On the participation of cannabinoid receptors in the reconsolidation of spatial long-term memory in male rats

Ruliam Queiroz, Crisleine Marchiori, Heitor Kraushaar Rosas, Isabella Sayuri Shiguehara Medeiros, Henrique Quilante Ferreira, Juliana Sartori Bonini, Weber Cláudio da Silva

Corresponding author: Weber Cláudio da Silva (wwwclaudion@gmail.com)

Abstract

To date, there is insufficient evidence to explain the role of cannabinoid receptors in the reconsolidation of long-term spatial memory. In this work, we elucidate the role of the cannabinoid receptor family (CB1 and CB2) in this process. We demonstrated that when infused bilaterally into the hippocampal CA1 region immediately after an early non-reinforced test session performed 24 hours post-training in the Morris water maze task, anandamide (arachidonoylethanolamide, AEA) and oleamide (cannabinoid CB1 receptor agonist) can cause anterograde amnesia for early (just oleamide) and late long-term spatial memory. This effect on spatial memory reconsolidation was blocked by CB1 receptor antagonist, showing that this effect occurs through CB1 receptor. Finally, we showed that the amnesic effect of oleamide on spatial memory reconsolidation depends on the occurrence of protein degradation and that the amnesic effect of inhibition of protein synthesis on spatial memory reconsolidation can be blocked by the inverse activation of CB1 receptor, implying this receptor participates in the protein turnover process regulation.

Keywords: rats, hippocampus, spatial memory, cannabinoid, reconsolidation

1. Introduction

The reconsolidation of long-term memories is a process of stabilising the mnemonic trace that occurs shortly after the labilization of this trace, triggered by the unreinforced evocation of a previously consolidated memory [1,2]. It is important to emphasize that the reconsolidation process occurs only through the absence of reinforcing stimulus during learning. In most cases, it occurs promptly the first few times that memories are retrieved [3]. In addition, if the reinforcing stimulus is present during evocation, the process of labilization, followed by reconsolidation, is impaired. Instead, only a reinforcement of the consolidated mnemonic trace occurs. This is a subtle but important distinction between the consolidation and reconsolidation processes [4].

Reconsolidation is underlain by a gene transcription- and protein synthesis-dependent mechanism, without which memory traces become progressively weaker [5,6,7,8,9]. It is considered to take place mainly as a memory-updating process [10], the biochemical mechanisms of which are in the process of being unraveled [11]. For example, it has already been shown that memory reconsolidation requires the activation of L-subtype voltage-dependent calcium channels (L-VDCCs) [12], protein kinase A [13,14], protein kinase C [15], and extracellular signal-regulated kinase subtypes 1 and 2 (ERK1/2) [16]. It has also been proven that reconsolidation requires the activation of transcription factors to occur [17,18].

The endocannabinoid system (ECS) comprises two cannabinoid receptors - CB1 and CB2 - which belong to the family of G protein-coupled receptors (GPCRs); the ligands for the cannabinoid receptors are the endocannabinoids (eCBs) anandamide (arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG), both derived from arachidonic acid [19]. The ECS is highly conserved evolutionarily in vertebrates, is widely

distributed throughout the body, and occupies a central position in the regulation of numerous biological processes in both neural and peripheral tissues. In addition, it is a system intertwined with many neurotransmitters and lipid signalling systems, thus integrated into broad functional networks [20]. ECS plays a role in enhancing the physiological processes that maintain the body in a state of homeostasis. In humans, several polymorphisms in ECS components are associated with neuropathophysiological processes, suggesting that they promote susceptibility to the development of neuropsychiatric disorders [21].

Detailed analyses of expression at the cellular level in the central nervous system (CNS) have revealed that the CB1 receptor is present in virtually all regions of the brain and in all major cell types, i.e. neurons [22], glial cells (oligodendrocytes and astrocytes) [23] and CNS immune cells (microglia) [24]. CB1 receptors are also present in the neurons of the main neurotransmitter systems (glutamate, GABA, serotonin, noradrenaline, acetylcholine) [25]. Expression levels can vary greatly depending on the region of the brain and the cell type. For example, high levels of CB1 receptor messenger RNA (mRNA) have been detected in GABAergic interneurons of the hippocampus and neocortex. Glutamatergic neurons generally contain low levels of CB1 receptors [26].

The expression of CB2 receptors is predominantly observed in both peripheral and central immune cells, but they have also been detected in neurons, indicating their potential impact on neural functions [27]. In the central nervous system (CNS), CB2 receptor expression has been reported in activated microglia, brainstem cells, hippocampal glutamatergic neurons, and ventral tegmental region dopaminergic neurons. CB2 mRNA transcripts are around 100 to 200 times less abundant than CB1 receptor mRNA but are strongly positively regulated in response to various events such as chronic pain, neuroinflammation and stroke [25].

In the mammalian central nervous system, CB1 receptors, when activated, couple to the Gi/o protein, which inhibits the production of cAMP stimulated by forskolin, inhibits N-type and P/Q-type calcium channels, resulting in a decrease in calcium influx shortly after stimulation by action potentials, and activates G-protein-activated inward rectifier potassium channels (GIRKs); CB2, which couples to the Gi protein, which inhibits the enzyme adenylyl cyclase (AC), activates the MAPK (mitogen-activated protein kinase) pathway and activates the PI3K/AKT (phosphatidyl inositol 3 kinase) and JNK (c-Jun N-terminal kinase) pathways [28].

The location of the CB1 receptor binding site is the presynapse, where activation of the CB1 receptor can suppress the release of the neurotransmitter from the presynapse. This mechanism is very well detailed for glutamatergic and GABAergic synapses, where eCB 2-AG is generated in the postsynapse and, diffuses retrogradely to the presynapse and stimulates the CB1 receptor. This can result in a short-term decrease in calcium influx at the presynaptic terminal. This signalling can typically lead to processes called depolarization-induced suppression of excitation (DSE) at glutamatergic synapses and depolarization-induced suppression of inhibition (DSI) at GABAergic synapses [29].

The endocannabinoid system is believed to play an important role in various aspects of learning and memory, controlling neuronal responses to environmental challenges [19], as well as being considered a robust system in the modulation of emotional memory processes [30].

Depending on expression, cell type and synaptic location, eCB can mediate different forms of short- and long-term plasticity. In the hippocampus, postsynaptic depolarization of pyramidal cells leads to the release of eCBs and pre-synaptic activation of the CB1 receptor in inhibitory interneurons, resulting in DSI, a form of short-term plasticity [31]. The induction of DSI is mediated by the activation of voltage-gated calcium channels (VGCC) in the hippocampus and is involved in the modulation of gamma oscillations during the organization of neuronal ensembles in the formation of localization fields with place cells, as well as in hippocampus-dependent learning [32]. Endocannabinoid signaling is also linked to other forms

of plasticity, such as DSE, as well as forms of long-term plasticity such as long-term depression (LTD) in both excitatory and inhibitory synapses [33]. The induction of these different forms of plasticity is probably linked to the activation pattern of postsynaptic neurons, which, in turn, modulates the synaptic concentration of eCBs, the activation time of the CB1 receptor and its downstream effects [34].

Endocannabinoids are related to the consolidation of fear memory through the activation of CB1 receptor [35]. The bilateral injection of AM251, a CB1 receptor inverse agonist, into the dorsal hippocampus after fear conditioning impaired the retrieval of fear memory [36,37] and suppressed the LTP in the dorsal CA1 of male rats [37]. Regarding CB2 receptor, CB2 receptor knockout (KO) mice showed a decrease in fear response 24 h after fear conditioning [38].

