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Intra-CA1 administration of FK-506 (tacrolimus) in rat impairs learning and memory in an inhibitory avoidance paradigm

Amirmasoud Hadjiasgary [1], Hamid Reza Banafshe [1, 2], Abolfazl Ardjmand [1*]

[1] Physiology Research Center, Kashan University of Medical Sciences, Kashan, Iran
[2] Department of Pharmacology, Kashan University of Medical Sciences, Kashan, Iran

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ABSTRACT

Objective(s): Calcineurin (CN) is a main phosphatase and a critical regulator of cellular pathways for learning, memory, and plasticity. The FK-506 (tacrolimus), a phosphatase inhibitor, is a fungal-derived agent and a common immune suppressant extensively used for tissue transplantation. To further clarify the role of CN in different stages of learning and memory the main aim of this study was to evaluate the role of FK-506 in an inhibitory avoidance model.

Materials and Methods: Using different doses of FK-506 (0.5, 5, and 50 nM) in the CA1 of hippocampus at different times (before, after the training and also before the test), the effect of drug was evaluated in a step-through inhibitory avoidance paradigm. The latency of entering to the dark compartment was considered as a criterion for memory.

Results: The pre-training intra-CA1 injections of FK-506 impaired inhibitory avoidance (IA) learning acquisition. In addition, the post-training intra-CA1 injections of FK-506 at 1, 2, and 3 hr relative to training impaired memory consolidation. Moreover, the pre-test intra-CA1 injections of FK-506 impaired memory retrieval.

Conclusion: These findings suggest that the FK-506 selectively interferes with acquisition, retention, and retrieval of information processing in CA1 of hippocampus. Given the crucial role of CN in common signaling pathway of higher functions such as memory performance and cognition, in future it would be a probable therapeutic target in the treatment of a wide verity of neurological conditions involving memory.

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Introduction

Long-term potentiation (LTP) and long-term depression (LTD), as forms of plasticity consist of a series of cellular and molecular mechanisms to strengthen the synaptic transmission at different hippocampal synapses (1-3). The dominant theory prevailing over decades in the field of plasticity is that the role of memory suppressing proteins were nearly equivalent to memory enhancing ones (4). It is known that protein kinases and phosphatases, phosphorylate and dephosphorylate the proteins involved in different stages of learning, memory, (5) and different forms of plasticity.

Calcineurin (CN) is a main phosphatase in plasticity and memory (5) and a critical mediator of cellular pathways for the behavioral study of learning and memory (6). The regulatory role of CN in transmitter release, LTD, LTP, and vesicular exocytosis (7) is well documented.

FK-506 (tacrolimus) is a fungal-derived agent from bacteria *streptomyces tsukubaensis* and a ubiquitous immunosuppressing drug. In clinical practice FK-506 is widely used for tissue transplantation (8).

Diverse central nervous system side effects (e.g. memory impairment) were reported in more than 10% of clinically prescribed FK-506 which clarify the effect of drug on this system (8, 9). FK-506 exerts its effects following binding to the protein domain of FK-binding proteins (FKBPs) and the inhibition of CN (10), thereby the inhibitory effect of CN is mediated by the secondary proteins, FKBP12 (11).

Considering the physiologic relation between CN and FKBPs and the notion that the density of these proteins is highest in the hippocampus (especially CA1), a 10-50 time higher concentration of immunophillins (FKBPs) in the brain than the immune system signifying its significance of neural roles (12).

By using different experimental/animal models for learning and memory, diverse effects are reported following the administration of FK-506, for instance: ameliorating learning in cerebral hypo-

*Corresponding author: Abolfazl Ardjmand. Physiology Research Center, Kashan University of Medical Sciences, Kashan, Iran. Tel: +98-31-55550021-5(514); Fax: +98-31-55621157; email: ardjmand_ab@kaums.ac.ir

perfusion model (13); increasing dendritic branching and spine density (14); memory loss (11); restoring associative learning (15); reversal of long-term recognition memory (5); and enhancing spatial memory (16).

