The bidirectional effect of prelimbic 5-hydroxytryptamine type-4 (5-HT4) receptors on ACPA-mediated aversive memory impairment in adult male Sprague-Dawley rats

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Objective(s): This study aimed at investigating the effect of serotonergic 5-HT4 receptor agonist/antagonist on memory consolidation deficit induced by ACPA (a potent, selective CB1 cannabinoid receptor antagonist) in the pre-limbic (PL) cortex.

Materials and Methods: We used the step-through passive avoidance test to evaluate memory consolidation of male Sprague-Dawley (SD) rats. Bilateral post-training microinjections of the drugs were done in a volume of 0.6 μl/rat into the PL area (0.3 μl per side).

Results: The results showed a significant interaction between RS67333 hydrochloride (5-HT4 receptor agonist) or RS23597-190 hydrochloride (5-HT4 receptor antagonist) and ACPA on consolidation of aversive memory. RS67333 hydrochloride (0.5 μg/rat) enhanced consolidation of memory and its co-administration at the ineffective dose of 0.005 μg/rat with ineffective (0.001 μg/rat) or effective (0.1 μg/rat) doses of ACPA improved and prevented impairment of memory caused by ACPA, respectively. In other words, RS67333 had a bidirectional effect on ACPA-caused amnesia. While RS23597-190 hydrochloride had no effect on memory at the doses used (0.005, 0.01, 0.1, or 0.5 μg/rat); but its concomitant use with an effective dose of ACPA (0.1 μg/rat) potentiated amnesia. None of the drugs had an effect on locomotor activity.

Conclusion: This study revealed that activation or deactivation of the 5-HT4 receptors in the PL may mediate the IA memory impairment induced by ACPA indicating a modulatory role for the 5-HT4 serotonergic receptors.

Introduction

Serotonin or 5-hydroxytryptamine (5-HT), as an important brain neurotransmitter and neuromodulator, has a pivotal role in various functions (1). A broad range of studies presents the interplay between serotonergic neurotransmission and multiple other neurotransmitters including glutamate (Glu), γ-aminobutyric acid (GABA), dopamine (DA), acetylcholine (ACh), and cannabinoids (CBs). The interplay is involved in a variety of cognitive functions such as learning and memory processes (2, 3). The 5-hydroxytryptamine type-4 receptor (5-HT4 R) has a heterogeneous distribution pattern throughout the brain with high densities in limbic structures linked to memory and cognition (4, 5). Based on previous studies, 5-HT4 Rs could be a promising therapeutic target for the treatment of cognitive deficits (6, 7). The contribution of 5-HT4 Rs in learning and memory processes has been reviewed in the scientific literature (4, 8-10).
function by interacting with neurochemical systems. Growing evidence indicates a potential interaction between serotonergic and endocannabinoid systems. In the PFC, alterations in serotonergic transmission occur in response to cannabinoind administrations (20, 21). Cannabinoid modulation of neuronal activity is largely mediated by cannabinoid type 1 receptors (CB1Rs) (22). CB1Rs are expressed in serotonergic fibers and synapses (3, 23). In addition, co-expression of CB1Rs and various types of serotonin receptors have been shown in the forebrain areas (24). Ferreira et al. showed that functional presynaptic CB1Rs were localized in frontocortical serotonergic nerve terminals and mediated as a modulator of serotonin neurotransmission (25). Furthermore, Balázsa et al., indicated that CB1Rs were implicated in nonsynaptic release modulation of [3H]serotonin in the hippocampus (26). In the CNS, endocannabinoid (eCB) signaling is implicated in the release of serotonin (27, 28), as well as modulation of the activity and expression of different serotonin receptors (3, 29, 30). Likewise, serotonin receptor activation may evoke eCB release (31). Based on the above evidence, it is expected that serotonergic and eCB systems cross-control the activity of each other. However, there is no evidence supporting the presence of functional interaction between CB1Rs and 5-HT4Rs in mediating the IA memory function in the PL. Therefore, this study was performed to evaluate the possible role of prelimbic 5-HT4Rs in IA memory function in the PL. Therefore, the animals were allowed to recover for at least 7 days before behavioral testing.