The role of eCBs in the reconsolidation of fear memory depends on the brain regions. The bilateral injection of AM251 into the retrosplenial cortex after the retrieval test increased the fear response 24 h after retrieval in male rats [39]. The bilateral injection of AEA into the dorsal hippocampus after the retrieval test reduced the fear response 24 h after the retrieval, and the reduction was prevented by the preinjection of AM251 in male rats [40]. Thus, CB1R in the retrosplenial cortex and dorsal hippocampus inhibits the reconsolidation of fear memory.

Here, we analyzed the effect of the intra-CA1 infusion of AEA and other cannabinoid agents, including cannabinoid-receptor subtype-specific antagonists and agonists, on the reconsolidation of spatial memory. To that end, we utilized the highly validated, hippocampal-dependent Morris water maze (MWM) task [41,42,43,44].

2. Materials and Methods

2.1. Subjects

All experiments were conducted blindly to the animals' treatment condition and following the National Institutes of Health guidelines for animal care and use and were approved by the Animal Care and Ethical Committee of the University of Center-West of Paraná. Furthermore, all methods are in accordance with ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments. Three-month-old male Wistar rats weighing 220–280 g and raised in our animal facilities were used in the experiments. Animals were housed four or five in a cage and maintained at 21–23 °C under a 12-h light/12-h dark cycle (lights on at 07:00 hours) with free access to food and water.

2.2. Experimental Design

The effect of intrahippocampal infusion of either AEA (10 nmol/side) or cannabinoid agonists (10 nmol/side) or antagonists (50 nmol/side) on reconsolidation of early and late long-term spatial memory in rats was evaluated. First, these rats went through stereotaxic surgery to implant guide cannulas, and after three days of recovering, the rats were randomly divided into the following experimental groups: AEA dose curve, AEA plus antagonists, antagonists, agonists, agonist CB1 plus lactacystin, and finally inverse agonist CB1 plus rapamycin. For the reconsolidation experiments, the rats were trained in the Morris water maze task (MWM) for five consecutive days (8 trials per day). Then, they received an intrahippocampal bilateral infusion of the drug(s) under scrutiny, immediately after the first test achieved 24 hours after the last training day. Then, the rats were retested after saline/drug infusion for 24 hours (early long-term spatial memory), five and ten days (late long-term spatial memories). There were

also four experimental control groups (infusion, reinforcement, temporal and structure controls) with other sets of rats.

2.3. Stereotaxic surgery for cannula implants

To implant them with indwelling cannulae, rats were deeply anaesthetized with 75 mg/kg ketamine (König) plus 10 mg/kg xylazine (Coopers), and 27-gauge 9.0-mm guide cannulae were stereotaxically aimed to the pyramidal cell layer of the dorsal CA1 region, using coordinates (–4.2 anterior, ±3.0 lateral, –2.0 ventral from bregma) taken from the atlas of Paxinos and Watson [45]. The animals were allowed to recover from surgery for 3 days before submitting them for any other procedure.

2.4. Microinfusion procedures

At the drug delivery time, 30-gauge 10.0-mm infusion needles (extending 1.0 mm beyond guide cannulae) were tightly fitted into the guides. Infusions (1.0 μL per side) were carried out over 60 s, and the infusion cannulae was left in place for 30 additional seconds to minimize backflow. Cannulae placement was verified postmortem: 2–4 h after the last behavioural test, 1.0 μL of a 4% (mass/vol) methylene-blue solution was infused as described earlier, and the extension of the dye 30 min after that was taken as indicative of the presumable diffusion of the vehicle or drug previously given to each animal. Only data from animals with correct implants were included in the statistical analysis.

2.5. Drugs

AEA (anandamide, A0580-25MG), oleamide (agonist CB1 receptor, O2136-100MG), HU 308 (agonist CB2 receptor, SML3128-5MG), AM4113 (antagonist CB1 receptor, SML1804-25MG) and SR 141716A (inverse agonist CB1 receptor, SML0800-25MG) were purchased from Sigma. COR 170 (inverse agonist CB2 receptor, Tocris 4756), lactacystin (Tocris 2267) and rapamycin (Tocris 1292) were obtained from Tocris. Cannabinoids agonists and antagonists were first dissolved in vehicle solution (saline/polyethylene glycol/Tween80, 90/5/5, v/v [46]) and stored frozen at −20 °C until the moment of use. Lactacystin and rapamycin were first dissolved in saline solution and stored frozen at −20 °C until the moment of use.

2.6. Morris water maze task

The water maze was a dark blue circular pool (180 cm in diameter) conceptually divided into four equal imaginary quadrants for data analysis. The water temperature was 21–23 °C. There was a black circular platform (12 cm in diameter) two centimeters beneath the water's surface and hidden from the rat's view. It had a rough surface, allowing the rats to climb onto it once detected easily. The swimming path of the animals was recorded by using a video camera mounted above the center of the pool and analyzed using a video tracking and analysis system. The water maze was located in a well-lit white room with several posters and other distal visual stimuli hanging on the walls to provide spatial cues. Rats were handled 5 min per day for 3 d before training. Training using the spaced training protocol was carried out for five successive days [6]. On each day, rats received eight consecutive training trials, during which the hidden platform was kept in a constant location. A different starting location was used on each trial, which consisted of a swim followed by a 30-s platform sit. Any rat that did not find the platform within 60 s was guided to it by the experimenter. To evaluate the effect of drugs given after memory reactivation, rats were trained for 5 days, as indicated earlier, before being submitted

to the first probe test without the escape platform 24 h after the last training session. Then, immediately after this test session, rats received intra-CA1 infusions of the drug under scrutiny or vehicle. Memory retention was evaluated in successive probe tests carried out at 24, 5 d and 10 d after the first one.

2.7. Data analysis

The normality test Shapiro-Wilk was made, and the behavioural data are presented as mean and mean standard error. As appropriate, data were analyzed by either two-tailed Student t-test or one-way ANOVA followed by Dunnett's post hoc tests. Values lower than 0,05 were considered significant ones.

3. Results

3.1. AEA impairs late spatial memory reconsolidation

To investigate whether AEA affects spatial memory reconsolidation, rats trained for 5 d in the spatial version of the MWM were submitted to a probe test without the escape platform 24 hours after the last training session. Those rats received an intra-hippocampal bilateral infusion of either vehicle or AEA (0.1, 1.0, 10 or 100 nmol/side) immediately after this first probe test. The rats were then tested 24 h post-infusion.

Initially, we verified that the rats learned the task over the training days, which was evidenced by the reduction in the average latency time during the training, and by the fact that the rats consolidated a recent long-term memory for the task, as evidenced by the time spent in the target quadrant (Figure 1) in the 24 h post-training test.

We verified that when AEA was infused at a dose of 10 nmol/side or 100 nmol/side immediatelty after the first probe test, there was a reduction in the time spent in the target quadrant in the 5 and 10 days post-first-test tests, therefore showing that reconsolidation of late long-term memory was impaired in these groups, and that this effect is dose-dependent (Figure 1).

