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Dissertação apresentada ao Programa de Pós-graduação em Ciências Farmacêuticas, área de concentração: Produtos Naturais e Sintéticos Biologicamente Ativos da Universidade Estadual de Maringá – UEM, para obtenção do grau de Mestre em Ciências Farmacêuticas.

Orientador:

Prof. Dr. Humberto Milani

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Dedico este trabalho

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SUMMARY:

RESUMO

Antecedentes: Recentemente introduzimos o Labirinto Radial Aversivo (AvRM) para mensurar os efeitos da Isquemia Cerebral Global e Transitória (TGCI) sobre a aprendizagem e memória de ratos. Subsequentemente, reprojetamos o AvRM para dificultar o uso de pistas cinestésicas intralabirinto pelo rato, melhorando portanto a natureza espacial do AvRM. **Objetivo:** O presente estudo é dividido em duas partes, as quais têm os seguintes objetivos: 1) avaliar os efeitos de diferentes modelos animais de hipóxia/isquemia sobre o desempenho de aquisição e retenção medidos na versão confinada do AvRM (Parte I), e 2) avaliar se o efeito neuroprotetor do tacrolimus (FK506), usualmente observado no hipocampo, poderia ser acompanhado por uma redução nos déficits comportamentais induzidos pela isquemia no AvRM (Parte II). **Métodos:** Ratos wistar, machos foram submetidos a a) isquemia cerebral global e transitória (TGCI, modelo 4-VO agudo); b) isquemia cerebral focal e transitória (TFCI, modelo de oclusão da artéria cerebral média); ou c) hipoperfusão cerebral crônica (CCH, modelo 4-VO crônico). As sessões de treinamento e retenção começaram 20 dias após a isquemia cerebral e o desempenho comportamental foi examinado durante 9 (experimento da TFCI) ou 15 dias consecutivos (experimentos da TGCI e CCH), e expressos por latência para encontrar o esconderijo, número de erros referenciais e operacionais. FK506 (1.0 mg/kg) foi administrado intravenosamente no início da reperfusão, seguido de doses intraperitoniais aplicadas 6, 24, 48 e 72 h pós-isquemia. A perda celular na região CA1 do hipocampo ou o tamanho do infarto foram avaliados ao final dos testes comportamentais. **Resultados:** (Parte I) Durante as sessões de treinamento, a latência para encontrar o abrigo e o número de erros referencias e operacionais aumentaram após TGCI ($p < 0.0001$- 0.05), CCH ($p< 0.0001$ - 0.01) e TFCI ($p < 0.05$ – “0.068”). Foi obtido um significativo efeito de “treino” para todos os grupos e parâmetros ($p <0.0001$). Um prejuízo, aparentemente, irreversível e estável para aquisição foi observado no grupo submetido a CCH, com oclusão das artérias carótidas

comuns mais artérias vertebrais (CCH/CCA, $p < 0.01 – 0.05$). Nenhum prejuízo na aquisição foi encontrado quando as artérias carótidas internas mais as artérias vertebrais foram gradual e permanentemente ocluídas (CCH/ICA, $p > 0.05$). A TGCI também afetou acentuadamente a capacidade de retenção da tarefa aprendida antes da isquemia, um efeito expresso por todos os três parâmetros, ou seja, latência, números de erros referencias e operacionais ($p< 0.0001 – 0.001$). Estes efeitos da TGCI, CCH ou TFCI sobre a aquisição e retenção também foram fortemente manifestados quando os parâmetros foram analisados como latência total e número total de erros, a soma ao longo de todo o período de treinamento ou retenção ($p < 0.001 – 0.05$). Um profundo dano hipocampal foi observado após TGCI (81%, $p<0.0001$), enquanto que a CCH ocasiou uma perda celular na região CA1 de leve a moderada, embora significativa (23.2% e 37%, $p < 0.001 – 0.01$). Infarto cerebral, unilateral (23%) foi visto após TFCI ($p < 0.0001$ *vs.* contralateral hemisfério). No estudo com FK506, os ratos submetidos a TGCI e tratados com veículo revelaram um déficit significativo na aquisição e retenção. (Parte II) O tratamento com tacrolimus não reduziu os efeitos da TGCI sobre o desempenho na aquisição tampouco na retenção ($p > 0.05$). **Conclusão:** As presentes descobertas provêem evidências adicionais a favor da utilidade do AvRM na avaliação dos efeitos do dano isquêmico cerebral sobre a aprendizagem e memória em ratos. Uma vez que o modelo exclui a necessidade de privação alimentar ou imersão dos animais em água, ele serve como uma alternativa sensível e prática de teste comportamental para avaliar os efeitos neuroprotetores de fármacos após o dano cerebral isquêmico ou outros tipos de disfunções cerebrais. Estudos adicionais são necessários a fim de melhor investigar os efeitos do FK 506 sobre o prejuízo cognitivo causado pelos diferentes modelos animais de isquemia cerebral.

Palavras-chave: Isquemia Cerebral, Dano Neuro-cerebral, Labirinto Radial Aversivo, Prejuízo de aprendizagem e memória, Tacrolimus (FK506), Neuroproteção.

ABSTRACT

Background: Recently, we introduced the aversive radial maze task (AvRM) to measure the effects of transient, global cerebral ischemia (TGCI) on learning and memory of rats. Subsequently, we redesigned the AvRM to hinder the rat of using intra-maze, kinesthetic cues, thus improving the spatial nature of the AvRM. **Objective:** The present study is divided in two parts, which has the following objective: 1) to evaluate the effects of different animal models of hypoxia/ischemia on the acquisition and retention performances, as measured in the confined version of the AvRM (Part I), and 2) to evaluate whether the neuroprotective effect of tacrolimus (FK506) usually observed in the hippocampus could be accompanied by reduction of the ischemia-induced behavioral deficit in the AvRM (Part II). **Methods:** Male, Wistar rats were subjected to a) transient, global, cerebral ischemia (TGCI, acute 4-VO model); b) transient, focal, cerebral ischemia (TFCI, middle cerebral artery occlusion model); or c) chronic, cerebral hypoperfusion (CCH, chronic 4-VO model). Training or retention trials begun at least 20 days after cerebral ischemia and behavioral performance was examined for 9 (TFCI experiment) or 15 consecutive days (TGCI and CCH experiments), and expressed by latency to find the goal box, number of reference memory errors, and number of working memory errors. 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. Hippocampal, CA1 cell loss or infarct size were assessed at the end of behavioral testing. **Results:** (Part I) Over the training sessions, the latency to find the goal box, and the number of reference and working memory errors increased after TGCI ($p < 0.0001$- 0.05), CCH ($p< 0.0001$ - 0.01) and TFCI ($p < 0.05$ – “0.068”). A highly significant ‘Session’ effect was found for all groups and parameters ($p <0.0001$). An apparently irreversible, steady state acquisition impairment resulted in the group subjected to CCH, with occlusion of the common carotid arteries plus the vertebral arteries (CCH/CCA, $p < 0.01 – 0.05$). No acquisition impairment was found

when the internal carotid arteries plus vertebral arteries were gradually and permanently occluded (CCH/ICA, $p > 0.05$). TGCI also affected markedly the capacity of retention of the pre-ischemic acquired task, an effect expressed by all the three parameters, i.e., latency, number of reference errors and number of working errors ($p< 0.0001 – 0.001$). These effects of TGCI, CCH or TFCI on acquisition and retention were also strongly manifested when the parameters analyzed were total latency and total number of errors, summed over the entire training or retention sessions ($p < 0.001 – 0.05$). Profound, hippocampal damage was observed after TGCI (81%, $p<0.0001$), while mild-to-moderate, although significant, CA1 pyramidal cell loss was caused by CCH (23.2% and 37%, $p < 0.001 – 0.01$). Unilateral, cerebral infarct (23%) was seen after TFCI ($p < 0.0001$ *vs.* contralateral hemisphere). In the study with FK506, the rats subjected to TGCI and treated with vehicle revealed a significant acquisition and retention deficit. (Part II) The treatment with tacrolimus did not reduce the effects of TGCI on neither acquisition nor retention performances ($p > 0.05$). **Conclusion:** The present findings provide additional evidence in favor of the usefulness of the AvRM in assessing the effects of ischemic brain damage on spatial learning and memory in rats. Since the method excludes the need for food deprivation or immersion of the subject in water, it should provide an alternative, sensitive and practical behavioral test to evaluate the neuroprotective effects of drugs after ischemic brain damage or other kinds of brain dysfunction. Further studies are needed to better investigate the effects of FK 506 on the cognitive disruption caused by different animal models of cerebral ischemia.

Keywords: Cerebral ischemia, Neuronal brain damage, Aversive radial maze, Learning an memory impairment, Tacrolimus (FK506), Neuroprotection.

1. INTRODUCTION

Learning and memory impairments are major outcomes of cerebral ischemia, an event that occurs during cardiac arrest and cardiopulmonary bypass surgery (Cummings et al., 1984), stroke (Gavrilescu & Kase, 1995), and chronic, cerebral hypoperfusion (Farkas & Luitten, 2001). The use of chronic animal models of neuropsychological symptoms resulting from ischemic brain damage is necessary to understanding the pathophysiology of such symptoms, and to develop protective drug therapies (Nunn & Hodges,1994; Hunter et al.,1998; Corbett & Nurse, 1998).

In rodent models, the nature of the cognitive disturbances following cerebral ischemia has been widely studied using Morris's water maze task or the 8-arm, radial maze task (Nunn & Hodges 1994; Hunter et al. 1998; Corbett & Nurse 1998; Okada et al., 1995; Block & Schwarz, 1995; Hodges 1996; Cain & Boon, 2003). In evaluating drugs potentially useful in treating the outcome of cerebral ischemia, the combined use of histological and behavioral end-points is highly recommended (Corbett & Nurse 1998; STAIR 1999). Thus and owing to difficulty with the water maze model in our laboratory, in a previous study we employed the circular platform task (CPT) to provide a base line for the effects of transient, global forebrain ischemia (TGCI) in rats (Milani et al., 1998). The CPT was first used by Barnes (1979) to examine the effect of aging on spatial learning and memory function, and is considered to be conceptually similar to the water maze task, with the advantage that it does not require immersion of the subjects in water. The CPT was developed on the basis of the rat's natural behavior of avoiding aversive places, mainly open and illuminated areas, and its preference for a darkened, enclosed shelter (Barnes 1979, 1988). Inspired by our previous experience with the CPT (Milani et al., 1998) we developed a non-food rewarded version of the 8-arm radial maze, referred to as the "aversive" radial arm maze (Paganelli et al., 2004; see also Appendix A), since it uses the same principle as the CPT. In contrast to the appetitive

(conventional) radial maze paradigm, the aversive radial maze (AvRM) does not require food deprivation. Differently from the water maze, the AvRM does not need immersion of the rat in water. In principle, these characteristics may represent an important advantage of the AvRM model. Therefore, the sensitivity of the AvRM to different animal models of brain injury and the reproducibility of data should be investigated to ensure its applicability.