At the end of the tests, to ensure the accuracy of the infusion site (Figure 1), the animals were anesthetized with a high dose of ketamine/xylazine dilution. Next, methylene blue solution was injected into the PL area (1%, 0.3 μl/side) and animals were decapitated using a guillotine. The brains of animals were removed and stabilized in formalin solution (10%, 7 days) and sliced. The fixed brains were then sliced directly across the cannulae placements, and the placements were histologically verified using the rat brain atlas of Paxinos and Watson coordinates (33). Animals with incorrect cannulae placement (about 5% of total animals) were excluded from the analysis.

Experimental procedures

Animals
In order to perform the experiments, adult male rats with the scientific name of Rattus Norvegicus Allivias of Sprague-Dawley breed, weighing approximately 250 to 290 g at the time of surgery, were used. The animals were purchased from the Comparative and Experimental Medical Center, Shiraz University of Medical Sciences, Shiraz, Iran. Animals were kept (4/cage) in an animal house under the temperature of 22 ± 2 °C and 12/12 hr light/dark cycle (lights on at 07:00 hr) and had free access to water and food, except in the limited times of trials. Eight rats were used in each group and each animal was tested only once. The experiments were conducted during the light phase of the cycle. Animal care and behavioral tests were done in accordance with the Guide for the Care and Use of Laboratory Animals (32).

Surgery
All surgical procedures were performed under ketamine (70 mg/kg) – xylazine (7 mg/kg) anesthesia in a stereotaxic surgical apparatus (Stoelting Co, Illinois, USA) with a skull-flat orientation. Two stainless steel, 22-gauge guide-cannulas were bilaterally implanted 1 mm above the PL area according to the atlas of Paxinos and Watson. Stereotaxic coordinates of the PL were Anterior/Posterior (AP) equally +3.4 mm from the bregma, Medio/Lateral (ML) equally ±0.9 mm from the midline, and Dorso/Ventral (DV) equally −3 mm from the skull surface. The cannulas were secured to the skull bone using dental acrylic cement. Stainless steel stylets (dummy cannulae, 27 gauge) were inserted into the guide cannulae to prevent possible obstruction until the animals received the drugs. Following surgery, the animals were allowed to recover for at least 7 days before behavioral testing.

Intra-PL injection
In order to inject the drugs, rats were softly maintained by hand; then dummy cannulas were removed and substituted by 27 gauge infusion cannulas (1 mm below the tip of the guide cannulas). The infusion needle was joined to the Hamilton syringe (2-μl) via polyethylene tubing (PE-20). Intra-PL microinjections of the drugs were performed with the volume of 0.6 μl per rat (0.3 μl per side) in a 60 sec period. Following the injections of drugs, the injectors were left in place for an additional 60 sec to allow the drugs to diffuse into the tissue. The interval time between the two injections was 5 min. One microinjection took about 9 min to complete (34, 35).

Inhibitory (passive) avoidance task
The passive avoidance (IA) task is an associative learning test, based on negative reinforcement used to assess memory (36).

Memory testing and apparatus
The step-through passive avoidance device (Figure 2) included two same size chambers isolated by a sliding guillotine door (7 × 9 cm); a light chamber (30 cm × 20 cm × 20 cm) made of white opaque plexiglass
Assessment of locomotor activity

Motor activity was also assessed immediately after the retention trial session. In this regard, locomotion was recorded using an Animex activity meter device (Type DS, Farad electronics, Sweden). Rats separately were placed on the measurement platform and permitted to freely explore for a duration of 5 min. Each movement produced a signal which was automatically converted to numbers (37). Locomotor activity was evaluated by measuring the number of movements. Motor activity was evaluated by measuring the number of movements.

