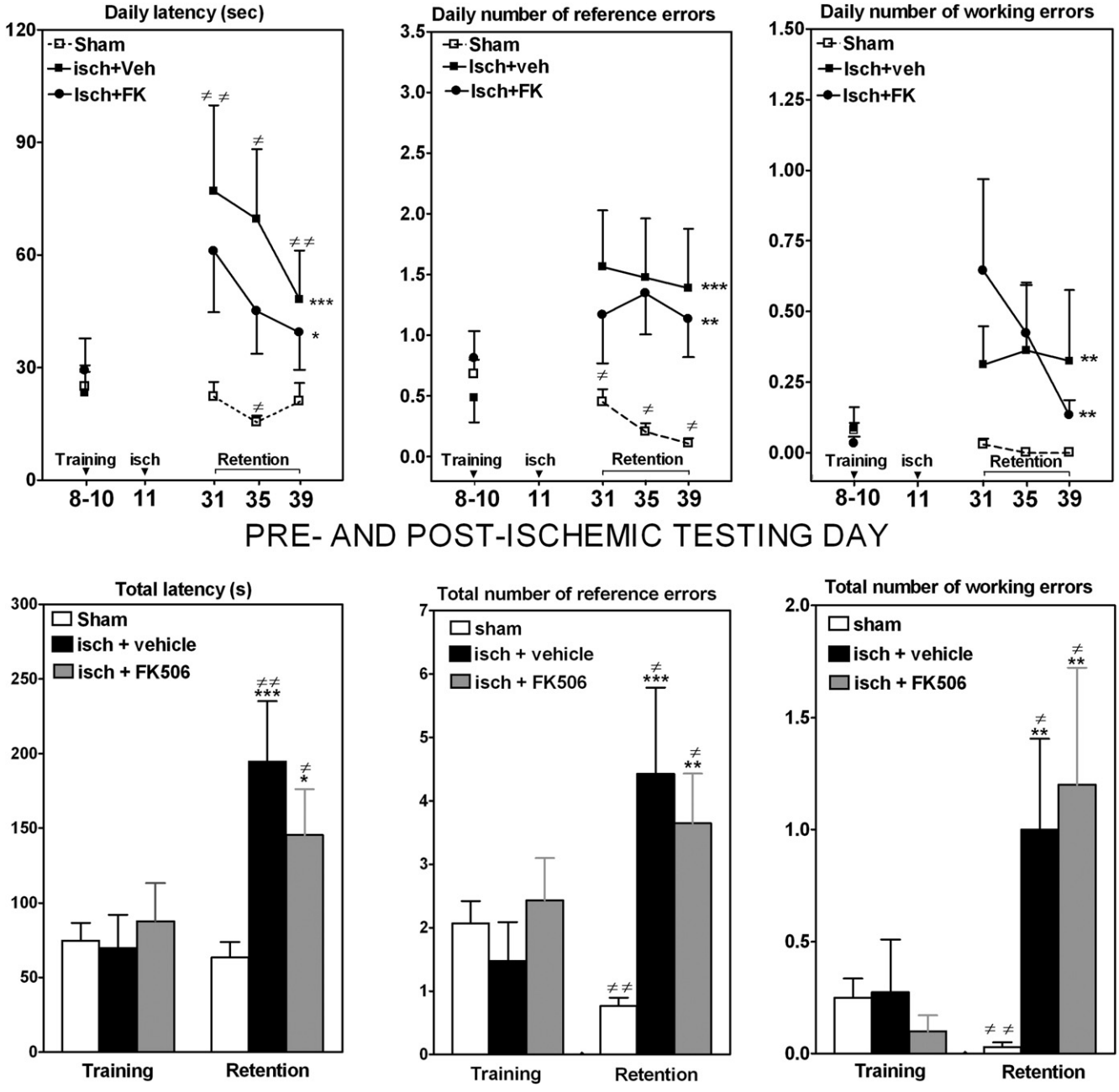

Fig. 3. The effect of FK506 (1.0 mg/kg, 1 injection i.v.+4 injections i.p.) on retention performance in rats subjected to TGCI (15 min) and tested in the confined AvRM. Ischemia was induced after 10 days of training (on day 11), and retention performance was assessed on days 31, 35 and 39 post-ischemia (*upper panels*). Pre-ischemic performance is given as the mean for the last three days of training (days 8–10, in block). The total latency and total number of errors (mean±SEM), summed (Σ) over the last 3 days of training and the entire retention period, are represented in the *lower panels*. Retention of cognition was disrupted by TGCI, an effect unchanged by FK506 (*$p<0.05$; **$p<0.01$; ***$p<0.001$ *vs* Sham, 'Between Group' comparison; $^{\#}p<0.05$ and $^{\#\#}p<0.01$, training *vs* retention performance, 'Within Group' comparison). Values are the group mean±SEM. Sample sizes: Sham=20; Vehicle=8; FK506=9.

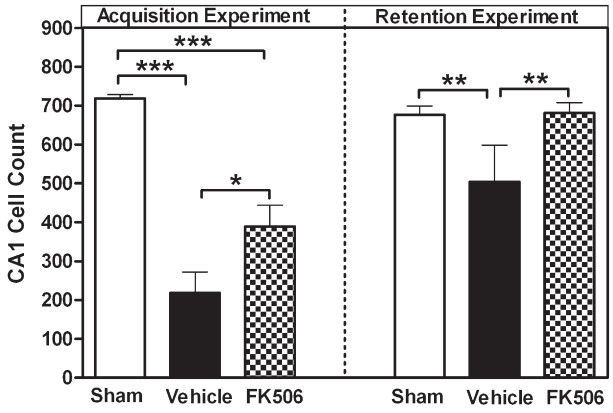

Fig. 4. Effect of FK506 on ischemia-induced, hippocampal pyramidal CA1 cell loss. Cells were counted along a transect 1.35 mm in length. Histological analysis was performed one day after the end of behavioral testing. Values are the mean±SEM. *$p<0.01$; ***$p<0.001$. Sample sizes: Acquisition experiment: Sham=24; Vehicle=21; FK 506=26. Retention experiment: Sham=20; Vehicle=8; FK506=9.

ischemia; during this phase, the groups assigned to each treatment did not differ from each other for the parameters 'latency' and 'number of errors' (Group effect: $F_{2,36}=0.21–0.68$, $p>0.05$, full curve not shown). In the daily trial analysis (*upper panels*), the pre-ischemic, asymptotic performances are provided as the mean of the final 3 days of training (days 8–10), which did not differ form each other, neither between nor within the groups. This was done to allow 'Within Group' comparisons between pre- and post-ischemic performances (3 days of training *vs* 3 days of retention, see below). Examining retention performance across days 31, 35 and 39 post-ischemia (*upper panels*), the repeated measures ANOVA revealed a highly significant 'Group' effect from 'latency' ($F_{2,34}=15.77$, $p<0.0001$), 'number of reference errors' ($F_{2,34}=16.52$, $p<0.0001$) and 'number of working errors' ($F_{2,34}=9.24$, $p<0.001$). The HSD *post-hoc* test revealed that retention performance in the vehicle-treated group was significantly disrupted by TGCI in all parameters used (*upper panels*: $p<0.001–0.01$, Vehicle *vs* Sham). A 'Session' effect was revealed