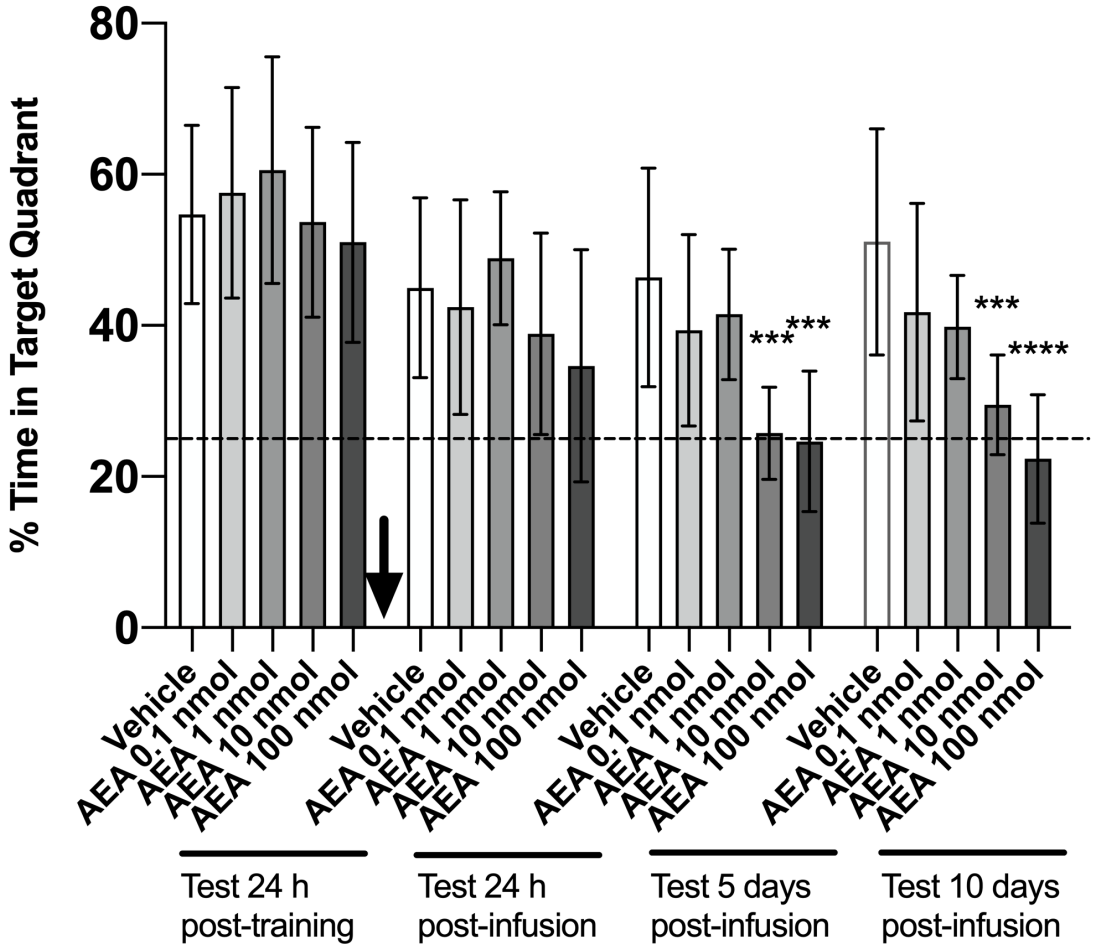

Figure 1. Intrahippocampal infusion of AEA immediately after nonreinforced retrieval hinders late spatial memory retention as measured 5 d and 10 d after infusion. Male Wistar rats with infusion cannulae implanted into the CA1 region of the dorsal hippocampus were trained during 5 d in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were submitted to a 60-s probe test in the absence of the escape platform. Immediately after this first test, the animals received intrahippocampal bilateral infusions (black arrow) of either vehicle solution (Vehicle) or AEA (AEA 0.1, 1, 10 or 100 nmol). Memory retention was assessed in sucessive 60-s probe tests carried out 24 h, 5 d and 10 d after the first test. Data are expressed as means (± SEM) of the percentage of swimming time spent in the target quadrant (TQ). Hashed line: theoretical mean of 25% of test time spent in TQ at random. ***$P < 0.001$, ****$P < 0.0001$ vs. respective Vehicle group, one-way ANOVA: test 24h post-first-test, $F(4, 50) = 1.693$; test 5d post-first-test, $F(4, 50) = 8.197$; test 10d post-first-test, $F(4, 50) = 10.09$; Dunnett's post test; n = 9–15 per group.

Moreover, this observed effect was indeed on reconsolidation of spatial memory since there were not any significant differences in all the tests in the time spent in the target quadrant compared to the saline group when AEA 10 nmol/side was infused either 24 hours post-training, in the absence of a immediately preceding probe test (Figure 2A). This result precludes the possibility that the previous effects verified after the bilateral infusion of AEA (Figure 2) had been independent of the fact that their infusion had been achieved immediately after a reactivation session test without reinforcement (the test 24 hours post-training), or due

to some long-term alteration triggered by AEA, which would be observed in any post-infusion test. Also, there was not any significant difference in all the tests in the time spent in the target quadrant compared to the vehicle group when AEA was infused immediadely after a reactivation test with reinforcement (test 24 hours post-training with the presence of platform in the same local as it used to stay during training days) (Figure 2B). This result restricts the effects caused by bilateral hippocampal infusion of AEA to the absence of reinforcement (escape platform) during the reactivation test session (test 24 hours post-training). The absence of reinforcement is necessary for triggering the labilization process, which precedes the reconsolidation process. Moreover, there was not any significant difference in all the tests in the time spent in the target quadrant compared to the vehicle group when AEA was infused 3 hours after the first probe test (Figure 2C), showing that the amnesic effect of AEA on spatial memory reconsolidation is time-dependent. Finally, there was not any significant difference in all the tests in the time spent in the target quadrant compared to the vehicle group when AEA was infused immediately after the first probe test into the occipital cortex, which is 1.0 mm above the pyramidal cell layer of the dorsal CA1 region, using coordinates (–4.2 anterior, ±3.0 lateral, –1.0 ventral from bregma) taken from the atlas of Paxinos and Watson [45] (Figure 2D). This result shows that the effects caused by bilateral hippocampal infusion of AEA on retrieval of late long-term spatial memory are structure-dependent. So, those effects only happen if AEA is infused immediately after the non-reinforced reactivation test (first test 24 hours post-training) into the hippocampal CA1 region.