In our original study (Paganelli et al., 2004), the AvRM was used as an unconfined maze, i.e., the central arena and the radial arms constituted a single compartment (see Appendix A for figure of the unconfined AvRM). This framework provides the animal with free and immediate access to each of the eight arms, at any time-point within a training session, and allows the animal to use egocentric guidance strategies to solve the task. Perhaps one of the most efficient tactics that many subjects employ to find the shelter or other reward in the unconfined radial maze entails a series of sequential entries into arms adjacent to that previously visited. After returning from an arm to the central area of the maze, most animals entered the arm adjacent to that previously visited and so on (Hodges 1996; Dale 1986; Crusio et al. 1987; Lassale et al. 1994; Roullet & Lassale 1995). This behavioral response was also observed and quantified by us in the unconfined, aversive radial maze (Paganelli et al., 2004; see Figure 3 in Appendix A). The rate of sequential entry increased progressively across training, reaching a maximum when the rat performed a series of sequential entrances and maintained this behavior uninterruptedly until finding the goal box. This behavior clearly indicates that the animal learns about the efficacy of this egocentric strategy and takes advantage of it. Therefore, it has been argued that when the rat adopts such a sequential entry strategy, it may use sensory (kinesthetic) cues rather than spatial navigating capabilities to guide behavior (Roullet & Lassalle, 1995).

Despite of this, and considering our previous findings showing an neuroprotective effects of tacrolimus (FK506) in the hippocampus (Giordani et al., 2003; Appendix B), in a

subsequent study we used the unconfined AvRM to evaluated whether the immunosuppressant agent tacrolimus (FK506, Prograff®) could improve the ischemia-induced learning and memory impairments (Benetoli et al., 2004; see Appendix C for results). Tacrolimus is a fungal-derived macrolide exhibiting a potent immunosuppressant action, and has been recently introduced in clinical practice to prevent allograft rejection. A more detailed description about the neuroprotective properties of tacrolimus is found in Appendix C.

In the Benetoli's study, however, interpretation of the effect of tacrolimus (FK506) on acquisition performance by ischemic rats was hindered since ischemia did not affect learning acquisition compared to sham-operated rats. This finding did not reproduce our previous one (Paganelli et al., 2004), probable because the influence of the extra-maze cues, which number, nature and spatial location were changed randomly and inadvertently in the Benetoli's study, as compared to that used in the Paganelli's study. This may have affected the sensitivity of the behavioral task between the two studies, since performance in the radial maze is highly influenced by the presence and distribution pattern of the cues.

In view of this apparent difficulty with the unconfined AvRM, we have made efforts to improve it, mainly by abolishing that strategy of sequential entry adopted by many rats. It is well known that the sequential entry behavior observed in the unconfined radial maze can be abolished by introducing a door at the entrance to each arm of the maze (Olton et al., 1977; Schwegler & Crusio, 1995). This arrangement allows the experimenter to confine the animal in the central arena before releasing it to explore the various arms and find the reward (pellets), or the shelter as in AvRM. Thus, the unconfined AvRM was changed to the confined version by adding guillotine-doors at the entrance of each arm. The figure 1 shows an illustration of the confined AvRM as developed in our laboratory (compare it with figure 1 in the Appendix A). A detailed description of the confined AvRM is given below (section 2.6).

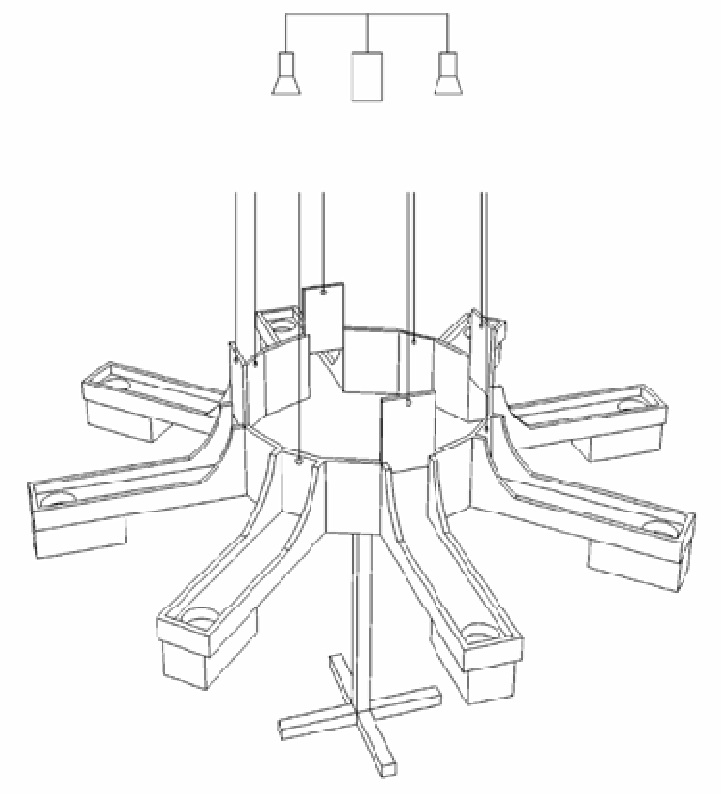


Figure 1. Schematic representation of the confined version of the AvRM. Each arm has a box just beneath the opening at the distal extremity; however, only one is the true goal box (close-ended box). In the remaining seven arms, the boxes are open-ended, i.e., they have walls like the true goal box, but 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. The sides located between arms are also walled. Above the central area are two spotlights which render the maze well illuminated.

Thus, the objective of the present work is to demonstrate, firstly, that the confined AvRM is a simple and sensitive behavioral test to measure the effects of ischemic brain damage on learning and memory function in rats. To this end, part of the results obtained previously in our laboratory was reorganized and the analysis of the data was expanded. These experiments were carried out to evaluate the effects of different models of ischemic brain damage on learning and memory of rats tested in the AvRM. These models include: a) transient, global cerebral ischemia (TGCI, acute 4-VO model); b) chronic, cerebral hypoperfusion (CCH, chronic 4-VO model; see Appendix D) and c) transient, focal cerebral ischemia (TFCI, middle cerebral artery occlusion model). In addition, we used the confined AvRM to reassess the effects of the immunosuppressant agent tacrolimus (FK 506, Prograf®) on the ischemia-induce behavioral and histological changes (see Discussion for a more detailed description on the neuroprotective properties of FK506).

2. MATERIALS AND METHODS

2.1. Subjects

Male, Wistar rats, arrounding 3 months of age at the time of surgery were used. The rats were housed at a controlled temperature (22 ± 1°C) on a 12 hour-alternate light/dark cycle (lights on at 0700 h). Food and water were provided *ad libitum* until the day of surgery. The experimental procedures described below adhere to the ethical principles set down by the Brazilian College of Animal Experimentation (COBEA, http://www.cobea.org.br), 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. Transient, global cerebral ischemia (TGCI)

The 4-vessel occlusion (4-VO) model (Pulsinelli & Brierley, 1979) was used with modifications. Under anesthesia (ethyl ether), the vertebral arteries were permanently electrogoagulated, and a silk thread was loosely snared around the common carotid arteries. Five to six hours later the carotid arteries were occluded for 15 minutes by carefully tightening the thread. Throughout occlusion and also during the first hour of reperfusion, the rats were maintained normothermic in a warming box with the temperature set to 30°C (Seif el Nasr et al., 1992). Loss of the righting reflex within 2 minutes of carotid occlusion, unresponsiveness to gentle touch, mydriasis, and tonic extension of the paws were considered indicative of effective ischemia. These signals were registered and the rat was excluded from the study if during occlusion it recovered the righting reflex. Rectal temperature was monitored during and after ischemia with a digital thermometer (Minipa APPA, MT-520, São Paulo, Brazil), using a rectal probe inserted to approximately 6 cm depth. Animals assigned to the sham-operated group were submitted to the same surgical procedure without vertebral and carotid occlusion.

2.3. Chronic cerebral hypoperfusion (CCH)

Using the same anesthesia and surgical techniques, rats were subjected to permanent, 3-stage 4-VO, according the following sequence of vessel occlusion: bilateral vertebral arteries (VA), right common carotid artery (CCA) and left CCA, i.e., VA → CCA → CCA, with 1-week inter-stage intervals. These rats were kept alive for 4 weeks after the final occlusion stage or sham operation. In a second group of rats, instead of occluding the CCA, the internal branches of the carotid arteries (ICA) were ligated at a point posterior to the origin of the pterygopalatine arteries (PPA), i.e., VA → ICA → ICA. In this group, the rats survived for 8 weeks after the last stage of 4-VO. Animals assigned to the sham-operated group were submitted to the same surgical procedure without vessel occlusion.

2.4. Transient, focal, cerebral ischemia (TFCI)

Focal, cerebral ischemia was induced by proximal occlusion of the left, middle, cerebral artery (MCA) according to the method described by Longa et al. 1989, with modifications. Under ether anesthesia, the MCA was occluded for two hours by inserting a 4-0 nylon monofilament suture (Ethicon) into the ICA, via the external carotid artery (ECA), and advancing gently until weak resistance was felt. Once the monofilament was inserted into the ECA and advanced close to the ICA/ECA bifurcation, anesthesia was interrupted and 3 to 4 minutes later the filament was advanced to occlude the MCA. Core temperature was controlled only during the period of surgery, and maintained at around 37.5 ^{o}C by a heating blanket. During ischemia and also during the first hour of reperfusion, the rats were maintained normothermic in a warming box with the temperature set to 30°C (Seif el Nasr et al., 1992). At the end of ischemia, the rat was briefly re-anesthetized and reperfusion initiated by retracting the filament from the ICA, into the ECA. The suture was closed and the animal

was kept alive for 35 days before behavioral testing. The rectal temperature was monitored each 30 minutes in the first seven hours post-ischemia, and once a day during the next 7 days of reperfusion.

2.5. Drug treatment

FK506 (1.0 mg/kg) was given intravenously (i.v.) at reperfusion, followed by intraperitoneal (i.p.) injections applied 6, 24, 48 and 72 hours post-ischemia. 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 the Fujisawa Pharmaceutical Co., Osaka, Japan.

2.6. Behavioral testing apparatus

A schematic representation of the confined version of the aversive, 8-arm radial maze is illustrated in Figure 1. Eight arms (55 cm X 15 cm) radiate outward 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 x11 x 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 contained the true refuge, i.e., a close-ended box, that can be shifted from one arm to another. In the remaining seven arms, the boxes were 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 bordered

each arm to prevent the animal from falling. The central arena is separated from the arms by transparent, acrylic guillotine doors (19-cm height). The rotatable maze was elevated 90 cm above the floor on a metal stand. From a separate room, a pulley system connects to each individual guillotine door, allowing the experimenter to confine the animal in the central arena before release to explore the arms. Several extra-maze cues (e.g., posters on the walls, a closed door, a window and some three-dimensional 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, 40W 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.

2.7. Behavioral Procedures

2.7.1. Post-operative acquisition trial

The effects of ischemia on the performance of ACQUISITION (here defined as the process of acquiring information and processing it to memory formation, i.e., learning) was evaluated in rats subjected either to TGCI, CCH or TFCI. Once the animals recovered from surgery, they were habituated to the testing apparatus before starting the training sessions. During habituation, all the arms were opened and the rat was placed in the center of the maze and allowed to explore until it found the shelter, or until a 4-min period had elapsed. If the rat did not find the goal box within 4 min, it was placed into the arm containing the correct goal box into which it was gently introduced by the experimenter. The rat was left for 4 min in the goal box, then returned to its home cage. The extra-maze cues were removed during habituation, and the spatial position of the goal box was randomly changed between subjects and trials.

This procedure was repeated for 3 days. On the day subsequent to habituation, training for acquisition of the task was begun for 9 (TFCI experiment) or 15 days (TGCI and CCH experiments). The rats were trained using three trials/session, one session per day. For training, the 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 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 simultaneously lowered. After returning 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 in 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 changed to another arm, maintaining its spatial position unchanged in relation to the extra-maze cues.