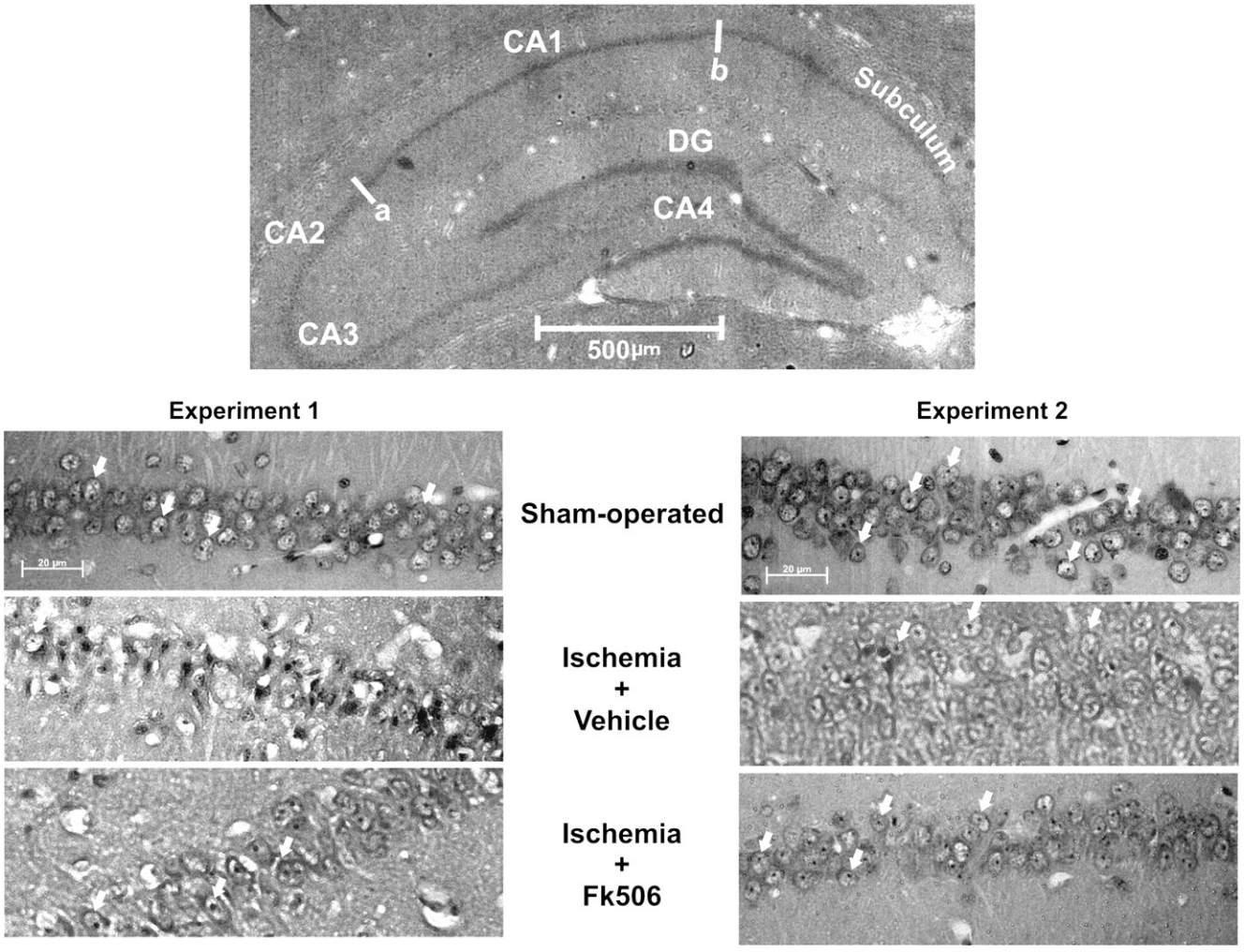

Fig. 5. *Upper panel*: low magnification (50×), celestine blue/acid fuchsin stained coronal brain section at the intermediate level of the hippocampus (4.52 mm posterior to bregma, approximately), obtained from a sham-operated rat to show the subiculum, CA1, CA2, CA3, CA4 and dentate gyrus (DG) subfields. Normal-appearing neurons were counted bilaterally between the CA2 border zone (trace 'a') and the CA1 apex (trace 'b'), along a transect 1.35 mm length. A sharp thickening of the pyramidal cell layer distinguishes the CA1/CA2 border zone. *Lower panels*: high magnification (400×) illustrating the CA1 pyramidal cell density in brains of animals assigned to sham-operation, ischemia+vehicle or ischemia+FK506, and tested either in the Experiment 1 or Experiment 2. Examples of counted, normal-appearing neurons are indicated by arrows.