Emotional memory is an accepted model for studying the mechanisms of different phases of learning and memory (17), of which IA is mostly used in rodents (18). The advantages of one-trial inhibitory avoidance (IA) learning are that it is acquired in a few seconds and evaluates each phase of learning and memory dependently specially on hippocampus (19). Thence administrating a drug in a specific time relative to each stage of learning and memory is a precious tool for monitoring the process of emotional memory (16).

However, it would be novel and interesting that the phosphatase inhibition by FK-506 in CA1 region of hippocampus has some effects on IA learning and memory in rat model that have yet to be fully explored. So the objective of this study was to evaluate the effect of microinjection of FK-506 in the hippocampus on different stages of learning and memory in IA model.

Materials and Methods

Animals

Adult male Wistar rats (weight range: 220-240 g) are provided by the animal room of Physiology Research Center, Kashan University of Medical Sciences (KAUMS), Kashan, Iran. The experiments were carried out on 20 groups; each consisted of 8 rats. One week before the experiments the animals were housed in their cages with the freely access to food and water. The environmental conditions were kept constant in terms of temperature (22±2°C) and the light/dark cycle (12 hr/12 hr). All experiments were compiled with the international guideline for animal care and were approved by the Ethics Committee of Deputy of Research, KAUMS.

Surgery

The animals were anesthetized with an intra-peritoneal injection of cocktail of ketamine+xylazine (100 and 10 mg/kg), respectively, and were fixed on a stereotaxic frame (Stoelting Instruments, US). On making an incision on skin and removing the debris, with a stereotaxic coordinates of AP: -3 to -3.5 mm posterior to bregma; L: ±1.8–2 mm from midline; V: -2.8 to -33 to dura (20), the bilateral stainless steel cannulae (22G) were placed in the CA1 of hippocampus, 1 mm above the site and were fixed with the dental cement. Stainless steel stylets in different sizes were used to prepare them patent for drug injections.

Drugs and microinjection

The drugs used were: FK-506 (Tocris, Bristol, UK) which was diluted in dimethyl sulfoxide (DMSO) as vehicle and were injected using a 27-gauge cannula needle into the target site. The injection volume was 0.5 µl/side which was done over 30 sec with an additional 30 sec for the diffusion of the remained drugs in cannula.

IA apparatus

The IA apparatus was a dual chamber box (20 cm×20 cm×30 cm) with a guillotine door separating the dark and light compartments. The floor of the chamber consisted of stainless steel grids for delivering the shock (50 Hz, 3 sec, 1 mA) by a constant current stimulator (MazeRouter, Tabriz, Iran).

Behavioral procedures

Training: Prior to the experiments the habituation was done for 30 min. Five sec after the animal was placed in the light compartment, the guillotine door was lifted to allow crossing to the dark compartment. On crossing with all four paws to the dark compartment, the door was closed which then the animal was returned to its cage. After another 30 min and placing again the animal in light compartment and allowing it to enter the dark compartment a shock was delivered to the feet of the animal. After 2 min in a similar manner the animal was retested and in the case of not entering to the dark compartment a successful acquisition was recorded. The pre-/post- training injections of the drug were done before and after a successful acquisition, respectively.

Testing: On test day which was carried out 24 hr after the training, no shock was used after placing the animal in light compartment. The latency of crossing to the dark compartment with a cut-off time of 300 sec was recorded as a criterion for learning.

Data analysis

Data analysis was done using one-way ANOVA followed by Tukey's *post-hoc* tests. The Mean±SEM for step-through latencies of rats were used for analysis. *P*-value <0.05 was considered as the significance level.

Histology

On terminating the experiment, rats were anesthetized and a volume of 0.5 µl methylene-blue (1%) was injected into the CA1. The animals were then decapitated and the brain was removed and preserved in formalin (10%). After the completion of the fixation the brain was sliced and the site of injection was verified based on Paxinos and Watson stereotaxic atlas (20). All data of the rats with an incorrect cannula placement were excluded.