The latency to find the goal box, the number of reference memory errors, and the number of working memory errors were the parameters used to measure behavioral performance. For the rat tested in mazes, such as the radial maze, 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 distinguishable from reference memory, which is a memory that would typically be acquired with repeated training, and would persist from days*

to month…Working memory, in contrast, is typically a delay-dependent representation of stimuli that are used to guide behavior within a task" (Dudchenko, 2004). In the present experiments, a reference memory error was registered when the rat visited an wrong arm for the first time within a trial. However, if the rat returned to an arm which had been previously visited during that trial, a working memory error was recorded. An arm was considered to be visited when the rat entered half way down its length. The animal was considered to have left an arm when its entire body, including the tail, returned the central area of the maze.

2.7.2. Post-operative retention trial

The effect of ischemia on the performance retention (here defined as being the process of memory consolidation) was evaluated in rats subjected to TGCI. 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 (11^{th} experimental day) the rats were subjected to TGCI and allowed to recover from surgery for 20 days, when they were tested for retention of cognition on days 31, 35 and 39 post-ischemia.

2.8. Histological analysis of the hippocampus (TGCI and CCH experiments)

On the day after behavioral testing, the animals were deeply anesthetized and the brain fixed by intracardiac perfusion or immersion in the fixative solution as described previously for rats subjected to TGCI (Appendix A) or CCH (Appendix D), respectively. After fixation, paraffin-embedded, coronal sections of 5-μm thickness were taken from each brain (-4.52 mm) and stained with celestine blue/acid fuchsin. Three sections were used for bilateral counts of normal-appearing neurons in the hippocampus CA1 subfield. The number of intact-appearing pyramidal cells with a distinct nucleus and nucleolus, in each hemisphere, was counted along a transect of 1.35 mm length (magnification 400x, field diameter: 450 μm,

Olympus). For each individual, the number of pyramidal cells was expressed as the mean of the three coronal sections. The identity of the groups was not revealed during histological assessment.

2.9. Quantification of Infarct Size (TFCI experiment)

On the day after behavioral testing (45 days after MCAo), the rat was deeply anesthetized with ether, decapitated and the brain quickly removed and immersed in ice-cold saline (1-2^{o}C) for 10 min. Using a chilled brain matrix, the brain was sectioned coronally into eight or nine, 1.5-mm thick slices starting from the frontal pole, and processed for histochemical staining by TTC as described in detail elsewhere (Bartus et al., 1994). The infarcted, necrotic tissue (white in color) was separated from the adjacent, intact tissue (carmine red in color) using a using a #11 microscalpel blade. After separation, the normal tissues from both the intact and the affected hemispheres, and the ischemic tissue, were transferred to Eppendorf tubes containing 10% buffered formalin and held at 0 ^{0}C until weighing. For weighing, the tissue portions were transferred to Petri dishes, and excess formalin on the tissue was carefully blotted with a swab. The ischemic tissue was then weighed using an analytical balance (Mettler-Toledo GmbH, Mod. AB204-S, Switzerland) (Schilichting et al., 2004). The hemispheric, infarct size was calculated using the equation: $L\ (\%) = 100(P_c - P_i) / P_c$, where: L = infarct size (%), expressed as the amount of lost tissue; P_c = weight of the contralateral (intact) hemisphere; P_i = weight of the ischemic hemisphere (remaining intact tissue) (adapted from ref. Swanson et al., 1990). Microscopic, histological analyses of the hippocampus or other brain regions were not performed in the rats subjected to MCA occlusion. A comparative analysis of the weighing method for quantification of infarct size was published (Schilichting et al., 2004).

2.10. Data Analysis

Multifactorial Analysis of Variance for repeated measures (MANOVA) was used to quantify acquisition and retention performance, with Groups as the ‘between’ and Sessions as the ‘within’ subjects factors. Therefore, the parametric Student’s t-Test or the Duncan’s Multiple Comparison Test were used as *post-hoc* tests, when appropriate, to locate the time point at which the ischemic and control groups were significantly different, or to distinguish the effects of different treatments (drug experiments). The Mann-Whitney, or the Kruskal-Wallis Analysis of Variance, followed by Dunn´s *post-hoc* test were used to compare the groups when the parameters ‘total latency’ and ‘total number of errors’ were computed. Simple ANOVA (one-way) was used to quantify the results of the histological analysis. Statistical significance was defined as a P value ≤ 0.05.

3. RESULTS

3.1 Part I: The effects of TGCI, CCH or TFCI on the behavioral performance measured in the AvRM task

3.1.1 Overall Behavior.

Independently of the model of cerebral ischemia, both the control and ischemic rats seemed well motivated to avoid the illuminated, central arena of the maze, and to search for the enclosed, darkened shelter after the guillotine doors were raised. In the present, confined AvRM, the behavior of sequential entry into the adjacent arms, prominent in our previous study with the unconfined maze, was not observed. After returning from an arm to the central arena, the rat encounters the other arms closed, thus disrupting the chaining response. During the final training sessions (Days), however, some subjects tend to circle close to the walls of the central arena until the guillotine doors are raised.

3.1.2 Effects of transient, global cerebral ischemia on acquisition and retention performance.

The upper panels of Figure 2 show the effects of TGCI on acquisition performance as measured daily in the confined AvRM. Acquisition was registered from day 23 to day 38 after TGCI and plotted as trial blocks (three days per block). Fifteen minutes of 4-VO disrupted acquisition performance as revealed by a significant ‘Group’ effect on latency ($F_{1, 200} = 6.65$, $p < 0.001$), number of reference memory errors ($F_{1, 200} = 6.25$, $p < 0.0001$) and number of working memory errors ($F_{1, 200} = 4.10$, $p < 0.05$).

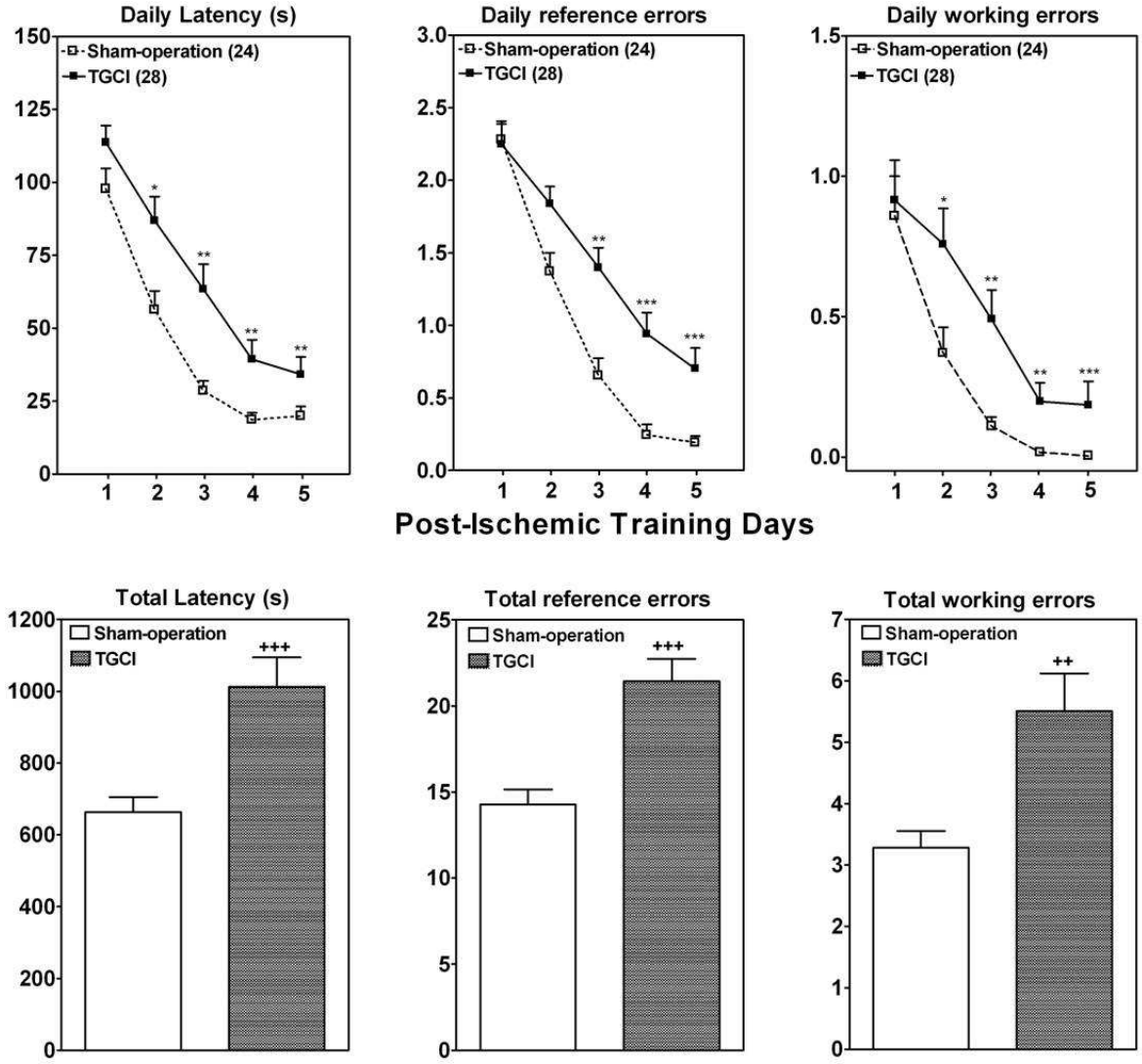


FIGURE 2. Upper panels: the effect of 15-min TGCI on acquisition in the AvRM . Acquisition performance was registered for 15 consecutive days. For each subject, the mean value measured in three trials within each session (day) was used to express performance in terms of latency (left), number of reference memory errors (middle) and number of working memory errors (right panel). Values are the group mean (± SE) plotted as trial blocks (three days per block). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, for point-to-point comparisons (Student's t-Test). The numbers in parentheses indicate sample size. Lower panels: total latency and total number of errors summed ($\sum$) over 15 days training (p** < 0.01; ***p < 0.001). This data is derived from the study reported in the Appendix E.

A highly significant 'Session' effect was evident ($F_{4, 200} = 26.34 – 49.90$, $p < 0.0001$), and a Group *vs.* Session interaction appeared for the parameter 'reference errors' ($F_{4, 200} = 2.09$, $p < 0.01$). The *post hoc* test (Student t-test, point-to-point comparison) revealed that the ischemic group differed from the control group during at least three consecutive training blocks ($p <$

0.01 – 0.0001), indicating that although the ischemic rats do benefit from training, their performance was significantly inferior to that of their sham-operated counterparts.

The lower panels of Figure 2 show the effect of ischemia on acquisition performance expressed as total latency and total number of errors, i.e., the sum ($\sum$) across the entire training period (Student's t-test, $p < 0.0001 – 0.01$). We include this analysis since the overall extent to which the ischemic group differs from its non-ischemic counterpart becomes more quantitatively palpable when the total latency and total number of errors are provided. Moreover, it may reveal additional information such as group differences that cannot be seen in the daily performance analysis (see other results below). Rectal temperature was unaffected by TGCI, at least up to 3 and half hours of reperfusion ($37.3 \pm 0.08 – 37.8 \pm 0.11$, figure not shown).

The effect of TGCI on retention performance is shown in Figure 3. Compared to the performance acquired on the last 3 days of pre-ischemic training (days 8, 9 and 10 in average), 15-minutes ischemia disrupted retention measured 31, 35 and 39 days later (paired t-test: $p < 0.001$-0.05, for the parameters "latency" and "number of reference errors". Corrected $\alpha^* = 0.017$). The sham-operation procedure did not affect retention performance at all. The effects of ischemia on retention of cognition were also evident when the ischemic group was compared to the control group across the entire retention test period (MANOVA: $F_{1, 66} = 13.61 – 22.42$, $p < 0.0001 – 0.001$; Student's t-test, $p < 0.05 – 0.001$ for point-to-point comparison of latency and number of errors). The lower panels of Figure 3 show the effect of ischemia on retention performance measured by total latency and total number of errors registered both before ischemia (the last three days of training) and after ischemia. Also by this analysis, the disruptive effect of ischemia on retention was clearly evident for all the three parameters (Sham-operation vs. TGCI: $p < 0.001 – 0.05$), while the sham-operation procedure did not affect retention negatively.