for the parameter 'working memory errors' alone ($F_{2, 82}=3.24$, $p<0.05$). The amnesic effect of TGCI was also clearly evident when the results are examined using 'total latency' and 'total number of errors' during the retention phase (*lower panels*: K–W=16.13–17.62, $p<0.01$, Vehicle *vs* Sham, retention phase). FK506 failed to reduce the amnesic effect of TGCI as measured by all three parameters, if the groups were compared during the retention phase, that is, on days 31, 35 and 39 (*upper panels*, MANOVA/*post-hoc* test: $p>0.05$, vehicle *vs* FK506). Similar results were obtained by examining the data as 'total latency' and 'total number of errors' (*lower panels*, MANOVA/*post-hoc* test: $p>0.05$, vehicle *vs* FK506). This failure of FK506 is also expressed when 'total latency' and 'total number of errors' measured pre-ischemia (training phase) are compared to those obtained post-ischemia (retention phase). All parameters increased significantly from the pre- to the post-ischemic phases in both vehicle- and FK506-treated groups (*lower panels*: $p<0.01$–0.05, Wilcoxon's signed rank test), indicating retention deficit in both groups. In the sham-operated group, performances did not change or even improve ($p<0.01$). In contrast, when pre- and post-ischemic performances are compared within group (*upper panels*: day 8–10 *vs* day 31), the parameter 'latency' increased significantly after ischemia in the vehicle group ($p<0.01$), but not in the group treated with FK506 ($p>0,05$), indicating a possible beneficial effect of FK506. By this analysis, however, the amnesic effect of ischemia measured by the parameters 'number of reference errors' and 'number of working errors' did not reach statistical significance (vehicle: $p>0.05$, pre- *vs* post-ischemia).

Fig. 4 illustrates the effect of tacrolimus on ischemia-induced neurodegeneration in the CA1 sector of the hippocampus. In the acquisition experiment, 15 min 4-VO caused a 70% neuronal loss in the vehicle-treated group ($F_{2, 70}=32.0$; $p<0.001$ Sham *vs* Vehicle). FK506 reduced this neurodegenerative effect of ischemia by 24% ($p<0.05$). Unexpectedly, only 25.5% cell death was found in the vehicle group assigned to the retention experiment ($F_{2, 36}=6.55$, $p<0.05$ Sham *vs* Vehicle). Even so, the neuroprotective effect of FK506 was statistically significant ($p<0.05$, Vehicle *vs* FK506) and the number of intact-appearing neurons approached that of the sham-operated group ($p>0.05$, Sham *vs* FK506). This finding may derive from the unfortunate fact that the warming box to be used during ischemia and the first hour of reperfusion was unavailable for the animals assigned to the retention experiment (see discussion for details). However, neither TGCI nor FK506 affected rectal temperature in either experiments, at least up to 3.5 h reperfusion (36.4±0.05–37.8±0.14 °C, data not shown). Fig. 5 illustrates representative photomicrographs from the hippocampus of rats receiving one of each treatment and tested for acquisition (Experiment 1) or retention performances (Experiment 2).

4. Discussion

Here, we reevaluate whether the immunosupressant tacrolimus (FK506) might facilitate the recovery from learning and memory disturbances caused by TGCI, since in a previous study we suggested this capability based on the apparent efficacy of FK506 to reduce the TGCI-induced retention deficit, although the effects of FK506 on acquisition performance could not be known in that occasion (Benetoli et al., 2004). The results of the present histological analysis confirm our previous findings (Giordani et al., 2003; Benetoli et al., 2004) that FK506 notably reduces, in a sustained manner, the CA1 pyramidal cell death caused by TGCI, an effect in agreement with findings from other contemporaneous studies (Katsuta et al., 2003; Furuichi et al., 2003a). FK506 failed, however, to reduce the TGCI-induced acquisition and retention deficits, as measured in the confined AvRM task. Since the present study did not include a sham-operated, FK506-treated group, it could be argued that a detrimental effect of FK506 on learning and memory, by itself, might have accounted for its failure to improve acquisition and retention performance after ischemia. However, FK506 has been shown to inhibit learning and memory in chicks when applied immediately, but not 10 or 20 min after training, suggesting that FK506 may affect learning and memory formation only when administration take place relatively close to the time of training (Bennett et al., 2002). In another study, calcineurin was inactivated by antisense oligodeoxynucleotides delivered continuously into the lateral ventricles of rats for 28 days. This treatment did not change the performance of rats tested in the water maze task, despite the concomitant administration of FK506 (Ikegami and Inokuchi, 2000). In the present study, FK506 was given around 3 weeks before behavioral testing.