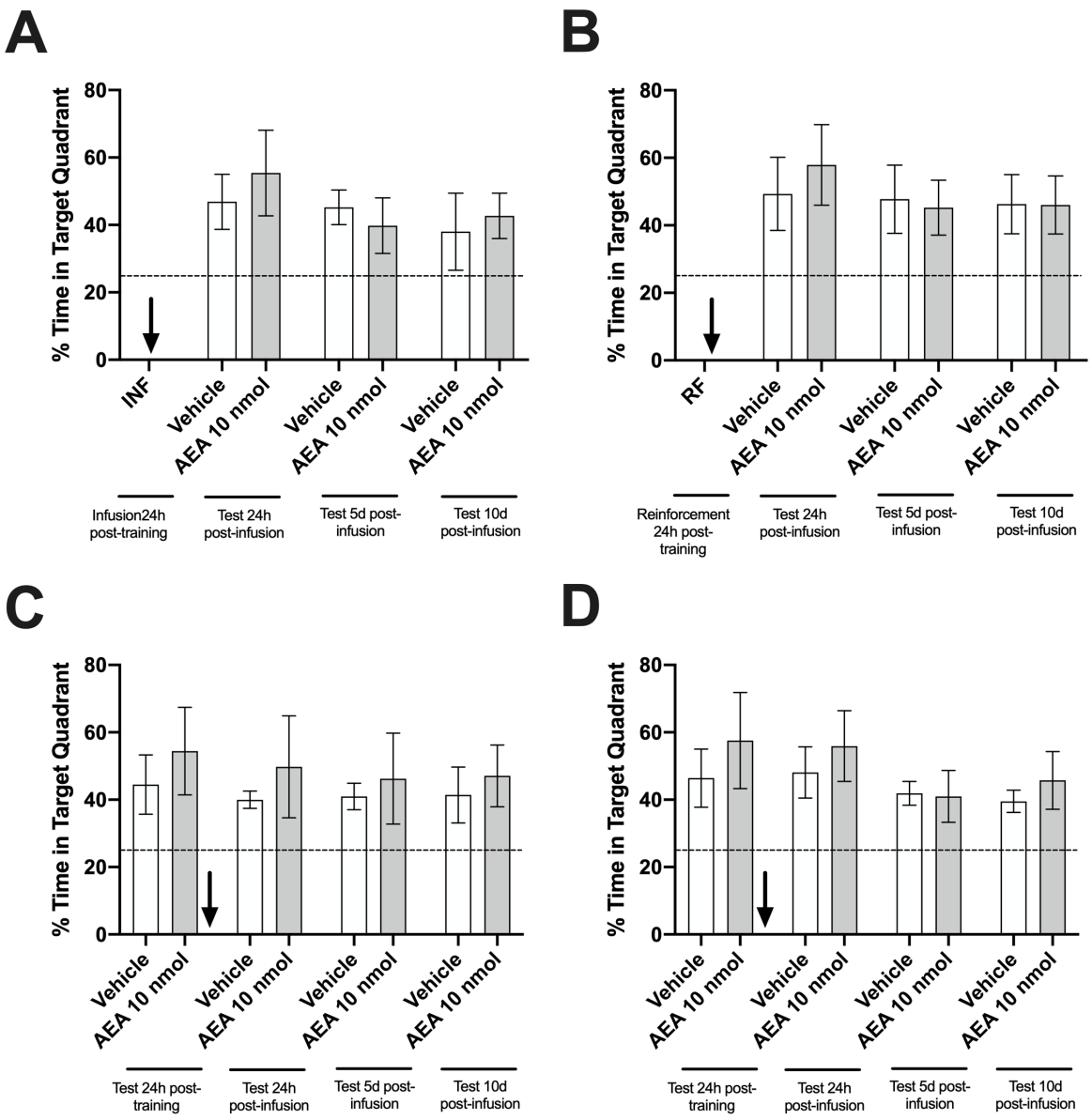

Figure 2. **A**. Intrahippocampal infusion of AEA after the last day of training does not affect early and late spatial memory retention as measured 24 h, 5 d and 10 days after infusion. Male Wistar rats with infusion cannulae implanted into the CA1 region of the dorsal hippocampus were trained during 5 days in the spatial version of the MWM. Twenty four hours after the last training session, the animals received intrahippocampal bilateral infusions of either vehicle solution (Vehicle) or AEA 10 nmol (AEA 10 nmol). Memory retention was assessed in sucessive 60 s probe tests carried out 24 h, 5 d and 10 d after infusion (INF, black arrow). The dashed line represents theoretical mean of 25 % of test time to be spent at random in target quadrant (TQ). Data are expressed as means (± SEM) of the percentage of swimming time spent in the TQ. There were not any difference between vehicle and AEA groups, t-Student test: test 24h post-first-test, $t_{(14)} = 1.596$; test 5d post-first-test, $t_{(14)} = 1.560$; test 10d post-first-test, $t_{(14)} = 1.001$; n = 8 per group. **B**. Intrahippocampal infusion of AEA immediately after reinforced retrieval does not affect early and late spatial memory retention as measured 24 h, 5 days and 10 days after the first test. Male Wistar rats with infusion cannulae implanted into the CA1 region of the dorsal hippocampus were trained during 5 days in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were submitted to a 60 s probe test in the presence of the escape platform (R). Immediately after R, the animals

received intrahippocampal bilateral infusions (black arrow) of either vehicle solution (Vehicle) or AEA 10 nmol (A 10 nmol). Memory retention was assessed in sucessive 60 s probe tests carried out 24 h, 5 d and 10 d after R. The dashed line represents theoretical mean of 25 % of test time to be spent at random in target quadrant (TQ). Data are expressed as means (± SEM) of the percentage of swimming time spent in the TQ. There were not any difference between vehicle and AEA groups, t-Student test: test 24h post-first-test, $t_{(17)} = 1.639$; test 5d post-first-test, $t_{(17)} = 0.5824$; test 10d post-first-test, $t_{(17)} = 0.06166$; n = 8–11 per group. **C**. Intra-hippocampal infusion of AEA 3 h after nonreinforced retrieval does not affect early and late spatial memory retention as measured 24 h, 5 d and 10 days after the first test. Male Wistar rats with infusion cannulae implanted into the CA1 region of the dorsal hippocampus were trained during 5 days in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were submitted to a 60 s probe test in the absence of the escape platform. Three hours after this test, the animals received intra-hippocampal bilateral infusions (black arrow) of either vehicle solution (Vehicle) or AEA 10 nmol (AEA 10 nmol). Memory retention was assessed in sucessive 60 s probe tests carried out 24 h, 5 d and 10 d after the first test. The dashed line represents theoretical mean of 25 % of test time to be spent at random in target quadrant (TQ). Data are expressed as means (± SEM) of the percentage of swimming time spent in the TQ. There were not any difference between vehicle and AEA groups, t-Student test: test 24h post-first-test, $t_{(13)} = 1.684$; test 5d post-first-test, $t_{(13)} = 0.9970$; test 10d post-first-test, $t_{(13)} = 1.245$; n = 7–8 per group. **D**. Intra-mediolateral occipital cortex infusion of AEA immediately after nonreinforced retrieval does not affect early and late spatial memory retention as measured 24 h, 5 d and 10 d after the first test. Male Wistar rats with infusion cannulae implanted into the mediolateral occipital cortex were trained during 5 days in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were submitted to a 60 s probe test in the absence of the escape platform. Immediately after this test, the animals received intra-mediolateral occipital cortex bilateral infusions (black arrow) of either vehicle solution (Vehicle) or AEA 10 nmol (AEA 10 nmol). Memory retention was assessed in sucessive 60 s probe tests carried out 24 h, 5 d and 10 d after the first test. The dashed line represents theoretical mean of 25 % of test time to be spent at random in target quadrant (TQ). Data are expressed as means (± SEM) of the percentage of swimming time spent in the TQ. There were not any difference between vehicle and AEA groups, t-Student test: test 24h post-first-test, $t_{(14)} = 1.661$; test 5d post-first-test, $t_{(14)} = 0.3105$; test 10d post-first-test, $t_{(14)} = 1.804$; n = 7–9 per group.

3.2. The AEA impairment on spatial memory reconsolidation is blocked by antagonist CB1.

To investigate through which receptor(s) AEA exerts its amnesic effect on the reconsolidation of late spatial memory, rats trained for 5 d in the spatial version of the MWM were submitted to a probe test without the escape platform 24 hours after the last training session. Those rats received an intra-hippocampal bilateral infusion of either vehicle, AEA (10 nmol/side) or AEA plus cannabinoid receptor antagonist CB1, immediately after this first probe test. The rats were then tested 24 h post-infusion (for evaluating early reconsolidated memory), 5 d and 10 d post-infusion (for late reconsolidated memory) (Figure 3). In this experiment, we did not use a CB2 receptor antagonist because, to date, there is no substance with this property.

We observed that when AEA was co-infused with antagonist CB1, its amnesic effect on late spatial memory reconsolidation was blocked, as there was not any significant difference in these tests in the time spent in the target quadrant compared to the vehicle group. These results show that AEA exerts its amnesic effect through its action on CB1 receptor.