Drugs

The drugs which were utilized in this research were: ketamine hydrochloride/xylazine (Alfasan Chemical Co, Woerden, Holland) in order to anesthetize the animals. ACPA (arachidonylcyclopropylamide; a potent, selective agonist for CB receptor; in amounts of 0.001, 0.01, and 0.1 μg/rat), RS67333 hydrochloride (1-((4-amino-5-chloro-2-methoxyphenyl)-3-[1-butyl-4-piperidinyl]-1-propanone hydrochloride; a potent and highly selective partial agonist for 5-HT4 receptor; in amounts of 0.005, 0.01, 0.1, and 0.5 μg/rat), and RS23597-190 hydrochloride (3-(piperidine-1-yl) propyl 4-amino-5-chloro-2-methoxybenzoate hydrochloride; a high affinity, selective competitive antagonist for 5-HT4 receptor; in amounts of 0.005, 0.01, 0.1, and 0.5 μg/rat) acquired from Tocris (Tocris Bioscience Bristol, United Kingdom). All drugs were resolved in sterile 0.9% saline, with the exception of ACPA, which was prepared dissolved in anhydrous ethanol in the amount of 5 mg/ml and was diluted to the needed volume with saline. All of the drugs were made ready freshly just previous to testing. The injection timing and choice of drug dosages were based on the pilot and published studies in scientific journals (35, 38).

Experimental design and drug treatment

At first, the role of post-training, micro-infusion of the drugs in various dosages was separately examined on the consolidation of emotional memory in the pre-limbic area, and curves of dose-response were plotted. Next, the probable interplay between a sub-threshold dose of 5-HT4 receptors agonist or antagonist plus ACPA in various dosages was evaluated. Eight rats were employed in each experimental group and each rat was examined just once. Bilateral intra-PL microinjection of the drugs was conducted immediately after a training session in a volume of 0.6 μl/rat (0.3 μl/side). The animals received one or two injections in the experiments. The Interval time between two drug injections was 5 min. Behavioral tests (passive avoidance & locomotor activity) were assessed in all experiments, as described in previous sections. The test session was performed 24 hr later, following the drug microinjection(s).

Experiment 1: Evaluating the effect of post-training intra-PL microinjections of RS67333 hydrochloride (5-HT4 receptor agonist) and RS23597-190 hydrochloride (5-HT, receptor antagonist) on IA memory consolidation

Ten groups of animals (n=8/group) received saline (0.6 μl/rat, two groups), RS67333 (a 5-HT4 Rs agonist; 0.005, 0.01, 0.1, or 0.5 μg/rat) or RS23597-190 (a 5-HT4 Rs antagonist; 0.005, 0.01, 0.1, or 0.5 μg/rat) immediately after training.
Experiment 2: Evaluating the effect of post-training intra-PL microinjection of saline, RS67333 hydrochloride, or RS23597-190 hydrochloride on IA memory impairment induced by ACPA

Twelve groups of animals were utilized. The animals were distributed into three four-group sets. Rats were initially injected with saline (0.6 μl/rat), the subthreshold dose of RS67333 (0.005 μg/rat), or RS23597-190 (0.5 μg/rat) immediately after training. Then after 5 min, rats were injected with vehicle (0.6 μl/rat) or different doses of ACPA (0.001, 0.01, and 0.1 μg/rat).

Statistical analysis
Kolmogorov–Smirnov test showed normal distributions of data in all groups. Therefore, data were analyzed using one- or two-way analysis of variance (ANOVA). One-way ANOVA was performed to assess the possible interactions between the drugs. Subsequently a significant F value, Tukey’s post-hoc analysis was done to evaluate paired-group comparisons. The results were presented as mean ± S.E.M. and P < 0.05 was considered as a statistically significant difference. SPSS software ver. 19 was used for statistical analyses.