Therefore, while the neurohistological effect of FK506 in the hippocampus is consistent with various studies (Ide et al., 1996; Tokime et al., 1996; Yagita et al., 1996; Drake et al., 1996; Giordani et al., 2003; Katsuta et al., 2003; Furuichi et al., 2003a,b; Benetoli et al., 2004), the lack of a behavioral improvement does not support our earlier claim that tacrolimus might facilitate functional recovery after transient forebrain ischemia. Since there have been no other investigations of the behavioral effects of FK506 in models of TGCI, including the use of other well known behavioral tests such as Morris' water maze or the conventional radial maze, the question arises whether the present results are more informative than our previous findings. This is a relevant point, since it is likely that the use of a confined or a non-confined radial maze would activate different processes that contribute to, or affect spatial learning. Hence, the 'spatial' abilities measured in one maze procedure might not resemble those engaged in the other, hampering the interpretation of drug- or lesion-induced deficits (Hodges, 1996) and perhaps drug-induced recovery of function.

The most important difference between our previous study and the present investigation concerns the use of the unconfined *vs* confined AvRM, respectively. In the unconfined maze, there is no barrier between the central arena and the radial arms, thus the entire maze being a single compartment. In the present, confined AvRM, the central arena is separated from the arms by transparent, acrylic guilloutine-doors. Therefore, in the unconfined maze the animal is provided with free and immediate access to each of the eight arms, at any time-point within a training session, and allows the animal to employ egocentric

guidance strategies to find the shelter (Paganelli et al., 2004). This type of behavior may render the parameters 'latency' and 'number of errors' less informative as a measure of spatial learning and memory abilities. In contrast, the confined AvRM abolishes that strategy of sequential entries into adjacent arms until the goal box is found. Therefore, confinement may render the radial maze task more difficult, thus requiring more complex behavior by using associative, spatially-guided responses. Accordingly, the use of confinement may have improved the sensitivity of the aversive radial maze as a spatial task, although we made no direct, comparative study between the unconfined and confined versions of AvRM. However, how such task-related aspects may have affected the action of FK506 on ischemia-induced acquisition and retention impairments cannot be known from the present study. We are assuming that if the confined maze task requires the rat to process distal, visuo-spatial relationships (allocentric-guided behavior) more than the confined maze can do, then it may be more appropriate for distinguishing the effects of drugs on the recovery of spatially-guided ability after brain damage. Thus, the lack of effect of FK506 on TGCI-induced acquisition and retention disruption observed here may be more informative than in our previous study, by showing that FK506 does not prevent the disruptive cognitive effects of TGCI, despite a certain degree of neuroprotective effect measured in a restricted area of the hippocampus.