Experimental design

Experiment 1

In this experiment the effect of pre-training intra-

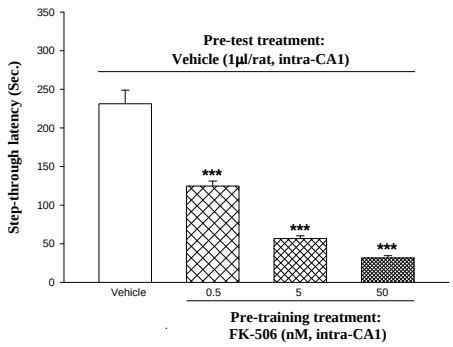

Figure 1. Effects of pre-training intra-CA1injections of vehicle or FK-506 on inhibitory avoidance learning acquisition. Intra-CA1 administration of vehicle (1 µl/rat, intra-CA1) or different doses of FK-506 (0.5, 5, and 50 nM/rat, intra-CA1) were done 30 min before the training. Each bar represents mean±SEM of rats per group. ***P-value <0.001 compared to the vehicle-vehicle control group

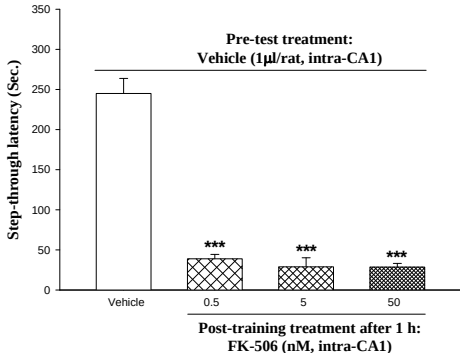

Figure 2. Effects of post-training intra-CA1injections of vehicle or FK-506 on inhibitory avoidance learning consolidation. Intra-CA1 administration of vehicle (1 µl/rat, intra-CA1) or different doses of FK-506 (0.5, 5, and 50 nM/rat, intra-CA1) were done 1 hr after the training. Each bar represents mean±SEM of rats per group. ***P-value <0.001 compared to the vehicle-vehicle control group

CA1injections of FK-506 on IA learning acquisition was examined. Four groups of animals received intra-CA1 administration of different doses of FK-506 (0.5, 5, and 50 nM) prior to training. As control group DMSO (20% v/v) was injected according to the experiment protocol for each stage of learning and memory.

Experiment 2

In this experiment the effect of post-training intra-CA1 injections of either DMSO or FK-506 on the consolidation of IA learning was examined in different time periods (i.e. 1, 2, and 3 hr) relative to training. Four groups of animals received intra-CA1 administration of either vehicle or different doses of FK-506 (0.5, 5, and 50 nM) after training.

Experiment 3

In this experiment the effect of pre-test intra-CA1 injection of either DMSO or FK-506 on the retrieval of IA memory was examined. Four groups of animals received intra-CA1 administration of either vehicle or different doses of FK-506 (0.5, 5, and 50 nM) prior to testing.

Results

Effect of pre-training intra-CA1 injections of FK-506 on inhibitory avoidance learning acquisition

The results of experiment 1 showed that pre-training intra-CA1 injections of FK-506 (30 min prior to training) altered IA learning acquisition of animals (F (3, 28) = 85.64, P-value <0.001). *Post-hoc* analysis with Tukey's test indicated that pre-training intra-CA1 injections of FK-506 at doses of 0.5, 5, and 50 nM impaired learning acquisition (Figure 1).

Effect of post-training intra-CA1 injections of FK-506 on inhibitory avoidance learning consolidation 1 hr after training

The results of experiment 2 showed that post-training intra-CA1 injections of FK-506 (1 hr after training) altered IA learning consolidation of animals (F (3, 28) = 137.33, P-value <0.001). *Post-hoc* analysis with Tukey's test indicated that post-training intra-CA1 injections of FK-506 at doses of 0.5, 5, and 50 nM impaired memory consolidation (Figure 2).

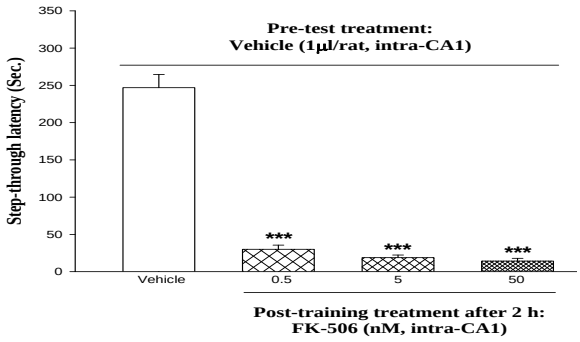

Figure 3. Effects of post-training intra-CA1injections of vehicle or FK-506 on inhibitory avoidance learning consolidation. Intra-CA1 administration of vehicle (1 µl/rat, intra-CA1) or different doses of FK-506 (0.5, 5, and 50 nM/rat, intra-CA1) were done 2 hr after the training. Each bar represents mean±SEM of rats per group. ***P-value <0.001 compared to the vehicle-vehicle control group