Results
Post-training intra-PL microinjection effects of RS67333 and RS23597-190 hydrochloride on memory consolidation and exploratory behaviors

One-way ANOVA analysis revealed that local intra-PL administrations of RS67333 altered consolidation of IA memory [F(4,35) = 11.042, P = 0.000 < 0.05, (Figure 3A, left panel)], while it did not alter locomotor activity behavior [F(4,35) = 0.408, P = 0.801 > 0.05, (Figure 3B, left panel)]. Moreover, Tukey’s post-hoc test showed that RS67333 at dose of 0.5 μg/rat significantly increased the step-through latency in passive avoidance learning task, during the test session. Based on the results, it appears that RS67333 has an enhancing effect on aversive memory consolidation. Moreover, one-way ANOVA indicated that post-training intra-PL administration of RS23597-190 at different doses (0.005, 0.01, 0.1, and 0.5 μg/rat) could neither alter the IA memory consolidation [F(4,35) = 0.248, P = 0.909 > 0.05, (Figure 3A, right panel)], nor the locomotor activity behavior [F(4,35) = 0.100, P = 0.982 > 0.05, (Figure 3B, right panel)], suggesting that RS23597-190 alone at the applied doses did not affect memory consolidation.

Effect of post-training intra-PL microinjection of RS67333 hydrochloride or RS23597-190 hydrochloride on the ACPA induced IA memory consolidation deficit

One-way ANOVA demonstrated that ACPA significantly altered memory consolidation [F(5,42) = 13.770, P = 0.000 < 0.05, (Figure 4A, left panel)] but did not affect locomotor activity [F(5,42) = 1.199, P = 0.326 > 0.05, (Figure 4B, left panel)]. Tukey’s post-hoc analysis showed that ACPA at a dose of 0.1 μg/rat impaired IA memory consolidation.

![Figure 3](image3.png)

**Figure 3.** Effects of post-training intra-PL microinjections of RS67333 and RS23597-190 on IA memory consolidation (A) and locomotor activity (B) in rats. Ten groups of rats (n=8/group) received either saline (0.6 μl/rat, two groups), or different doses of RS67333 (5-HT, receptor agonist; 0.005, 0.01, 0.1, and 0.5 μg/rat) and RS23597-190 (5-HT, receptor antagonist; 0.005, 0.01, 0.1, and 0.5 μg/rat), immediately after training. Step through latency and locomotor activity were evaluated in all groups after 24 hr. Each column shows mean ± SEM. **P < 0.001, as compared with the saline control group.

![Figure 4](image4.png)

**Figure 4.** Effects of post-training intra-PL microinjections of ACPA on IA memory consolidation (panel A) and locomotor activity (panel B) in the presence and absence of RS67333 (0.005 μg/rat) or RS23597-190 (0.5 μg/rat). Three four-group sets of rats were utilized. Rats were injected with saline (0.6 μl/rat), the subthreshold dose of RS67333 (0.005 μg/rat) or RS23597-190 (0.5 μg/rat) plus vehicle or ACPA at different doses (0.001, 0.01, 0.1 μg/rat). Step through latency (STL) and locomotor activity were evaluated in all groups, after 24 hr. Each column shows mean±SEM. ***P<0.001, different from vehicle/saline group. ++P<0.01 and +++P<0.001 different from respective ACPA/saline groups.
Furthermore, two-way ANOVA revealed a significant interaction between RS67333 plus ACPA on memory consolidation \( [F \text{ dose} (3,56) = 8.882, P=0.000 < 0.05; F \text{ drug} (1,56) = 0.256, P=0.609 > 0.05; F \text{ dose} \times \text{drug} (3,56) = 21.588, P=0.000 < 0.05, \text{(Figure 4A, middle panel)}] \), but not locomotor activity \( [F \text{ dose} (3,56) = .793, P=0.503 > 0.05; F \text{ drug} (1,56) = 0.162, P=0.751 > 0.05; F \text{ dose} \times \text{drug} (3,56) = 0.168, P=0.917 > 0.05, \text{(Figure 4B, middle panel)}] \). Tukey’s post-hoc test showed that the ineffective doses of ACPA (0.001 \( \mu \text{g/rat} \)) and RS67333 (0.005 \( \mu \text{g/rat} \)) when combined, significantly potentiated the emotional memory impairment. Curiously, RS67333 (0.005 \( \mu \text{g/rat} \)) when combined with the effective dose of ACPA (0.1 \( \mu \text{g/rat} \)) strengthened the ACPA effect. In addition, two-way ANOVA showed a significant interaction between RS23597-190 plus ACPA on memory consolidation \( [F \text{ dose} (3,56) = 43.195, P=0.000 < 0.05, \text{(Figure 4A, middle panel)}] \), but not locomotor activity \( [F \text{ dose} (3,56) = .221, P=0.882; F \text{ drug} (1,56) = 0.081, P=0.777; F \text{ dose} \times \text{drug} (3,56) = 0.661, P=0.580, \text{(Figure 3B, right panel)}] \). Tukey’s post-hoc test showed that post-training co-administration of a subthreshold dose of RS3597190 (0.5 \( \mu \text{g/rat} \)) plus the higher dose of ACPA (0.1 \( \mu \text{g/rat} \)) strengthened the ACPA effect.