Alternatively, FK506 may have failed to facilitate behavioral recovery since little histological protection was afforded. While this interpretation may make some sense in the acquisition experiment (24% neuroprotection), it does not apply to the retention experiment where FK506 failed to reduce the retention deficit, despite the absence of CA1 pyramidal cell loss. In the retention experiment, TGCI caused < 26% cell death in the vehicle-treated group. This is a diminished lesion compared to the 70% damage seen in the vehicle group used in the present acquisition experiment, and mentioned in our previous papers. This discrete CA1 lesion may have occurred because the warming box habitually used to prevent intracerebral hypothermia during ischemia was unavailable for the animals assigned to the retention experiment. In the context of experimental cerebral ischemia, the control of intracerebral temperature is an important variable, since hypothermia (33 °C or less) is neuroprotective when applied during ischemia (Busto et al., 1989; Ginsberg et al., 1992). Further, rectal temperature is not a reliable determinant of brain temperature, since during ischemia a large temperature gradient develops between body and brain (Busto et al., 1987). However, brain normothermia (36–37 °C) can be achieved during ischemia if the rat is maintained in a warming box held at 30 °C (Seif el Nasr et al., 1992). This method has been used routinely in our laboratory, but in the present retention experiment was unavailable for technical reasons (prolonged loss of electrical current) during the period in which the animals were ischemic. Possibly, brain temperature may have dropped sufficiently to reduce the effect of ischemia, despite rectal normothermia. Accordingly, the lack of CA1 cell loss seen in the FK506-treated group during the retention experiment may have resulted from a synergistic interaction between the first dose of FK506 given at the beginning of reperfusion and intracerebral hypothermia. That mild hypothermia (35 °C, temporal muscle) potentiates the neuroprotective effect of FK506 has been demonstrated systematically (Nito et al., 2004). The findings of the present retention experiment are interesting, however, in the sense that the failure of FK506 to reduce retention deficit, despite the absence of CA1 lesioning, suggests that the FK506-induced rescue of CA1 cells may be insufficient for recovery from retention performance after TGCI. Moreover, the robust retention deficit, despite the small CA1 cell loss seen in the vehicle-treated group, suggests that other brain mechanisms within or beyond the hippocampus may mediate the effects of ischemia on learning and memory, as well as the effects of drugs on recovery. Further, the drug-induced recovery of function may affect acquisition and retention processes differentially. Recently, we found that the Ginkgo biloba extract, EGb 761, facilitated recovery from acquisition, but not retention deficit after TGCI, although a similar degree of CA1 neuroprotection was present in both acquisition and retention experiments (Paganelli et al., 2006). In addition, the common difficulty in establishing a firm correlation between the extent of CA1 damage and the magnitude of learning and memory deficit measured in such tasks as the radial maze or water maze (Nunn et al., 1994; Hodges, 1996; Bachevalier and Meunier, 1999) further support this view. Dysfunction of complex behaviors and recovery of function may reflect alterations at the subcellular, synaptic or electrophysiological levels, 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). For example, while fenobarbital 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 establish broad behavioral impairments, whose nature, magnitude and drug-induced effects on recovery may vary according to both the behavioral task and the drug used.

Finally, considering that the confined AvRM may function as a more reliable spatial task compared to the unconfined maze, which allows the use of non-spatial, kinesthetically-guided responses, and that in the present investigation we expanded our evaluation of the behavioral data, requiring additional statistical analysis, we believe the present data set to be more reliable from a methodological point of view. Thus, we can conclude that FK506 is not effective in reducing the effects of TGCI on spatial learning and memory, at least as measured here. Unfortunately, apparently no other studies have investigated the use of tests like the conventional (appetitive) radial maze or the water maze to assess the efficacy of FK506 in alleviating the well documented, cognitive dysfunction observed after transient forebrain ischemia in the rat (Hodges, 1996). This contrasts with the large amount of data available using simple histological end-points to demonstrate the potential benefit of FK506 to treat cerebral ischemia (see citations above), although some studies note the efficacy of FK506 in minimizing the sensorimotor impairments measured in animal models of stroke (Sharkey et al., 1996; Furuichi et al., 2003b). Clearly, further studies using different behavioral testing methods, such as Morris' water maze, are

needed to better clarify the potential efficacy of FK506 in promoting functional recovery after transient, global forebrain ischemia. The Stroke Therapy Academic Industry Roundtable (STAIR, 1999) has emphasized the importance of functional measurements as one of the main steps in the pre-clinical evaluation of neuroprotective drug efficacy prior to beginning clinical trials. Together with histological data, the demonstration that a drug can or cannot protect against ischemia-induced behavioral disturbances is an important finding since a functional level of analysis renders the animal model used more pertinent in the clinical setting. Histomorphological protection may not imply recovery or preservation of function after brain damage (Green et al., 1992; Colbourne and Corbett, 1995, present data). This point is underscored by a recent, systematic review and meta-analysis of the efficacy of FK506 evaluated in animal models of stroke. Despite the findings demonstrating the substantial efficacy of FK506 in reducing infarct size and/or improving functional recovery, factors such as study quality and possible publication bias preclude a more conclusive decision in favor of the clinical efficacy of FK506 in the setting of ischemic brain damage (Macleod et al., 2005).

In conclusion, we show that FK506 failed to alleviate the acquisition and retention impairments caused by transient, global cerebral ischemia as measured in the confined AvRM, despite some neuroprotection in the hippocampus. Given the paucity of studies using behavioral end-points to assess the efficacy of FK506 in promoting functional recovery after TGCI, and given the novelty of the AvRM model, additional studies using other cognitive tests are needed to characterise more fully the effects of FK506 on functional recovery in models of TGCI.

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