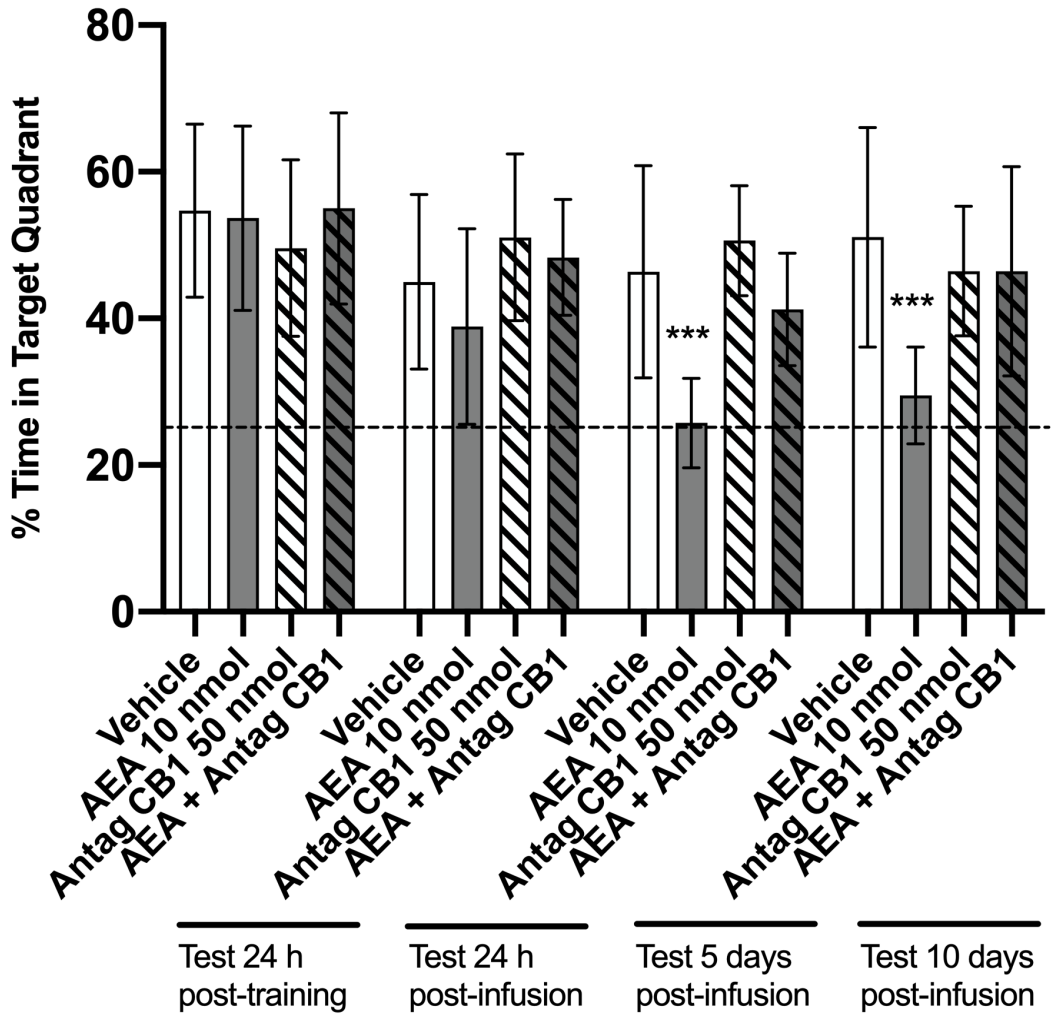

Figure 3. The amnesic effect induced by the intra-hippocampal infusion of AEA is blocked by CB1 antagonist. Male Wistar rats with infusion cannulae implanted into the CA1 region of the dorsal hippocampus were trained during 5 d in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were submitted to a 60-s probe test in the absence of the escape platform. Immediately after this first test, the animals received intrahippocampal bilateral infusions (black arrow) of either vehicle solution (Vehicle), AEA 10 nmol (AEA 10 nmol), antagonist CB1 50 nmol (Antag CB1 50 nmol) or AEA 10 nmol plus antagonist CB1 50 nmol (AEA + Antag CB1). Memory retention was assessed in sucessive 60-s probe tests carried out 24 h, 5 d and 10 d after the first test. Data are expressed as means (± SEM) of the percentage of swimming time spent in the target quadrant (TQ). Hashed line: theoretical mean of 25% of test time spent in TQ at random. ***P < 0.001 vs. respective Vehicle group, one-way ANOVA: test 24h post-first-test, $F(3, 33) = 1.885$; test 5d post-first-test, $F(3, 33) = 11.51$; test 10d post-first-test, $F(3, 33) = 6.517$; Dunnett's post test; n = 8–11 per group.

3.3. Cnnabinoid receptors do not participate individually in the spatial memory reconsolidation process.

To investigate whether cannabinoid receptors participate individually in the reconsolidation of early and late spatial memory, rats trained for 5 d in the spatial version of the MWM were submitted to a probe test without the escape platform 24 hours after the last training session. Those rats received an intra-hippocampal bilateral infusion of either vehicle or cannabinoid receptors inverse agonists (50 nmol/side), immediately after this first probe test. The rats were then tested 24 h post-infusion (for evaluating early reconsolidated memory), 5 d and 10 d post-infusion (for late reconsolidated memory) (Figure 4).

We observed that none of the inverse agonists caused any effect per se on both early and late spatial memory reconsolidation, as there was not any significant difference in all these group tests in the time spent in the target quadrant compared to the vehicle group. These results show that none of the cannabinoid receptors participates individually in the spatial memory reconsolidation process.