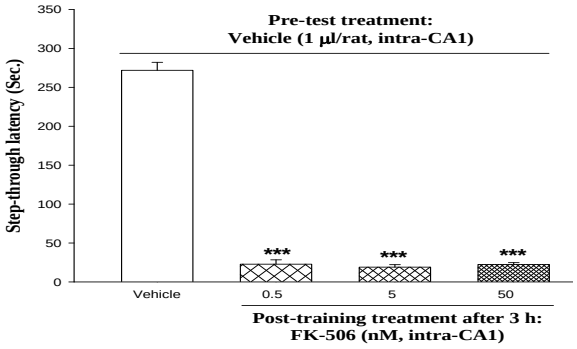

Figure 4. Effects of post-training intra-CA1injections of vehicle or FK-506 on inhibitory avoidance learning consolidation. Intra-CA1 administration of vehicle (1 µl/rat, intra-CA1) or different doses of FK-506 (0.5, 5, and 50 nM/rat, intra-CA1) were done 3 hr after the training. Each bar represents mean±SEM of rats per group. ***P-value <0.001 compared to the vehicle-vehicle control group

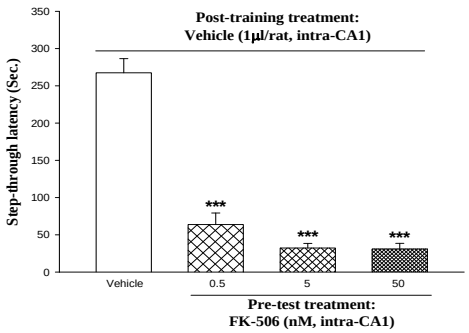

Figure 5. Effects of pre-test intra-CA1injections of vehicle or FK-506 on inhibitory avoidance memory retrieval. Intra-CA1 administration of vehicle (1 µl/rat, intra-CA1) or different doses of FK-506 (0.5, 5, and 50 nM/rat, intra-CA1) were done 30 min before the test. Each bar represents mean±SEM of rats per group. ***P-value <0.001 compared to the vehicle-vehicle control group

Effect of post-training intra-CA1 injections of FK-506 on inhibitory avoidance learning consolidation 2 hr after training

The results of experiment 2 showed that post-training intra-CA1 injections of FK-506 (2 hr after training) altered IA learning consolidation of animals (F (3, 24) = 74. 50, *P*-value <0.001). *Post-hoc* analysis with Tukey's test indicated that post-training intra-CA1 injections of FK-506 only at dose of 0.5 nM impaired memory consolidation (Figure 3).

Effect of post-training intra-CA1 injections of FK-506 on inhibitory avoidance learning consolidation 3 hr after training

The results of experiment 2 showed that post-training intra-CA1 injections of FK-506 (3 hr after training) altered IA learning consolidation of animals (F (3, 28) = 425.38, *P*-value <0.001). *Post-hoc* analysis with Tukey's test indicated that post-training intra-CA1 injections of FK-506 at doses of 0.5, 5, and 50 nM impaired memory consolidation (Figure 4).

Effect of pre-test intra-CA1 injections of FK-506 on inhibitory avoidance learning retrieval

The results of experiment 3 showed that pre-test intra-CA1 injections of FK-506 (30 min before training) altered IA memory retrieval of animals (F (3, 28)= 75.670, *P*-value <0.001). *Post-hoc* analysis with Tukey's test indicated that pre-test intra-CA1 injections of FK-506 at all doses impaired memory retrieval (Figure 5).