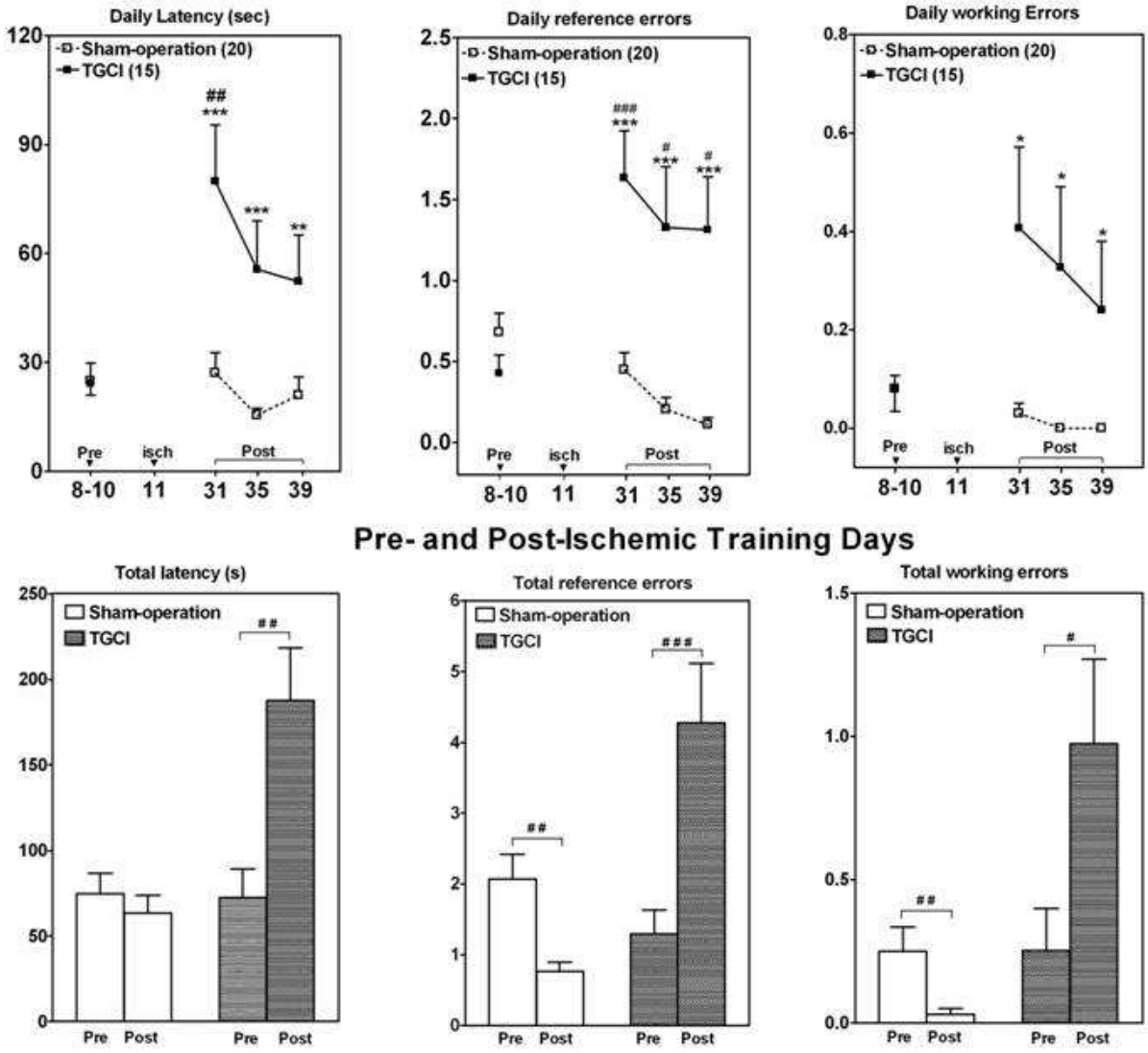


Figure 3. Upper panels: Daily analysis of the effect of TGCI on retention of acquired cognition. Before ischemia, the rats received 3 trials/day, for 10 days, and on day 11 they were subjected to TGCI. The pre-ischemic, asymptotic performance achieved by each group in the last three days of training (8^{th} -10^{th} day) was averaged and used for statistical comparisons to that measured post-ischemia on days 31, 35 and 39. Values are the group mean (± SE). #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ for paired t-test (day 8-10 *vs.* day 26, 30 or 34; corrected $\alpha = 0.017$); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for unpaired t-test (TGCI *vs.* Sham-operation, point-to-point comparisons during the retention test). The numbers in parentheses indicate sample size. Lower panels: total latency and total number of errors summed ($\sum$) over the last 3 days of training (Pre) and the entire retention period (Post), respectively (paired t-test, *$p < 0.05$; **$p < 0.01$ ***$p < 0.001$). This data derives partially from the studies with EGb 761 (Appendix E) and FK506 (see results below, unpublished), as they were pooled.

3.1.3 Effects of chronic, cerebral hypoperfusion on acquisition performance

Figure 4 shows the effect of chronic, cerebral hypoperfusion when the vertebral arteries (VA) plus the common carotid arteries (CCA) were permanently occluded following the sequence VA → CCA → CCA, with a 1-week inter-stage intervals. Four weeks after the last stage of vessel occlusion, a profound and sustained disruption of acquisition performance was revealed by a significant 'Group' effect on latency ($F_{1, 76}$ = 10.0, $P < 0.01$), number of reference memory errors ($F_{1, 76}$ = 19.30, $P < 0.001$) and number of working memory errors ($F_{1, 76}$ = 24.89, $P < 0.0001$). Although a global, significant 'Session' effect was detected ($F_{4, 76}$ = 5.41 – 15.72, $p < 0.0001$ - 0.001), the rats subjected to chronic 4-VO with CCA occlusion (4-VO/CCA) were profoundly and persistently impaired in learning the task ($p < 0.0001$ – 0.05; Student's t-Test, point-to-point comparisons). A significant 'Group' *versus* 'Session' interaction ($F_{4, 76}$ = 4.47 – 11.32, $P < 0.001$ – 0.0001) occurred for all parameters. The notable effect of chronic 4-VO/CCA on acquisition performance can be further visualized by using the total latency and total number of errors shown in the lower panels of Figure 3 (Student's t-test: $p < 0.0001$ – 0.01).

In contrast to CCA occlusion, however, Figure 5 shows the results when the internal carotid arteries (ICA) plus the vertebral arteries (VA) were occluded following the sequence VA → ICA → ICA (Figure 5). Even assessed 8-week after permanent 4-VO, the daily acquisition performance of "hypoperfused" rats did not differ significantly from their controls (upper panel, $F_{1, 64}$ = 0.11 – 2.50, $p > 0.05$ for all the three parameters). This result was unchanged when total latency and total number of errors were computed ($p > 0.05$, lower panel).

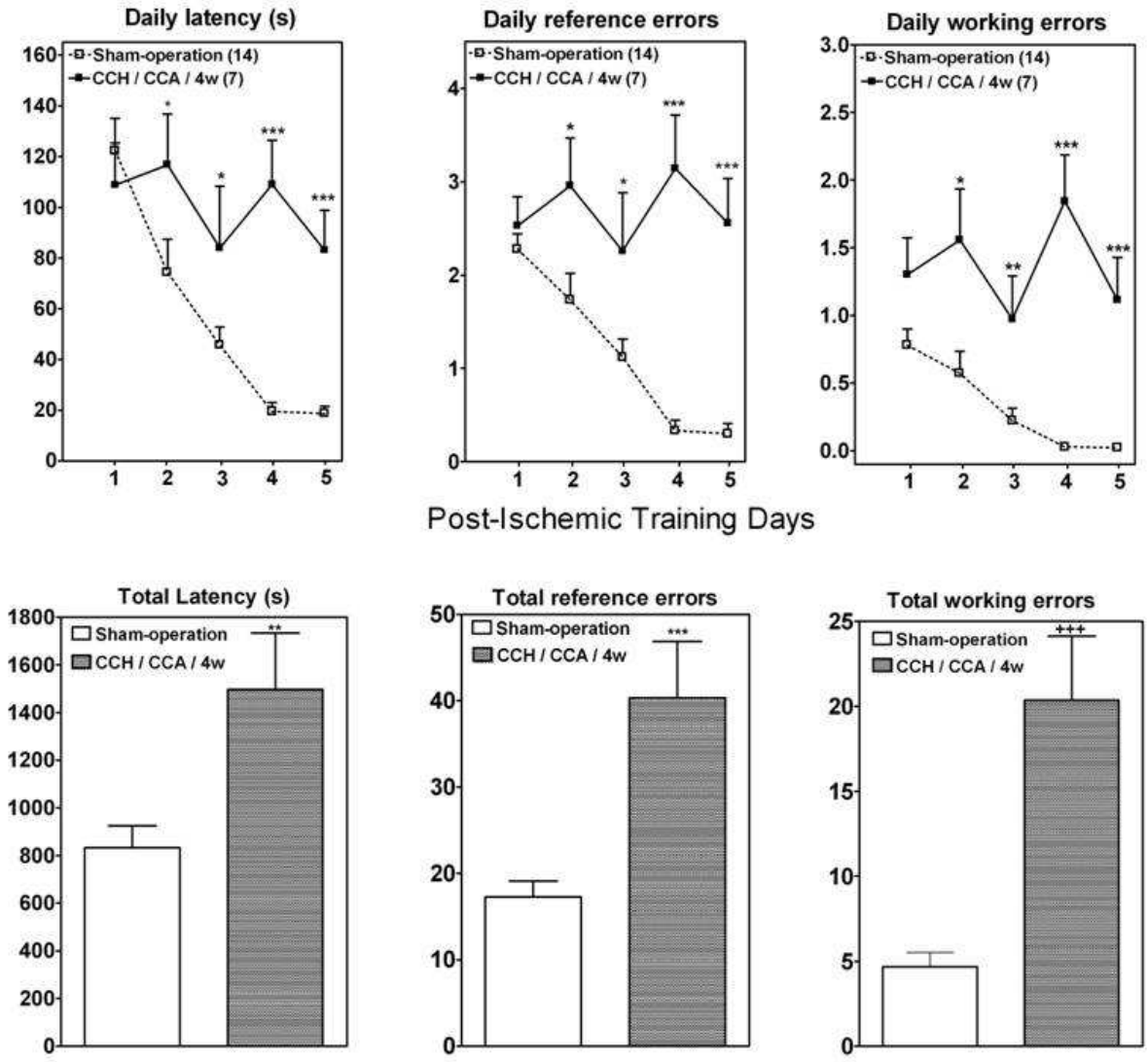


FIGURE 4. Upper panels: The effect of chronic, stepwise 4-VO on daily acquisition performance. The CCA were occluded, together with the VA, following the sequence VA → CCA → CCA, with a 1-week inter-stage interval. Acquisition performance was analyzed 4 weeks after the 4-VO stage, and extended for 15 consecutive days. For each subject, the mean value measured in three trials in each session (day) was used to express performance in terms of latency, number of reference memory errors, and number of working memory errors. Values are the group mean (± SE) plotted as trial blocks (three days per block). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ for point-to-point comparisons (Student's t-Test). The numbers in parentheses indicate sample size. Lower panels: total latency and total number of errors summed over 15 days training (** $p < 0.01$; *** $p < 0.001$).. (Modified from Appendix D).