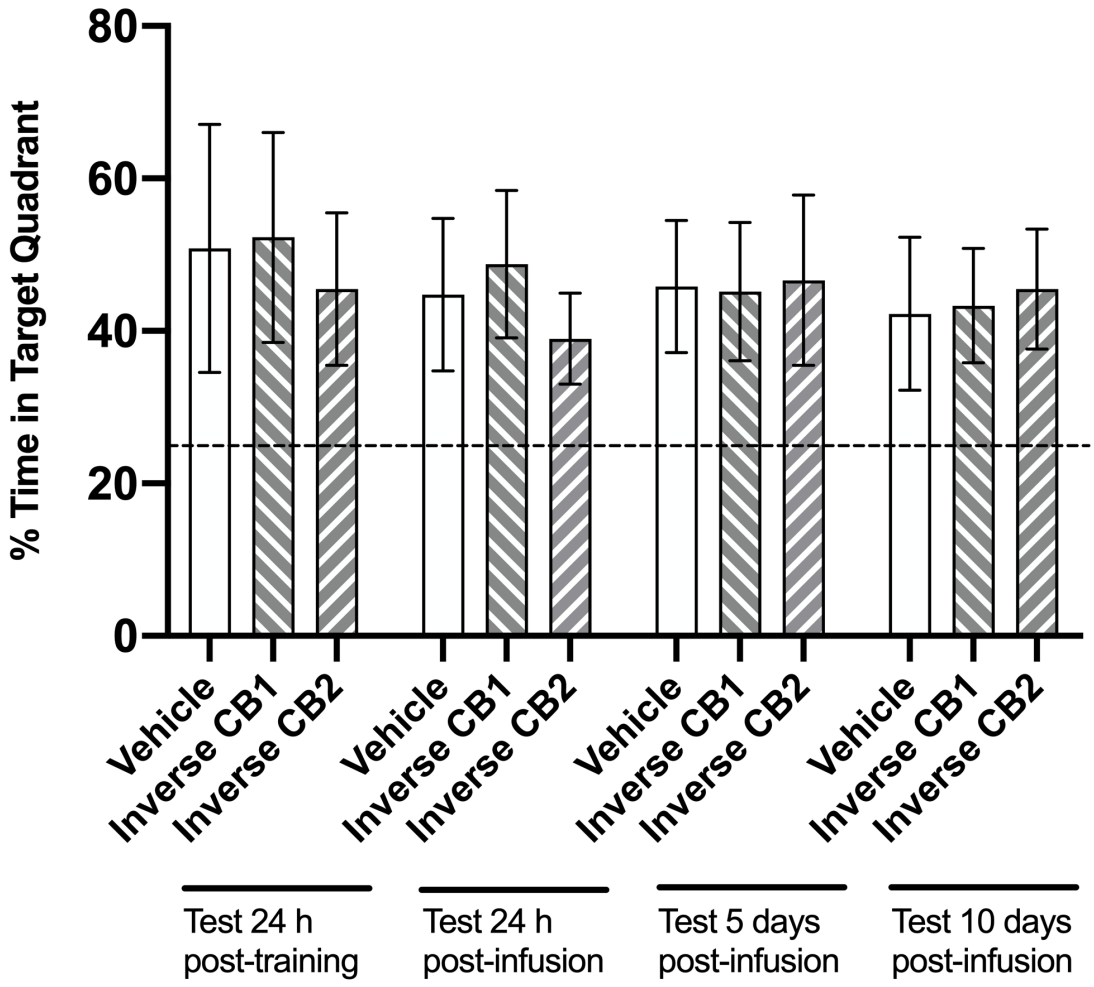

Figure 4. Inverse agonists of CB1 and CB2 receptors have no effect on spatial memory reconsolidation. Male Wistar rats with infusion cannulae implanted into the CA1 region of the dorsal hippocampus were trained during 5 d in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were submitted to a 60-s probe test in the absence of the escape platform. Immediately after this first test, the animals received intrahippocampal bilateral infusions (black arrow) of either vehicle solution (Vehicle) or inverse agonists CB1 (Inverse A1) or CB2 (Inverse CB2), 50 nmol each inverse agonist. Memory retention was assessed in sucessive 60-s probe tests carried out 24 h, 5 d and 10 d

after the first test. Data are expressed as means (± SEM) of the percentage of swimming time spent in the target quadrant (TQ). Hashed line: theoretical mean of 25% of test time spent in TQ at random. There were not any difference respective to vehicle group regarding distinct drug infusion: one-way ANOVA: test 24h post-first-test, $F(2, 32) = 3.169$; test 5d post-first-test, $F(2, 32) = 0.06589$; test 10d post-first-test, $F(2, 32) = 0.4073$; Dunnett's post test; n = 10–14 per group.

3.4. The amnesic effect of AEA on spatial memory reconsolidation is mimicked by activation of receptor CB1.

To investigate which cannabinoid receptor mimics the anandamide effect on the reconsolidation of early and late spatial memory, rats trained for 5 d in the spatial version of the MWM were submitted to a probe test without the escape platform 24 hours after the last training session. Those rats received an intra-hippocampal bilateral infusion of either vehicle or cannabinoid receptors agonists (10 nmol/side), immediately after this first probe test. The rats were then tested 24 h post-infusion (for evaluating early reconsolidated memory), 5 d and 10 d post-infusion (for late reconsolidated memory) (Figure 5).

We observed that only when CB1 receptors were activated, there was a significant difference in this group test in the time spent in the target quadrant compared to the vehicle group, similar to what occurred with AEA. Then AEA must act on receptor CB1 to impair late spatial memory reconsolidation. Notably, the receptor CB1 agonist also impaired early spatial memory reconsolidation.

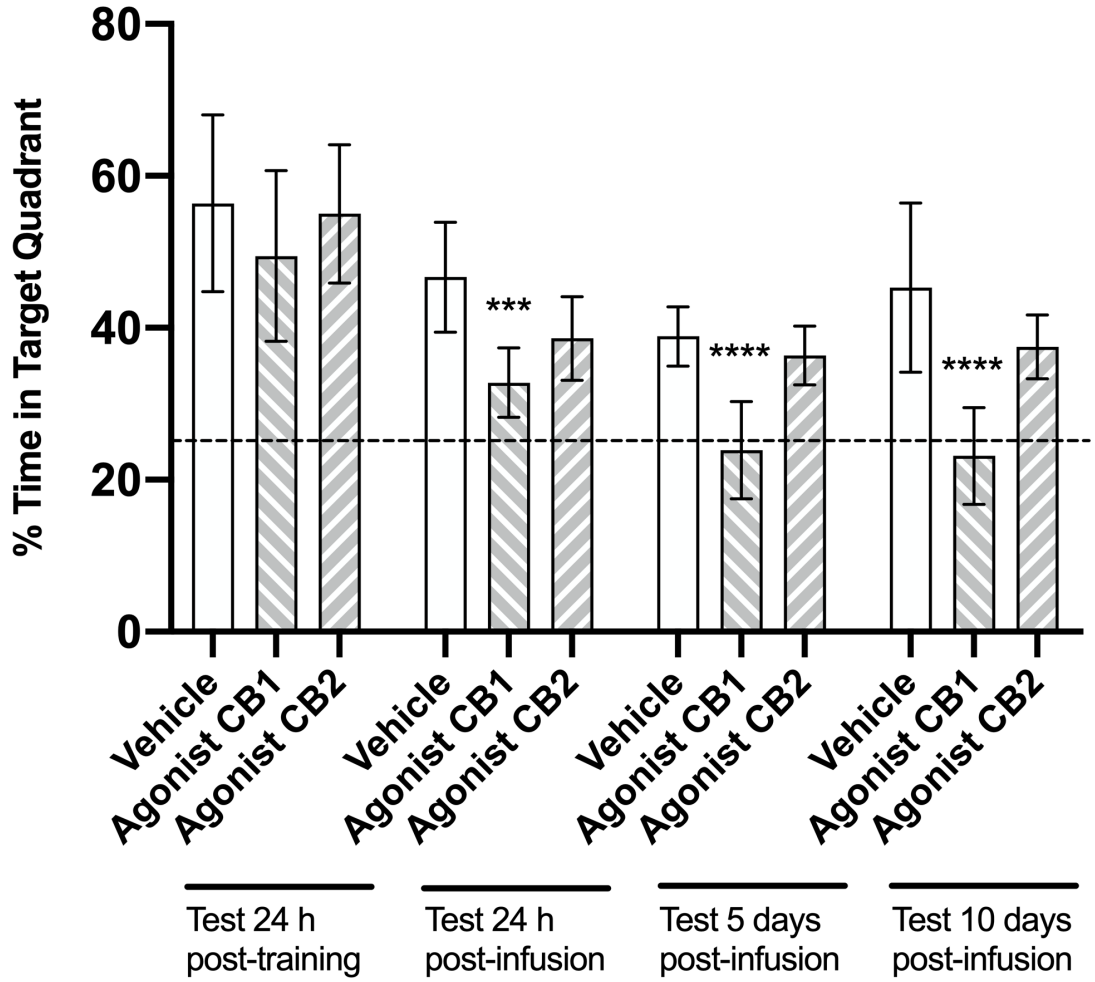

Figure 5. The amnesic effect of AEA is mimicked by CB1 receptor agonist. Male Wistar rats with infusion cannulae implanted into the CA1 region of the dorsal hippocampus were trained during 5 d in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were submitted to a 60-s probe test in the absence of the escape platform. Immediately after this first test, the animals received intrahippocampal bilateral infusions (black arrow) of either vehicle solution (Vehicle) or agonists CB1 (Agonist CB1) or CB2 (Agonist CB2), 10 nmol each agonist. Memory retention was assessed in sucessive 60-s probe tests carried out 24 h, 5 d and 10 d after the first test. Data are expressed as means (± SEM) of the percentage of swimming time spent in the target quadrant (TQ). Hashed line: theoretical mean of 25% of test time spent in TQ at random. ***P < 0.001, ****P < 0.0001 vs. respective Vehicle group, one-way ANOVA: test 24h post-first-test, $F(2, 18) = 10.78$; test 5d post-first-test, $F(2, 18) = 18.65$; test 10d post-first-test, $F(2, 18) = 16.52$; Dunnett's post test; n = 6–9 per group.