Discussion

The main aim of present study was to explore the effects of bilateral intra-CA1 administration of FK-506, as a phosphatase inhibitor, on learning and memory processes in an IA model. The results obtained from the study were as follows:

First, bilateral pre-training injection of different doses of FK-506 impaired learning acquisition firstly evident from 30 min pre-training. Second, bilateral post-training injection of the same doses of FK-506 impaired memory consolidation beginning 1 hr and lasting 3 hr post-training. Third, the bilateral pre-test injection of the mentioned doses of FK-506 (30 min before the test) also impaired memory retrieval as well.

Reportedly, the action of drug is initiated following binding to the secondary proteins, FKBPs (11). FKBPs can be considered as markers for the monitoring the temporal amnesic effect of FK-506 (21). Considering the similarity of time profile for post-training treatment between this study and previous experiments done by Bennnett *et al* (10), and given the notion that FK-506 affects protein folding in this time period (i.e. ~80 min) (22), the post-training effect of the drug observed in the present study may be attributed to the inhibition of protein synthesis that resulted in memory loss in this period.

In addition, in a series of studies reported by Bennett *et al* both the pre- and post-training administration of FK-506 impaired acquisition and retention in IA (21). Moreover, in another similar study the acquisition and retrieval of an aversive behavior were impaired in circular maze and Morris water maze (23) which was in line with our results. In these studies the pre-/post-training nanomolar concentrations of FK-506 in very small to high doses and different time points (either immediately or 60 min post-training) impaired the acquisition and retention of memory (21). This evidence may be of importance from the point of view that in spite of being neuroprotective for CA1 area of hippocampus, some reports (24), besides the mentioned study denote that FK-506 acts as a memory impairing agent.

Multiple histological/histopathological and pharmacological approaches on the effect of FK-506 provided sufficient support/evidence for the fact that instead of memory impairing in some brain regions (21, 23, 25, 26), the structurally and functionally impaired memory could be indicative of the behavioral/learning disturbances (24), similar to that seen in our study. However, the neuroprotective effects of FK-506 were also reported in some pathological models (6, 14, 21, 24). Moreover, the impairing effect of FK-506 on different stages of learning and memory in our study to some extent may be related to the specific regulatory function of CN in hippocampus which is in turn in line with the studies done by Lin *et al* (27, 28) denoting the main function of CN in the extinction but not consolidation or recall of aversive learning. The aforementioned are against the large body of evidence elucidating the memory enhancing effect of the drug (13,15, 29-31) in different paradigms.

On the other hand, a large corpus of findings has revealed the memory enhancing effect of drug on

learning and memory in different models which were in obvious contrast to our findings. These differences between our study and the findings cited by other researchers may be ascribed to the species, unspecific effects (mechanisms) of the drug, model's paradigm (12), and different patterns of CN inhibition at the cellular level in different brain regions or time of onset of drug effect (32). But what makes the present study distinguished from other similar studies is its timing design which is adopted from studying the temporal profile of similar previous experiences (15, 21, 33). In addition, the timing of memory loss observed in our study is also interesting and of particular interest given the available findings taken from our paradigm suggesting that the intermediate-term memory processes could be dissociated from both short-term and long-term processes. According to different reports, in rats the time range for an intermediate-term memory is manifested from ~15 min post-training that is paralelly and independently followed by a long-term stage lasting to about 75 min post-training (34). Worthy to note is that mentioned studies that designed only for evaluating the retention at only a single time relative to post-training, may be considered doubtful for the reasons that they may incompletely describe the transitional memory retention loss (10), but not a permanent loss as seen in our study. Considering this point, to our knowledge this work is among the rare studies that explores the chronological effect of drug on different memory stages in rat.

Among the others, another distinguished advantage of the present study is the site of injection. We selected the CA1 of hippocampus as a site for studying the effects of the drug. That was for the reason that the density of FKB12 in hippocampus (especially in CA1) is the highest (12), which signifies the physiological role of CN there (14). Hippocampus is one of the main structures of medial temporal lobe involved in learning and memory processes (35). CN has the potential of modulating synaptic plasticity and transmission thereby modulating the information storage (33). Given the bipotentiality of CN in regulating the efficacy of plastic modifications of synapses(33), its role in behavioral studies is paradigm specific. Reportedly essential role of CN for reversing the LTP is reported too (36). Pre- or post-synaptically, CN as a negative controller, exerts a constraining effect on LTP (36), proposing a cellular/molecular approach for the mechanism of acquisition and retrieval. In agreement with the cited studies, on administrating FK-506 in a culture which mimicked the pre-synaptic action of the drug, the restoration of LTP was seen (37).