**Discussion**

This study revealed that 5-HT4 Rs agonist (RS67333) in the presence of CB1 receptor agonist (ACPA) produced bidirectional effects on the consolidation of aversive memory in the PL area. The enhancing effect of RS67333 on memory consolidation is in agreement with the studies that showed that 5-HT4 Rs agonists improved the learning and memory process \( (4, 10, 39-41) \). However, some studies have reported that RS67333 impaired consolidation of memory \( (38, 42, 43) \).

Based on several lines of studies, serotonin influenced neuronal plasticity and memory formation through multiple intracellular signaling pathways which are implicated in diverse effectors such as cyclic adenosine monophosphate (cAMP) \( (8, 44) \). Emerging evidence suggests a flexible mechanism for 5-HT4 Rs to modulate synaptic transmission and neuronal excitability in the PFC networks \( (7) \). The 5-HT4 Rs are \( G_{\alpha}\)-protein-coupled receptors and positively coupled to adenylyl cyclase. It seems that agonist activation of these receptors by engaging the downstream signaling cascades probably activates the cAMP formation \( (45, 46) \) and participates in the new memory formation \( (9) \). In addition, 5-HT4 Rs agonists improved facilitation of the various neurotransmitter releases in the brain structures linked to memory function and enhanced synaptic transmission which might have affected the development of memory \( (47, 48) \). 5-HT4 Rs also represented constitutive (ligand-independent) activity which elucidates the differences between expected and observed effects of agonists and antagonists of the 5-HT4 Rs \( (48) \). 5-HT4 Rs splice variants have been identified both in rodents and humans \( (49, 50) \) with structural differences. It might be influenced and contributed to their functional diversity and involved in the fine-tuning of the receptor coupling to G-protein subtypes. Some of splice variants of 5-HT4 Rs are able to activate both \( G_{\alpha}\text{-}s \) and \( G_{\alpha}\text{-}i/os \) \( (51) \). These variants may interact with distinct or overlapping signaling machinery leading to differential intracellular responses \( (9) \). It may be possible that RS67333’s effect in this paradigm is being mediated in part or significantly by its action on other receptors such as sigma receptors which are also known to ameliorate anxiety-related responses and affect PFC neural transmission and memory function \( (52, 53) \). It has been shown that RS67333 \( (54) \) and RS23597-190 \( (55) \) had a high affinity for sigma-1 binding sites. It may indicate that the highest tested dose of RS23597-190 was showing an effect similar to the lower dose of RS67333, despite their antithetical pharmacological action. Therefore, RS67333 like other 5-HT4 agonists may interact with other receptors and its effect might not be mediated solely by action on 5-HT4 Rs \( (56) \).