3.5. The amnesic effect of CB1 receptor agonist on spatial memory reconsolidation is blocked by proteasome inhibition

To investigate whether the impairment of spatial memory reconsolidation by CB1 receptor agonist depends on protein turnover, rats trained for 5 d in the spatial version of the MWM were submitted to a probe test without the escape platform 24 hours after the last

training session. Those rats received an intra-hippocampal bilateral infusion of either vehicle, CB1 receptor agonist (10 nmol/side), lactacystin (200 pmol/side) or CB1 receptor agonist plus lactacystin, immediately after this first probe test. The rats were then tested 24 h post-infusion (for evaluating early reconsolidated memory), 5 d and 10 d post-infusion (for late reconsolidated memory) (Figure 6).

We have observed that when protein degradation is blocked, the amnesic effect of CB1 receptor agonist on spatial memory reconsolidation disappears, indicating that the action of CB1 receptor somehow implies a lower protein input to the reconsolidation process. If this is indeed the case, the already known effect of blocking protein synthesis on spatial memory reconsolidation [5;47;48] can be reversed by blocking this receptor. In the next section, we decided to test this hypothesis for the CB1 receptor inverse agonist.

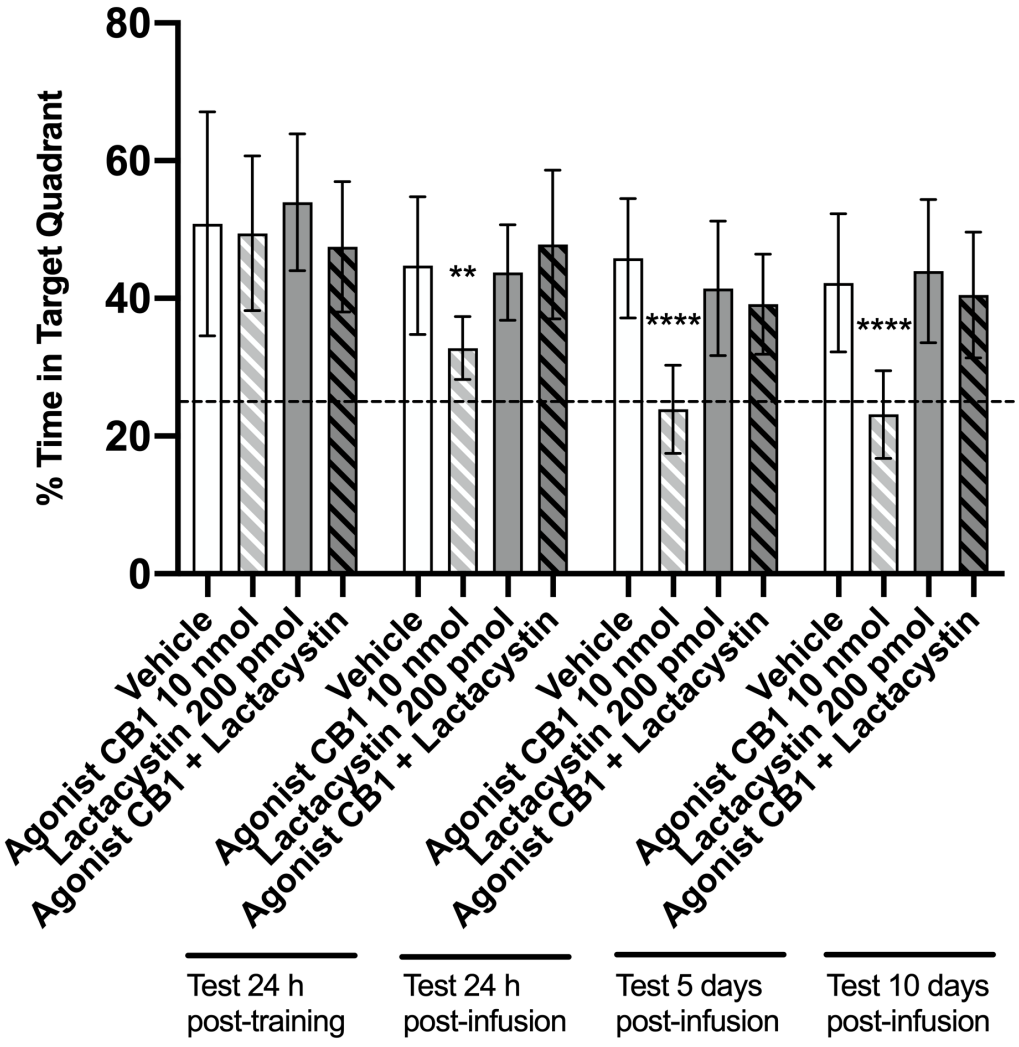

Figure 6. The amnesic effect on reconsolidation induced by the intra-hippocampal infusion of CB1 receptor agonist is blocked by the proteasome inhibitor lactacystin. Male Wistar rats with infusion cannulae implanted into the CA1 region of the dorsal hippocampus were trained during 5 d in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were submitted to a 60-s probe test in the absence of the escape platform. Immediately after this first test, the animals received intrahippocampal bilateral infusions (black arrow) of either vehicle solution (Vehicle), oleamide 10 nmol (Agonist CB1 10 nmol),

lactacystin 200 pmol (Lactacystin 200 pmol) or oleamide 10 nmol plus lactacystin 200 pmol (Agonist CB1 + Lactacystin). Memory retention was assessed in sucessive 60-s probe tests carried out 24 h, 5 d and 10 d after the first test. Data are expressed as means (± SEM) of the percentage of swimming time spent in the target quadrant (TQ). Hashed line: theoretical mean of 25% of test time spent in TQ at random. **P < 0.01, ****P < 0.0001 vs. respective Vehicle group, one-way ANOVA: test 24h post-first-test, $F(3, 37) = 5.253$; test 5d post-first-test, $F(3, 37) = 13.79$; test 10d post-first-test, $F(3, 37) = 10.16$; Dunnett's post test; n = 8–14 per group.

3.7. The amnesic effect of protein synthesis inhibition on spatial memory reconsolidation is blocked by inhibiting the cannabinoid CB1 receptor.

To investigate whether the impairment of spatial memory reconsolidation caused by inhibition of protein synthesis can be reversed by blocking the cannabinoid CB1 receptor, rats trained for 5 d in the spatial version of the MWM were submitted to a probe test without the escape platform 24 hours after the last training session. Those rats received an intra-hippocampal bilateral infusion of either vehicle, CB1 receptor inverse agonist (50 nmol/side), rapamycin (5 μg/side), or CB1 receptor inverse agonist plus rapamycin, immediately after this first probe test. The rats were then tested 24 h post-infusion (for evaluating early reconsolidated memory), 5 d and 10 d post-infusion (for late reconsolidated memory) (Figure 7).

As predicted previously, the inverse activation of the CB1 receptor was sufficient to reverse the amnesic effect on reconsolidation caused by the inhibition of protein synthesis by rapamycin. This result implies that endogenous cannabinoids, acting via CB1 receptors, is required for the inhibition of protein synthesis to affect protein turnover in a way that compromises the protein input required for spatial memory reconsolidation.