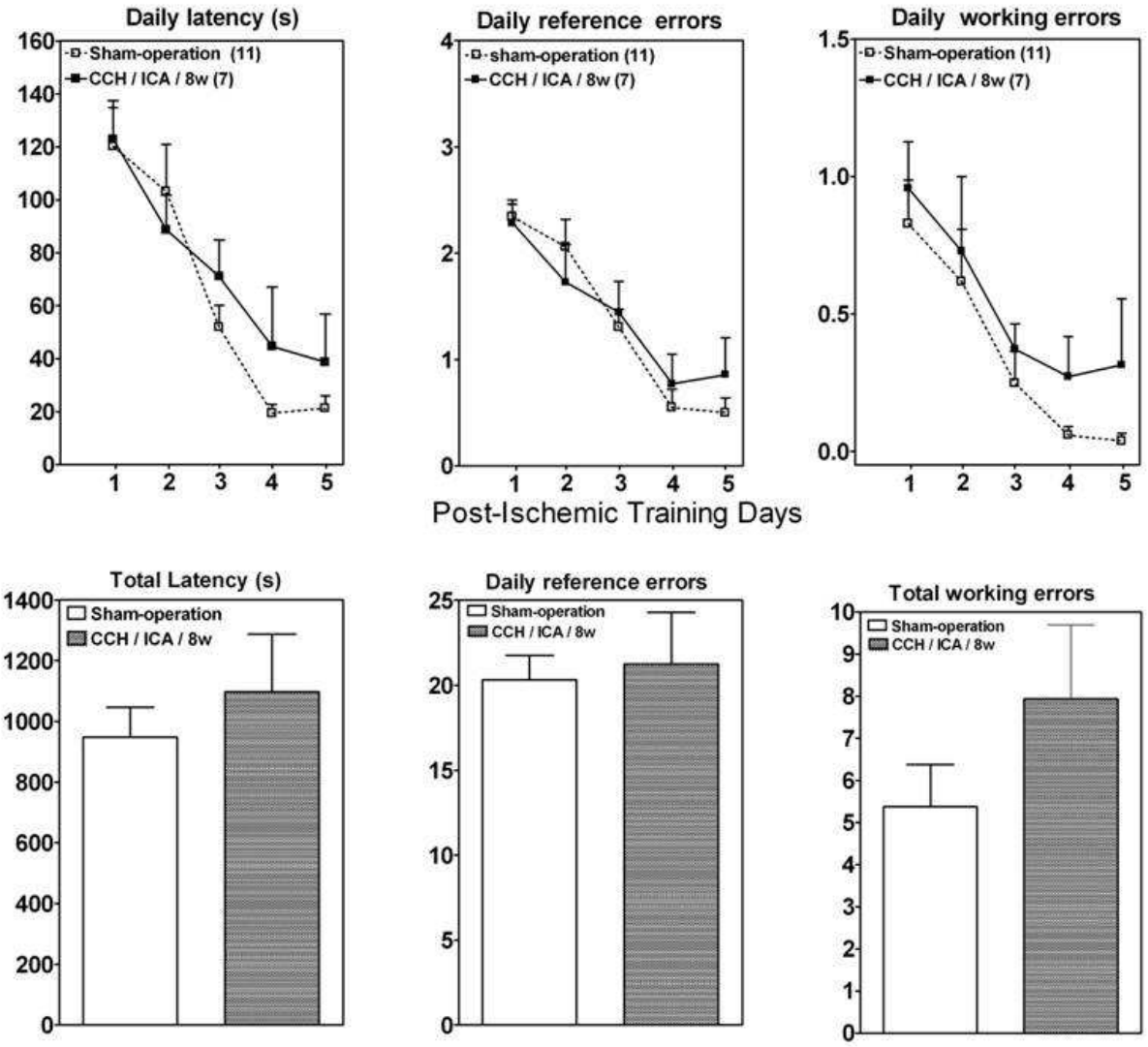


FIGURE 5. Upper panels: The effect of chronic 4-VO on daily acquisition performance, in rats with occlusion of the ICA and VA, following to the sequence VA → ICA → ICA. Lower panels: total latency and total number of errors summed over 15 days training. The numbers in parentheses indicate sample size (Modified from Appendix D). Additional details follow as described in figure 4.

3.1.4 Effects of transient, focal, cerebral ischemia (TFCI) on acquisition performance

The effect of regional brain ischemia on the capacity of the rats to solve the spatial task in the aversive radial maze is illustrated in Figure 6.

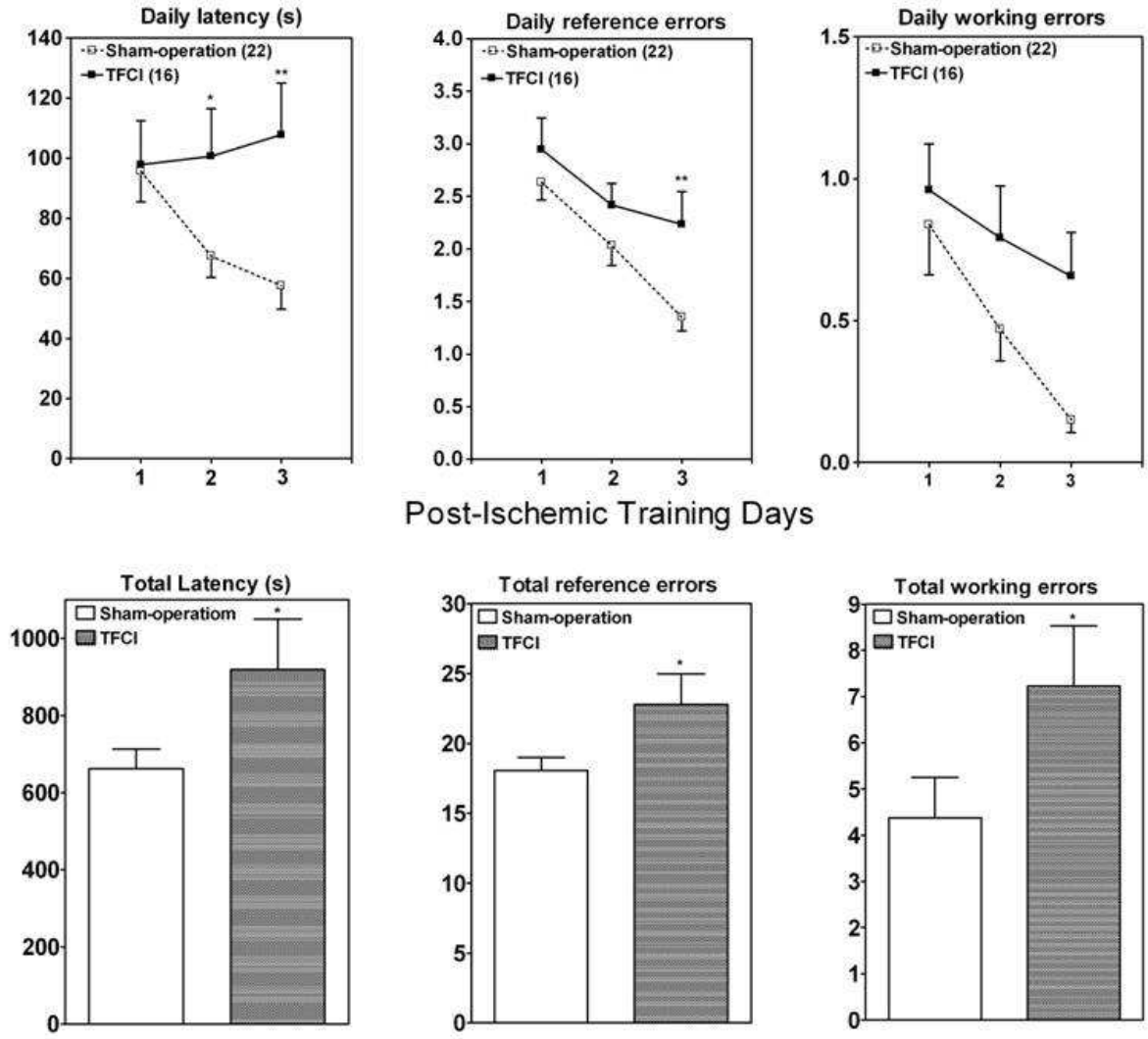


FIGURE 6. Upper panels: The effect of transient MCA occlusion (2 hours) on daily acquisition performance in the confined AvRM. The animals were tested from day 35 to day 44 post-ischemia. For each subject, the mean value measured in three trials in each session (day) was used to express performance in terms of latency, number of reference memory errors and number of working memory errors. Values are the group mean (± SE) plotted as trial blocks (three days per block). Sample size is indicated in parentheses. *$p < 0.05$; **$p < 0.01$ for point-to-point comparisons (Student's t-Test). Lower panels: total latency and total number of errors summed over 15 days training (**$p < 0.01$).

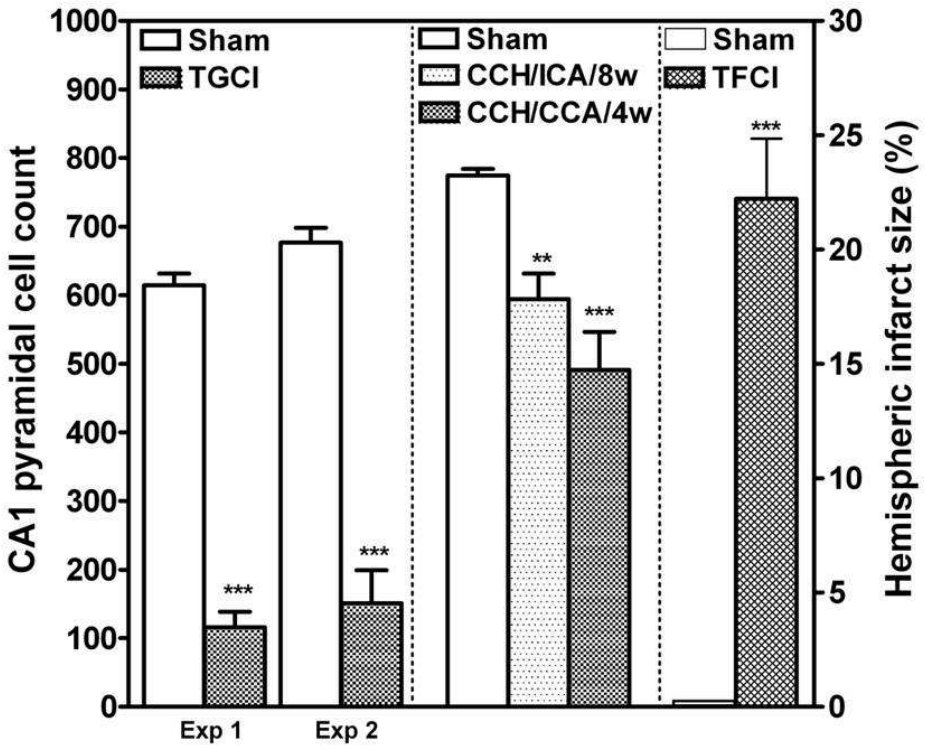
Acquisition performance was measured from day 35 to day 44 after unilateral MCAo. The group subjected to 2-h MCAo took longer to find the shelter ($F_{1, 72} = 4.14$; $p < 0.05$) and committed more reference memory errors ($F_{1, 72} = 4.84$; $p < 0.05$). Working memory performance was also affected, but not at 5% level ($F_{1, 72} = 3.55$; $p < 0.068$). Significant, global 'Session' effects were found for both reference and working memory errors ($F_{2, 72} =$

12.73 – 18.59, $p < 0.0001$), but not for latency ($F_{2, 72} = 1.92$, $p > 0.05$). A Group x Session interaction was found for latency ($F_{2, 72} = 4.75$, $p < 0.05$). Working memory performance was significantly impaired, however, when the total number of errors was considered (lower panel: $p < 0.05$).