The present results also revealed that there is a significant interaction between RS67333 or RS23597-190 plus ACPA on memory consolidation. Furthermore, RS67333 potentiated or reversed ACPA response (a bidirectional effect). In accordance with our findings, it has been shown that 5-HT4 Rs agonists such as RS67333 reversed memory impairment induced by diverse classes of pharmacological agents in different behavioral tasks \( (41, 57-61) \). However, some reports are describing the intensifying effect of RS67333 on ACPA-induced amnesia \( (38, 62) \).

The mechanisms underlying the roles of cannabinoid-based drugs are not fully known. Increasing evidence indicates the bidirectional interaction between the cannabinoid and the serotonergic systems which employed different direct and indirect mechanisms and brain structures \( (3, 23, 25) \). This is partially rationalized because of: 1. A high level of functional overlapping between these two systems in the regulation of several physiological functions \( (3) \). 2. Extensively overlapping distribution pattern of CB1Rs and 5-HT4Rs in the brain \( (63) \), and 3. Engaging both of these systems in creating the connections and the maturation of brain neocortical circuitry as well as in neuromodulation of glutamatergic and GABAergic transmission in the PFC \( (64) \). Co-expression of 5-HT and CB1Rs has been shown in the brain, representing possible interactions between them \( (24) \). Cannabinoids display CBR-independent activity and target non- CB1/CB2, GPCRs that may contribute to the pharmacological actions of CBs \( (65) \). On the other hand, some studies indicated the ability of CB1Rs to form homo- and heteromeric complexes with the 5-HT4Rs \( (66, 67) \). These interactions mediate different aspects of CB1R function. Based on previous studies, CB1Rs have unusual properties such as the dual capacity for inhibition or activation of adenylyl cyclase by linking to \( G_{\alpha}\text{-}s \) or \( G_{\alpha}\text{-}i/o\) proteins \( (69) \) and influencing the intracellular signaling pathways. It is the potential of CB1Rs to modulate the activity of the other receptor systems. In addition, CB1Rs are mainly expressed in the presynaptic glutamatergic and GABAergic neurons \( (70) \). It has been reported that CB1R activation is involved in the modulation of synaptic plasticity by controlling PKA activity in the GABAergic cells \( (71) \). On the other hand, dual effects of
5-HT4 Rs agonists have been shown on the GABAergic inhibitory postsynaptic currents (IPSCs) in the PFC pyramidal neurons. Its activation-induced enhancement or reduction of the GABAergic evoked currents (72) depending on the synaptic kinase A activation (7) and participated in modulation of synaptic transmission and neuronal excitability. In addition, PKA is known as a cAMP-dependent protein kinase and works through the cAMP signaling pathway (73). Therefore, it is possible that co-activation of CB1Rs and 5-HT4Rs has influenced the intracellular cAMP accumulation and participated in the memory process through engaging downstream signaling pathways. It can be said that this response “bidirectional effects of 5-HT4 Rs agonist in the presence of CB1Rs agonist on memory consolidation” is likely the result of CB1R switching from Gi to Gs signaling and neuronal excitability. In addition, PKA is known as a cAMP-dependent protein kinase and works through the cAMP signaling pathway (73).

**Conclusion**

In summary, this study showed that: 1. Intra-PL injection of RS67333 but not RS23597-190 increased IA memory consolidation., 2. There is a significant interaction between RS67333 or RS23597-190 plus ACPA on memory consolidation, 3. RS67333 potentiated or reversed ACPA response (a bidirectional effect), 4. RS23597-19 intensifies ACPA-induced impairment of memory consolidation. We suggest that activation or deactivation of 5-HT4 Rs in the PL area, presumably was involved in memory impairment induced by ACPA in the step-through IA task. Future studies are required to uncover the details.

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**Conflicts of Interest**

The authors have not declared potential conflicts of interest with respect to the Declaration of Conflicting Interests.

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