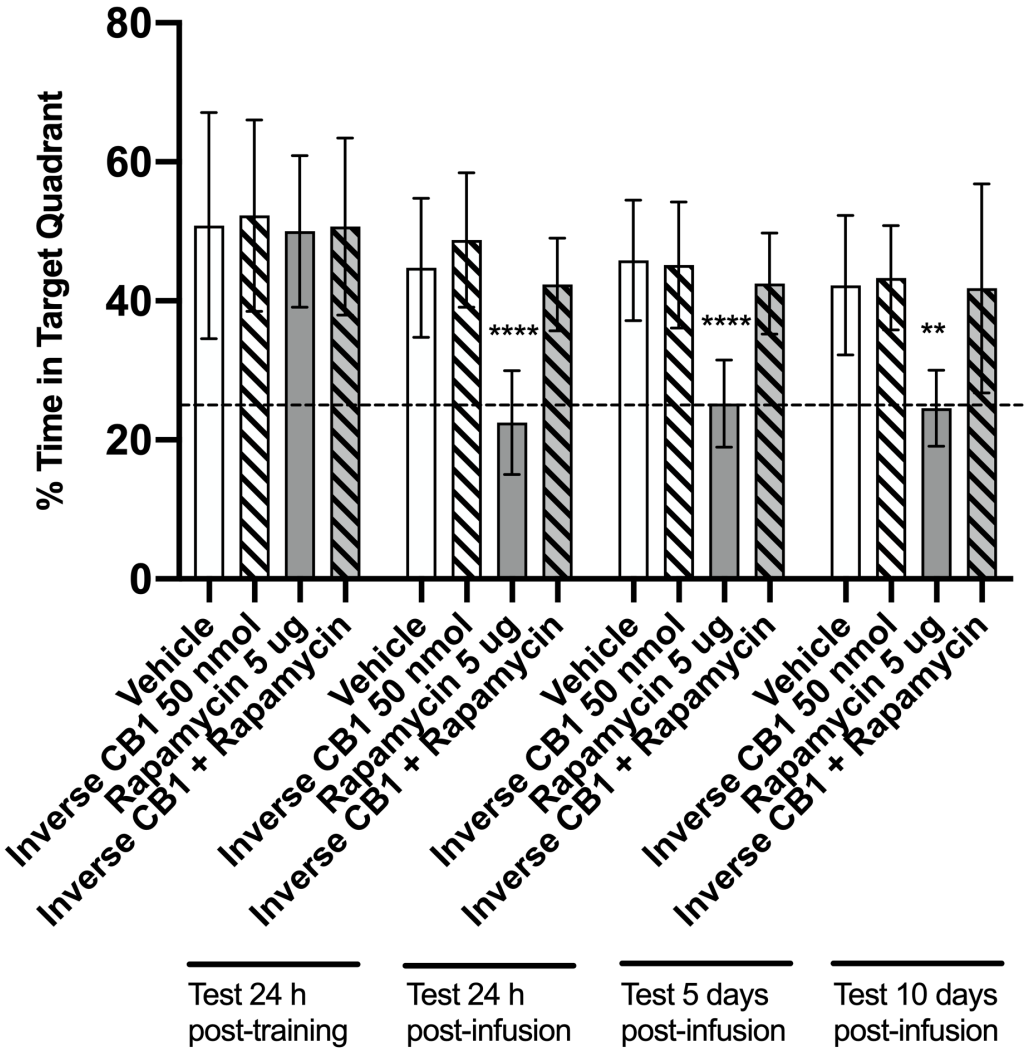

Figure 7. The amnesic effect on reconsolidation induced by the intra-hippocampal infusion of rapamycin is blocked by the CB1 inverse agonist. Male Wistar rats with infusion cannulae implanted into the CA1 region of the dorsal hippocampus were trained during 5 d in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were submitted to a 60-s probe test in the absence of the escape platform. Immediately after this first test, the animals received intrahippocampal bilateral infusions (black arrow) of either vehicle solution (Vehicle), CB1 receptor inverse agonist 50 nmol (Inverse CB1 50 nmol), rapamycin 5 µg (Rapamycin 5 ug) or antagonist A3 plus rapamycin 5 µg (Inverse CB1 + Rapamycin). Memory retention was assessed in sucessive 60-s probe tests carried out 24 h, 5 d and 10 d after the first test. Data are expressed as means (± SEM) of the percentage of swimming time spent in the target quadrant (TQ). Hashed line: theoretical mean of 25% of test time spent in TQ at random. **P < 0.01, ****P < 0.0001 vs. respective Vehicle group, one-way ANOVA: test 24h post-first-test, $F(3, 41) = 15.91$; test 5d post-first-test, $F(3, 41) = 13.03$; test 10d post-first-test, $F(3, 41) = 6.315$; Dunnett's post test; n = 8–14 per group.

4. Discussion

Our results show that AEA and cannabinoid receptor agonist CB1 given in the dorsal hippocampus immediately after a probe test in the absence of the escape platform performed

24-h post-training impairs spatial memory retention during subsequent probe tests carried out 24 hours (only CB1 receptor agonist oleamide), 5 days and 10 days later. The amnesic effect of AEA and CB1 receptor agonist is contingent on the non-reinforced reactivation of the mnemonic trace.

The fact that the AEA caused a verifiable effect only on the reconsolidation of late spatial long-term memory, but not on the early one, again shows us a difference in the level of biochemical mechanisms between these two types of long-term memories, early vs late. This kind of difference has already been found in other studies of ours [49,50]. The first time this difference in biochemical level was found between these two types of long-term memory was regarding the activation of CaMKII, which needs to be activated immediately after an unreinforced test in the MWM task to have a reconsolidation that generates a stable memory in the late-term (5 days post-test without reinforcement) [12]. These results, together with the fact that CB1 receptor activation inhibit a subset of voltage-gated calcium channels and activate inwardly rectifying potassium (GIRK) channels [51] and the fact that intracellular calcium levels are also a key factor in synaptic plasticity regulatory mechanisms [52], suggests an interesting line of investigation, aiming to discover some regulatory link between the CB1 receptor and calcium-dependent signal transduction pathways, and the participation of this link in mnemonic processes such as that of the reconsolidation of long-term memories, and in how they differ in determining the persistence of the mnemonic trace upon reconsolidation.

CB1 receptor is coupled to an inhibitory G protein [53], which, once activated, inhibits membrane adenylyl cyclase, thereby decreasing the intracellular concentration of the cAMP second messenger, which in turn leads to lower activation of PKA, and subsequently to lower activation of the transcription of immediate and late genes related to long-lasting synaptic plasticity [54,55,9]. All this converges to a decrease in the synthesis rate of new proteins, which implies a consequent impairment in long-term memory retention [5,56,57,58]. Corroborating with this successive description of events, we show that the amnesic effect of CB1 receptor activation on spatial memory reconsolidation disappears if there is no protein degradation. Without protein degradation, a decrease in the rate of new protein synthesis does not translate into a lower protein input required for the reconsolidation process. Furthermore, we show that the amnesic effect caused by inhibition of protein synthesis during spatial memory reconsolidation can be blocked by the inverse activation of CB1 receptor, implying this receptor participates in the protein turnover process regulation.

Several studies have highlighted the inhibitory - and therefore neuroprotective - function of endocannabinoids (eCBs) in response to cellular stress conditions [59,60,61]. This modulatory role appears to act as a defense mechanism against the cellular damage commonly associated with such states. Additionally, the involvement of eCBs in long-term spatial memory reconsolidation helps to bridge the connection between stress-induced cellular dysfunction and alterations in cognitive performance. Situations such as excitotoxicity [62,63], neuroinflammatory responses [64,65,66], and early neurodegenerative events marked by β-amyloid plaque buildup [67,68] and neurofibrillary tangle formation [67] are all contexts in which these effects have been observed. The eCB system is thus deeply involved in these pathological processes, underscoring the importance of advancing our understanding of its receptors in both normal and disease-related states, as this may inform novel therapeutic strategies.

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