Rectal temperature increased to 38.7 ± 0.12 ^{0}C at the end of MCAo (2-h period) ($p < 0.01$ vs. basal), but decreased abruptly to 38.1 ± 0.13 ^{0}C immediately after reperfusion, when the rat was briefly re-anesthetized to remove the filament. This temperature level was maintained at least for seven hours of reperfusion, and returned to basal values (36.3 ± 0.15) at 24 hours of reperfusion (figure not shown). This data on temperature reproduces our previous one (Schilichting et al, 2004; Lima et al., 2006).

3.1.5 Magnitude of brain damage after TGCI , CCH or TFCI

Figure 7 shows the magnitude of hippocampal, CA1 pyramidal cell loss induced by TGCI or CCH, and the size of hemispheric infarct (%) caused by TFCI. Fifteen minutes of acute, 4-VO caused marked neurodegeneration in the CA1 sector of the hippocampus (81%; $p<0.0001$). After chronic, cerebral hypoperfusion, the number of intact-appearing CA1 pyramidal cells was most reduced in the group subjected to 4-VO with CCA occlusion (group 4-VO/CCA/4w; 37% cell loss; $p < 0.001$ vs. Sham). A lesser degree of CA1 lesioning occurred after 4-VO with ICA occlusion (group 4-VO/ICA/8w; 23.2% cell loss; $p < 0.01$ vs. Sham)**.** Two hours MCAo caused around 23% hemispheric infarct ($p < 0.0001$), the location of which varied from a single striatal or cortical focus to a broad cortico-striatal zone of necrosis, enveloping the medio-frontal and dorso-lateral portion of the affected hemisphere.



FGURE 7. Left and middle portions: the number (mean ± SEM) of intact-appearing, pyramidal cells in the hippocampal CA1 subfield after 15 min global, cerebral ischemia (TGCI) or chronic, cerebral hypoperfusion (CCH), respectively. Right portion: Size of hemispheric infarct induced by transient (2-h), focal cerebral ischemia (TFCI). Histological analysis was performed 39 days after TGCI, 49 days after CCH (4-VO/CCA/4w) or 79 days after CCH (4-VO/ICA/8w), and 45 days after TFCI. In the TGCI and CCH experiments, the CA1 pyramidal cells were counted along a transect of 1.35 mm length in each hemisphere. For each subject, the number of cells represents the mean of three, coronal brain sections. In the TFCI experiment, infarct size was estimated by weighing the ischemic tissue, then expressed as a percentage of hemispheric infarct. Histological analysis of the hippocampus was not performed in the rats subjected to TFCI. **$p < 0.01$; ***$p < 0.001$, compared to the respective sham-operated group. (Four brains from vehicle-treated rats in the acquisition experiment were lost during histological processing).

There was no correlation between behavioral performance, as measured by total latency and total number of errors, and the extent of lesion induced either by TGCI (‘r’ = 1.45 – 1.67, $p > 0.05$), CCH (‘r’ = 0.036 – 0.23, $p > 0.05$) or TFCI (‘r’ = 0.08– 1.35, $p > 0.05$; figures not shown).

3.2 Part II: The effects of tacrolimus (FK506) on TGCI-induced behavioral and histological changes

Figure 8 shows the effects of tracrolimus on acquisition impairment resulting from transient, global cerebral ischemia (TGCI). Examining performances across training Sessions (*upper panels*), the repeated measures ANOVA revealed a significant 'Group' effect on 'latency' $F_{2,296} = 6.3$, $p < 0,01$) and 'number of working memory errors' ($F_{2,296} = 3.26$, $p < 0,05$). TGCI affected also the performance of reference memory, but not at the level of 5% ($F_{2,\ 296} = 2,70$, $p < \underline{0,07}$). A highly significant 'Session' effect was evident for all three parameters ($F_{4,\ 296} = 100.12 – 142.24$, $p < 0.0001$), indicating that all groups learned the task very well. A Group *vs.* Session interaction appeared for the parameters 'reference memory errors' ($F_{8,\ 296} = 2.68$, $p < 0.01$) and 'working memory errors' ($F_{8,\ 296} = 3.11$, $p < 0.01$). Duncan's multiple range test showed that acquisition performance was impaired for all three parameters in the group subjected to TGCI and treated with vehicle ($p < 0.01$- 0.05, Vehicle *vs.* Sham), thus indicating that ischemia disrupted acquisition in the AvRM. The disruptive effect of ischemia was also evident when the results were analysed as the '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 treatment with FK506 failed to reduce the disruptive effect of ischemia on acquisition as measured by all three parameters ($p > 0.05$, FK 506 *vs.* vehicle).

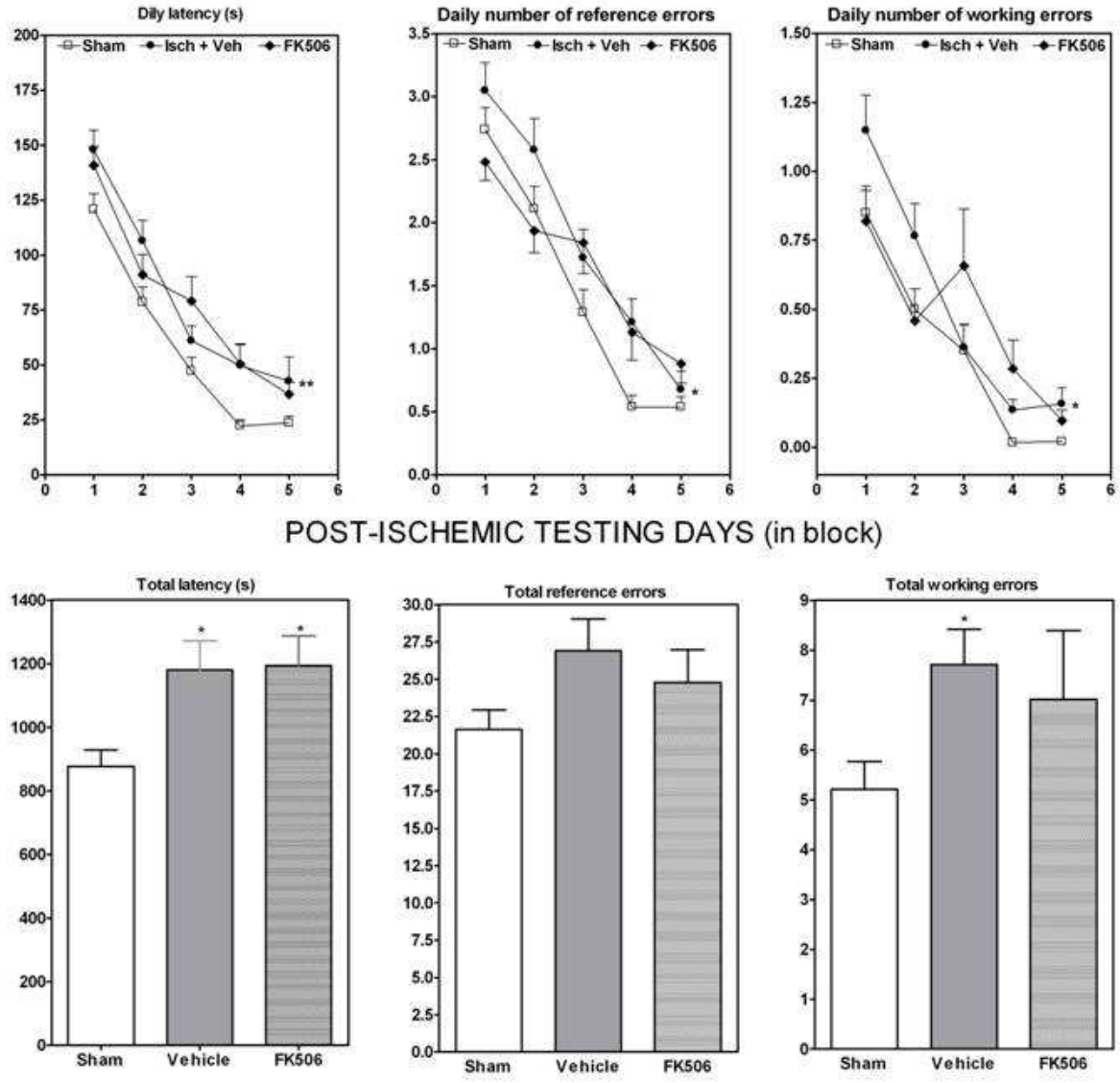


Figure 8. The effect of FK506 (1.0 mg/kg, 1 injection iv + 4 injections ip) on acquisition performance of rats subjected to TGCI (15 min) and tested in the confined AvRM. FK506 was given 0, 6, 24, 48 or 72 h postischemia. For each individual, the mean value obtained from three trials/day expresses performance 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). This effect was not reduced by FK 506 ($p > 0.5$, FK 506 *vs.* vehicle. Data are the group mean ± SEM. Sample sizes: Sham = 30; Vehicle = 21; FK 506 = 26.

The effect of TGCI on retention performance and the influence of tacrolimus (FK506) thereon is showed in Figure 9.