CN is also a negative controller of glutamate synaptic transmission and its function is carried out by a pre-synaptic mechanism (37). Moreover, given the involvement of CN in the modulation of glutamate exocytosis through a pre-synaptic mechanism (37), CN in turn regulates the glutamate release (38). It is therefore tempting to argue that the memory impairing effect of FK-506 in rat hippocampus may result from the decrease in glutamate release mediated by a pre-synaptic mechanism.

Generally, the matter of memory efficacy is also to some extent related to the CN activation in memory-specific substrates (29). As previously reported, CN which is also a main phosphatase vital for the plasticity and memory processes that in the transitional states between the different temporal profiles of the memory (15) acts as a molecular switch shifting between reconsolidation and extinction of fear-related memory (39). From this point of view CN activation has a significant role in the extinction of learning (39) which is termed as a form of learning dependent on the dissociation of a paired association (40), thereby making the learning behavior meaningless by the suppression of CN before the extinction of learning (40). In other words, a balanced state of kinase and phosphatase activity is essential for the efficacy of memory, the disturbance of this balance towards the stronger inhibition of CN can impair, instead of improving the memory (33).

On another side of matter, LTP and LTD as forms of synaptic plasticity can be considered the neuronal substrates of memory. Besides being required for synaptic depotentiation, reportedly, CN is necessary for the induction of both LTP and some forms of LTD (41). However, the antagonistic effect of FK-506 in the induction of LTP is also concerned (42). Altogether, in obvious agreement with these mechanisms, another approach for the impairing effect of FK-506 is that synaptic plasticity may be regulated through the neurotransmission by PKC and the de-phosphorylation of metabotropic glutamate receptors (43). This neuromodulatory effect is dependent on the N-methyl-D-aspartate (NMDA)receptor activation and may have pre- and post-synaptic elements (e.g. the diminished glutamate release) paralelly (38). In this manner CN recruitment done by LTD would have a regulatory effect on the expression of LTD (44).

Alternatively, another probable mechanism for the effect of drug can be proposed by nitric oxide (NO). The regulatory role of NO in the modulation of LTP is well documented. In this process the post-synaptic NO production is regulated by the pre-synaptic glutamate release and the post-synaptic CN (45). Thereby, the inhibition of CN by a CN-inhibitor, FK-506, can remove the modulatory effect of NO (45). Another alternative mechanism for the effects detected in this study may be related to the blocking

effect of FK-506 on the L-type Ca^{+} channels of hippocampus neurons (46). Considering the important role of these channels in LTP (47), the blockade of LTP in this region is accomplished by interfering with gene expression (48).

From the molecular point of view, FK-506 acts by binding to its binding proteins (49). By dissociating the FKBP-bearing complex or forming the new FKBP12 (FK-506-calcineurin complex), FK-506 affects different downstream memory cascades (50). One main target in downstream of LTP is cAMP-response element binding (CREB) (29). On downstream pathways, the effect of FK-506 on CREB depends on the period of its phosphorylation (51). For instance, in an odor preference model the peak time period for CREB phosphorylation was reported 10 min post-training followed by reaching to baseline 60 min afterwards, (52-54) as seen by McLean *et al* (55) in mitral cells of dorsolateral quadrant of the olfactory bulb. These evidence revealed that CREB phosphor-rylation/dephosphorylation regulated by CN (29) may modulate the LTP.

Apart from the mentioned mechanisms, a sort of unspecific effect of FK-506 was also proposed in some instances whereby the FK-506 may disrupt LTP (33). Thus the findings denoting the impairing effect of drug on memory done through disrupting LTP are supported electrophysiologically which in turn can be proved by some genetic tools (56-58).

Conclusion

In conclusion, these findings suggest that the FK-506 selectively interferes with acquisition, retention, and retrieval of information processing in CA1 of hippocampus. However another probable reason may be that FK-506 affects reconsolidation, hypothesized by some authors and to be achieved on accessing previously liable memory which in turn opens new horizons for future research.

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