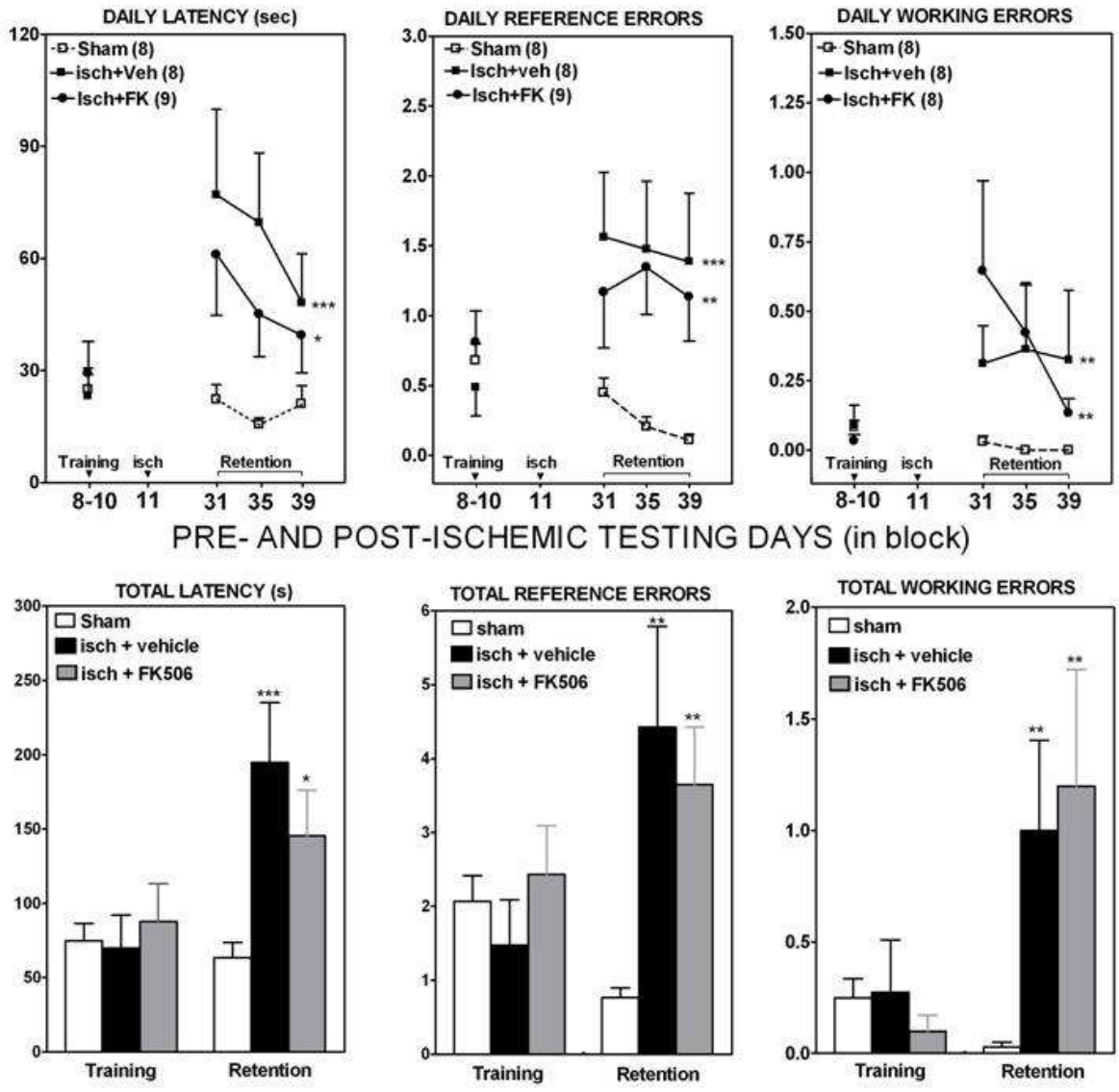


Figure 9. The effect of FK506 (1.0 mg/kg, 1 injection iv + 4 injections ip) on retention performance of rats subjected to TGCI (15 min) and tested in the confined AvRM. Ischemia was induced after 10 days of training (on day 11), and retention performance was assessed on days 31, 35 and 39 post-ischemia (*upper panels*). Pre-ischemic performance is given as the mean for the last three days of training (days 8-10). The total latency and total number of errors (mean ± SEM), summed (∑) 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 as measured by all three parameters; this amnesic effect of TGCI was lower in the FK506-treated group, but without reaching statistical significance. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Vehicle *vs.* Sham or FK506 vs. Shan). Values are the group mean ± SEM. Sample sizes: Sham = 8; Vehicle = 8; FK506 = 9.

Animals were trained for 10 days before 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). In the *upper panels*, the pre-ischemic, asymptotic performances are represented by the mean for the final 3 days of training (day 8-10). Comparing the groups during the retention trials on days 31, 35 and 39 (*upper panels*), the repeated measures ANOVA revealed a significant 'Group' effect for 'latency' ($F_{2,82}$ = 15, p < 0.0001), 'number of reference errors' ($F_{2,82}$ = 18.8, p < 0.0001) and 'number of working errors' ($F_{2,82}$ = 9.24, p < 0.0001). The *post-hoc* analysis revealed that retention performance in the ischemic, vehicle-treated group was significantly disrupted by TGCI (p < 0.001 – 0.01, Vehicle *vs.* Sham). The amnesic effect of TGCI is also clearly evident when the results are examined using 'total latency' and 'total number of errors' (*lower panels*, K-W = 22.52 – 35.82, p < 0.01, Vehicle *vs.* Sham). The treatment with FK506 did not prevent the amnesic effect of TGCI (p > 0.05, FK506 vs. Vehicle, for all three parameters).

Figure 10 illustrates the pyramidal cell density in the CA1 sector of the hippocampus of the several experimental groups. Fifteen minutes 4-VO induced 69.7% neuronal loss in the group used in the acquisition experiment ($F_{2, 76}$ = 39.91; p<0.001 Sham *vs.* Vehicle), but only 24.2% cell death during the retention experiment ($F_{2, 36}$ = 3. 82, p < 0.05 Sham *vs.* Vehicle). FK506 reduced CA1 cell death significantly in the acquisition experiment (p < 0.05), an effect which should be considered weak, since cell loss was reduced 23.7% only. In contrast, the degree of CA1 cell loss was minimal, but statistically significant in the group treated with vehicle during the retention experiment ($F_{2, 36}$ = 3.82, p < 0.05 sham vs. vehicle). In the group treat with FK 506, the number of intact-appearing cells was at the level to the sham-operated rats. On the basis of our previous findings, this result was unexpected and the influence of some uncontrolled variable may have occurred (see Discussion).

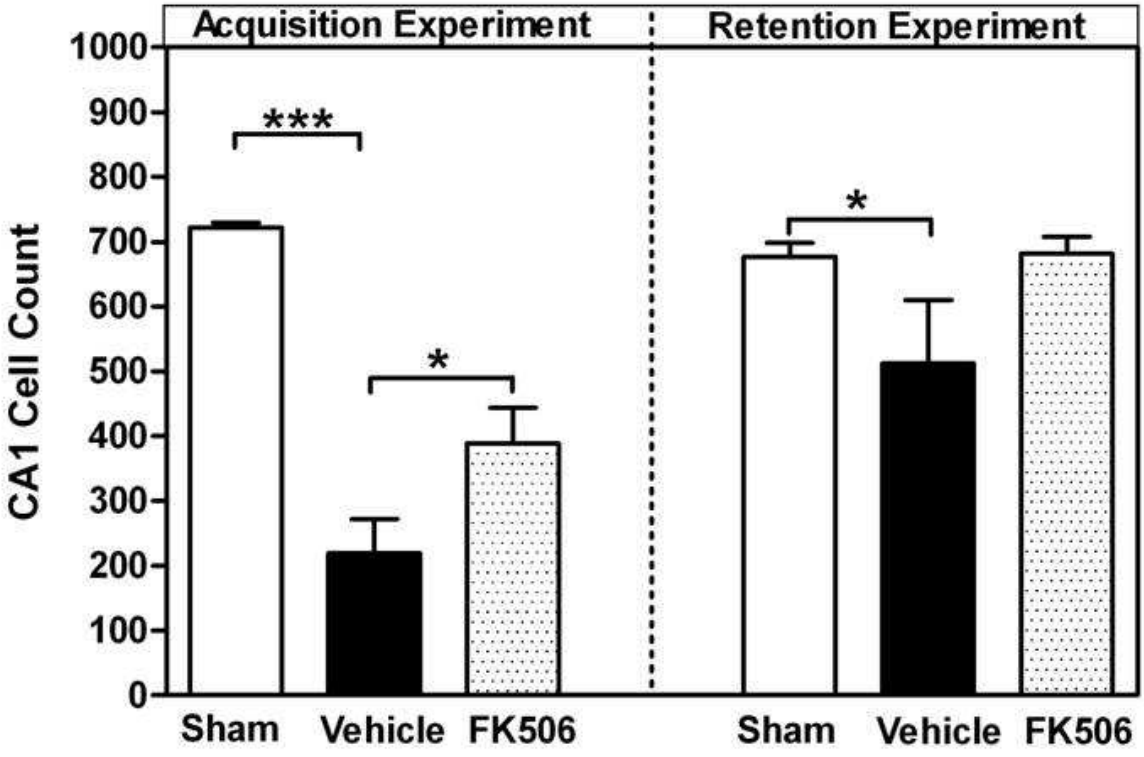


Figure 10. Effect of FK 506 on ischemia-induced, hippocampal pyramidal CA1 cell loss. Cells were counted along a transect 1.35 mm in length. Histological analysis was performed one day after the end of behavioral testing. Values are the mean ± SEM. *p < 0.01; ***p < 0.001. Sample sizes: Acquisition experiment: Sham = 30; Vehicle = 21; FK 506 = 26. Retention experiment: Sham = 8; Vehicle = 8; FK506 = 9.

Finally, the rectal temperature was unaffected by TGCI in both experiments, at least up to 3,5 hours reperfusion (37.3 ± 0.08 – 37.8 ± 0.11 ^{o}C, data not shown). This data reproduces that reported previously (Benetoli et al., 2004; see Figure 5 in Appendix C).

4. DISCUSSION

The present data extend our previous findings to demonstrate that the AvRM constitutes a useful model to evaluate spatial learning and memory in rats, and the effects of ischemic brain damage, with the advantages that it does not require neither food deprivation nor immersion of the animal in water, as is the case in the conventional, appetitive radial maze or water maze, respectively. The apparatus used here differs structurally from that reported originally (Paganelli et al, 2004) in that the central arena is separated from the radial arms by transparent, acrylic walls and guillotine doors placed at the entry to each arm. This allows the experimenter to confine the animal in the center of the maze before release to explore the arms and find the safe location (shelter). Although a systematic, comparative analysis was not made here, the use of confinement may have rendered the AvRM task more difficult. Comparing the groups subjected to TGCI in the present study with that examined previously in the unconfined maze (Paganelli et al., 2004; see also Appendix A), the number of training sessions (days) increased from eight days in the unconfined maze to fifteen days in the present, confined maze, before a similar level of performance was reached in both studies. Most important, however, the use of guillotine doors eliminated the sequential entry strategy adopted by many rats when they are tested in the unconfined maze. This behavior was quantified in our early study, in which we observed that some rats perform a vigorous, uninterrupted series of sequential entries until finding the shelter (Paganelli et al., 2004). This agrees with the notion that such a response may be a highly efficient strategy in the unconfined radial maze, and may involve superimposed, associative, tactile and/or olfactory modes of information processing (Hodges, 1996; Roullet & Lassalle 1995, Schwegler & Crusio, 1995). Despite the possible role of sensorial inputs for processing spatial information (Gallistel, 1989), it has been argued that when this type of response is well developed, the rat

may use sensory, kinesthetic cues rather than spatial navigating capabilities to perform the task (Dale, 1986, Roullet & Lassalle 1995, Schwegler & Crusio, 1995). Thus, the strategy of sequential arm choice used by some individuals in the unconfined radial maze may influence the nature and magnitude of the parameters used to quantify spatial learning and memory performance, particularly reference and working memory errors. This may partially explain why the number of reference memory errors measured in the unconfined AvRM after TGCI (Paganelli et al, 2004; see Figure 2 in Appendix A) was higher than that observed in the present, confined maze. Therefore, the implication is that such 'kinesthetically'-guided behavior renders the parameters less informative as a measure of spatial learning and memory processes. Accordingly, if the strategy of sequential arm choices demands kinesthetic rather than spatial capabilities, then the use of confinement may have improved the sensitivity of the aversive radial maze. Although the use of the confined radial maze was reported several years ago (Olton et al., 1977; Schwegler & Crusio, 1995) and has become routine in many laboratories, other recent studies have employed the unconfined, radial maze to assess the effects of ischemia for example (Block & Schwartz 1995, Sakai et al., 1996, Lee et al., 2003). This may be justified, given that sequential arm choice is not an efficient strategy in partially rewarded mazes, as for example when four arms are baited in the conventional, 8-arm radial maze (Hodges, 1996).

It is important to note that the AvRM was sufficiently sensitive to distinguish the effects of different animal models of cerebral hypoxia/ischemia. This is notorious in the experiment of chronic 4-VO, in which the occlusion of different vessels were combined. In the group subjected to permanent occlusion of the vertebral (VA) and common carotid arteries (CCA) (4-VO/CCA group), acquisition performance was persistently impaired, i.e., the animal did not learn the task throughout the training sessions (see Figure 4). In contrast, when occlusion

of the internal carotid arteries (ICA) and VA were combined (4-VO/ICC group), there was no acquisition deficit in the AvRM. A significant CA1 hippocampal lesion occurred in both permanent 4-VO/CCA and 4-VO/ICA groups, although in a lesser extent in the last group. In contrast, the degree of CA1 cell loss in the TGCI groups was too much greater than that in the chronic 4-VO/CCA group (see Figure 7 for comparison). Therefore, the CA1 lesion alone can not account for the profound, sustained learning deficit occurred after chronic 4-VO/CCA (compare Figure 4 with Figures 2 and 5). An additional, if not the principal factor may concern the disruption of visual acuity caused by permanent occlusion of the common carotid arteries (Slakter et al., 1984; Davidson et al., 2000). Occlusion of the common carotid arteries interrupts blood flow through the pterygopalatine arteries (PPA), the main source of blood to the eyes and optic nerves (Davidson et al., 2000). In contrast, permanent occlusion of the internal carotid arteries (ICA) does not result in visual disturbances (Ohta et al., 1997). Accordingly, chronic 4-VO with ICA occlusion (4-VO/ICA group) did not disrupt acquisition performance, even after 8 weeks of permanent 4-VO/ICA (see Figure 5). Whether acute, 15-min 4-VO (TGCI group) caused visual dysfunction and maze learning deficit is unknown. Therefore, and considering that spatial learning in the radial maze is highly dependent on visual, extra-maze cues, at least when these are available (Hodges, 1996), the steady-state deficit observed here after permanent, graded 4-VO with CCA occlusion, may result partially, if not largely, from visual dysfunction. This is further supported by other findings, since rats subjected to CCA ligation (2-VO model) for 3 months, and exhibiting important atrophy of the optic nerves, were impaired in the conventional, radial maze task, despite the absence of hippocampal lesioning (Ohta et al., 1997). Further studies are being carried out in our laboratory to better characterize the influence of chronic 4-VO/CCA or 4-VO/ICA on the visual structures and behavioral performance measured in the AvRM.

The aversive radial maze was also sensitive in detecting an apparently sustained impairment of acquisition after focal, ischemic brain damage (TFCI group, Figure 6). In animal models of regional brain ischemia, only a few studies have used the radial maze task to assess cognitive outcomes, perhaps because of the difficulties typically associated with the conventional paradigm, particularly the need for food deprivation and the requirement for long-term training. However, consistent results have been obtained from radial maze (Okada et al., 1995; Sakai et al., 1996, Lee et al., 2003) that indicate the sensitivity of the method in detecting the effects of experimental stroke on spatial learning and memory. The water maze model, which has been more frequently used, also detects consistent, sustained disruption of cognitive function after MCA occlusion in rats (Cain & Boon, 2003; Green et al., 1992; Olsen et al., 1994; Sun & Alkon, 2004; Markgraff et al., 1992; Dahlquvist et al., 2004). Our behavioral data agree with these studies, and further establish that the aversive radial maze (AvRM) may be as sensitive as both the conventional radial maze and the water maze tasks. However, the extent to which acquisition performance is associated with hippocampal damage after MCA occlusion cannot be concluded from the present experiment. Since the stroke model used here is currently being established in our laboratory, the method of dissecting and weighing the remaining, intact brain tissue was used for estimating infarct size. Percentage of infarct as measured by this method correlates very well with that assessed by computer-assisted image analysis (Schilichting et al., 2004); we recognize, however, this method prevents the detailed analysis of brain damage in terms of specific structures damaged by MCA occlusion. Although the hippocampus was not apparently affected in the present experiment, at least macroscopically, sporadic, hippocampal cell death has been observed after MCA occlusion in rats (Dahlquvist et al., 2004). However, the presence of long-term spatial memory impairment also occurs in the absence of both structural (Okada et al., 1995; Sakai et al., 1996) and biochemical (Okada et al., 1995) alterations of the hippocampus,

suggesting that other brain regions or disruption of larger neuronal networks determine the learning impairment after MCA occlusion (Okada et al., 1995; Sakai et al., 1996).

The effect of TGCI on retention performance is well consistent (see Figure 3) either by the daily analysis of performance or by computing the total performance. This further support our assumption that the AvRM may represent a suitable behavioral model to study learning and memory after brain damage in rats. These data are consistent with others described for the conventional, radial maze (Iwasaki et al., 1996). The present data indicate that the confined AvRM was sufficiently sensitive to detect the disruptive effect of ischemia on hippocampal-dependent memory retrieval processes, and/or on the mechanisms of memory consolidation shared by other brain regions. Extending our previous findings, here we observed that ischemia-induced retention deficit can be sustained for several days (‘steady-state deficit’), which is particularly useful to measure the neuroprotective effects of drugs.

The results of the present study (Part I) suggest that the aversive radial maze may incorporate characteristics of both the water maze and radial maze tasks. In fact, the use of aversive incentives, simple place learning, and ‘rapid’ acquisition rate are principal characteristics of the water maze task, and are shared by the present AvRM model. However, the need for learning a complex sequence of choices, the greater availability of associative mechanisms, the use of other intra-maze strategies either alone (e.g., sequential entry into adjacent arms in the unconfined maze) or together with the use of allocentric, visuospatial cues, are characteristics typically described for the appetitive, 8-arm, radial maze task (Hodges, 1996), and also may operate in the present, aversive radial maze. When compared to the water maze task, however, the AvRM task has an important, methodological limitation. In the AvRM, the rat starts from a constant location, i.e., the central arena. This procedure characterizes the so called ‘simple place learning task’. In the water maze, however, the

starting position can be changed randomly between sessions. This procedure characterizes the so called 'variable place learning task'. This is an important aspect of the task, since it has been observed that when the water maze is used as a 'simple place learning task, it is unable to detect the effect of ischemic (Auer et al., 1989; Gionet et al., 1991; Kiyota et al., 1991), traumatic (Hamm et al., 1993) or septal brain lesion (for review see Hodges, 1996). However, when the starting position is changed between sessions, the water maze is well sensitive to ischemia (Green et al., 1992; Markgraff et al., 1992; Olsen et al., 1994; Cain & Boon, 2003; Sun & Alkon, 2004; Dahlquvist et al., 2004). Although the AvRM is sensitive to ischemia even used as a 'simple place learning task', it would be very interesting to investigate the possibility to use it as a 'variable place learning task'. This is our plan for future studies.

Finally, the effect of tacrolimus (FK506) on the ischemia-induced learning and memory deficit, as measured in the confined AvRM, was evaluated. The first evidence on the neuroprotective effects of tacrolimus emerged from observations in *in vitro* model of glutamate-induced neurotoxicity (Dawson et al., 1993). Subsequent studies confirmed its action in *in vivo* models of focal (Sharkey and Bucher, 1994; Butcher et al., 1997; Arii, et al., 2001) or global, cerebral ischemia in the gerbil (Ide, et al., 1996; Tokime, et al., 1996; Yagita, et al., 1996) and rat (Drake et al., 1996). Recently, we extended these findings to the 4-VO occlusion model of TGCI in rats, and suggested that the neuroprotective efficacy of FK506 can be sustained over time (Giordani et al., 2003, Appendix B). The beneficial effect of FK506 in reducing the functional deficits that follow brain damage has also been observed after focal brain ischemia (Sharkey et al., 1994) or chronic cerebral hypoperfusion in rats (Tanaka et al., 2001). In a previous study we found that FK 506 reduced the TGCI-induced retention deficit as measured in the unconfined AvRM. The effect of FK 506 on the acquisition performance, however, could not be interpreted in that study, since the vehicle-

treated ischemic group did not differ from the intact, sham-operated animals (Benetoli et al., 2004; Appendix C).

Here, we reassessed the putative efficacy of tacrolimus in preventing the TGCI-induce learning and memory disruption. Compared to our previous study (Benetoli et al., 2004; Appendix C), here we extended the analysis by using the 'total latency' and 'total number of errors', summed across the entire acquisition or retention training session (Figures 8 and 9, lower panels, respectively). Also, the retention performance was measured for three consecutive sessions (Figure 9), in contrast to a single session in the previous study (see figure 2 in Appendix C). In the present study, the acquisition performance of the vehicle-treated group was significantly impaired after TGCI, although to a modest degree. The treatment with FK506 did not prevent the disruptive effect of TGCI on acquisition performance. FK 506 also failed to reduce significantly the effect of TGCI on the retention performance, despite an apparent tendency to improvement, mainly on the parameters 'latency' and 'reference errors'. This lack of efficacy by FK506 is better interpretable in the retention experiment, since the retention performance was clearly disrupted by TGCI, an effect expressed either by the daily analysis of performance or when examined as the total performance (see Figure 9). It is possible, however, that the small sample size used for both vehicle- and drug-treated groups may have accounted for the lack of statistical significance. Similar finding was obtained recently with the Ginkgo biloba extract, EGb 761, which reduced significantly the acquisition deficit (great sample size), but not the retention deficit (small sample size) (Paganelli et al., 2006; Appendix E).

The effect of FK506 on TGCI-induced hippocampal cell death reproduced those observed previously (Giordani et al., 2003, Appendix B; Benetoli et al, 2004, Appendix C), at least partially. In the group tested for acquisition performance, FK506 reduced the CA1 hippocampal

lesion slowly, with a modest 23.7% reduction of cell death. Unexpectedly, however, there was no cell death in the FK506-treated group tested in the retention experiment (100% neuroprotection?). In the same retention experiment, the effect of ischemia in the vehicle group was too small (24.2% cell loss). This is negligible when compared to 80-90% cell loss usually observed in our laboratory. This small degree of CA1 lesion in the vehicle-treated group suggest that 4-VO was either ineffective or some other factor have acted to reduce the effect of ischemia. Looking for a plausible explanation for these results, we detected an accidental, methodological failure occurred during ischemia with the animals assigned to the retention experiment. In that occasion, the warming box could not be used for the majority of animal, as described in materials and method. In the setting of experimental, cerebral ischemia, the control of intracerebral temperature is an important variable, since hypothermia (33^{o}C or less) is neuroprotective when applied during ischemia (Busto et al., 1989; Ginsberg et al., 1992). In addition, rectal temperature is not a reliable determinant of brain temperature, since during ischemia there is a large temperature gradient between body and brain temperature (Busto et al., 1987). It has been demonstrated, however, that brain normothermia (36-37^{o}C) can be achieved during ischemia if the rat is maintained in a warming box with inner temperature regulated at 30^{o}C (Seif el Nasr, et al., 1992). This method has been used routinely in our laboratory, but it did not work for the rats assigned to the retention experiment. It is possible; therefore, that cerebral hypothermia occurred in those animals. Therefore, the lack of CA1 cell loss observed in the group treated with FK506 may resulted from a synergistic interaction between the first dose of FK506 given at the beginning of reperfusion and intracerebral hypothermia. It should be noted, however, that despite the absence of hippocampal lesion in the FK506-treated group, the TGCI-induced retention deficit was not significantly improved.

In conclusion, the findings reported here confirm our assumption that the aversive radial maze is a reliable, sensitive and simple method to study learning and memory in rats, with the

advantages that it does not require food deprivation or immersion of the animal in water. It was sufficiently sensitive to distinguish e effects of different models of cerebral ischemia on learning and memory. Although the compound FK506 failed to protect against the ischemia-induced cognitive disruption measured in the AvRM task, other recent findings from our laboratory demonstrate that the AvRM model is useful to evaluate the effects of drugs in modifying the behavioral effect of ischemia.

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APENDIX A

APENDIX B

APPENDIX C

APPENDIX D

APPENDIX E

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