Interplay between serotonin and cannabinoid function in the amygdala in fear conditioning

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Abstract

The possible interactions between the cannabinoid and serotonin systems in the regions of the brain involved in emotional learning and memory formation have been studied by some researchers. In view of the key role of the amygdala in the acquisition and expression of fear memory, we investigated the involvement of basolateral amygdala (BLA) serotonin 5-HT₄ receptors in arachidonylcyclopropylamide (ACPA; selective CB₁ cannabinoid receptor agonist)-induced fear memory consolidation impairment. In our study, a context and tone fear conditioning apparatus was used for testing fear conditioning in adult male NMRI mice. The results showed that intraperitoneal administration of ACPA 0.5 or 0.05, 0.1 and 0.5 mg/kg immediately after training decreased the percentage of freezing time in context or tone fear conditioning respectively, suggesting a context- or tone- dependent fear memory consolidation impairment. Post-training intra-BLA microinjections of RS67333, as 5-HT₄ serotonin receptor agonist, at doses of 0.025 and 0.05 µg/mouse also impaired context or tone memory consolidation, while RS23597, as 5-HT₄ serotonin receptor antagonist, did not produce a marked difference in both fear memories as compared with the control group. Moreover, a subthreshold dose of RS67333 did not alter ACPA response in both fear conditionings. Interestingly, a subthreshold dose of RS23597 potentiated or reversed ACPA response at the dose of 0.01 or 0.05 respectively. It is concluded that BLA serotonin 5-HT₄ receptors are involved in tone-dependent fear memory consolidation impairment induced by CB₁ activation using ACPA, suggesting a modulatory role for serotonin 5-HT₄ receptor.

Keywords: ACPA; RS67333; RS23597; Fear memory conditioning; Mice

1. Introduction

The amygdala has long been known to play a key role in supporting memory for emotionally arousing experiences (Hermans et al., 2014). Neuroanatomical and the molecular basis of emotional learning and memory have been widely studied by Pavlovian fear conditioning. Sound, light or a specific context can be paired with foot shock as a neutral conditioned stimulus which will elicit a conditioned fear response in the absence of unconditioned stimulus. Conditioned context- and tone-dependent fear can be acquired in a single trial and can be studied independent of one another (Misane et al., 2013).

Although different areas of the brain have been suggested to be involved in fear conditioning, the amygdala may be the main control center for the acquisition and expression of auditory fear conditioning (Gale et al., 2004; Phelps and LeDoux, 2005; Rodrigues et al., 2004). It has been shown that fear learning depends on the induction of neural plasticity within the basolateral complex of the amygdala (BLA) (Miserendino et al., 1990; Rogan et al., 1997). The BLA receives sensory projections (Luskin and Price, 1983; Miki et al., 1998) from other brain regions that encode information about conditioned stimuli (CSs) and unconditioned stimuli (USs). The information, after entering the BLA, is transferred to the central amygdala (CeA) for output (Sah et al., 2003). The BLA plays a selective role in memory consolidation in a variety of training tasks, such as contextual fear conditioning (LaLumiere et al., 2003) and cued fear conditioning (Schafe and LeDoux, 2000).

Ample evidence indicates that the BLA expresses high densities of cannabinoid CB1 receptors (Campolongo et al., 2009). The endocannabinoid system has an essential role for the modulation of emotional conditions and also reducing the fear-conditioning phenomenon (Kamprath and Wotjak, 2004; Marsicano et al., 2002). Moreover, increased endocannabinoid signaling decreases fear-conditioned response in different behavioral tasks (Bitencourt et al., 2008; Chhatwal et al., 2005; Lin et al., 2009; Pamplona et al., 2008). In a fear conditioning

study, it has been shown that cannabinoidergic agents in the BLA can influence the various phases of learning and memory; For example, WIN 55, 212-2, as a CB1R agonist, reduces fear consolidation in the BLA (Kuhnert et al., 2013).

There are many studies supporting the idea that the endocannabinoid signaling modulates the behavioral responses, at least in part, via the regulation of the serotonin (5-HT) system (Haj-Dahmane and Shen, 2011). It has been suggested that endocannabinoids reduce 5-HT release in the amygdala- known to be specifically involved in mediating fear- via the activation of CB1 receptors (Ashton et al., 2006). The expression of 5-HT₄ receptors in limbic areas, including amygdala, is highly suggestive of a role for these receptors in emotional processes (Waeber et al., 1994). In addition, 5-HT₄ receptor expression changes in diverse brain areas during learning and memory tasks (Manuel-Apolinar et al., 2005). Cumulative evidence indicates that 5-HT₄ receptor agonists modulate synaptic plasticity within the hippocampus and amygdala by augmenting long-term potentiation, attenuating depotentiation and altering patterns of long-term depression (Huang and Kandel, 2007; Kemp and Manahan-Vaughan, 2005). Our previous investigations showed that serotonergic 5-HT₄ receptors are involved in the responses induced by the activation of CB1 cannabinoid receptors (e.g. anxiety-related behaviors and emotional learning and memory formation) in the different brain regions such as ventral hippocampus (Nasehi et al., 2015b), nucleus accumbens (Khodayar et al., 2015) and BLA (Chegini et al., 2014). Considering the critical role of the BLA in emotional memory formation and in view of the fact that there is an interaction between functions of cannabinoid and 5-HT receptors and availability of 5-HT₄ agents, the present study was designed to evaluate the role of serotonin 5-HT₄ receptors of the BLA in mediating ACPA fear memory consolidation impairment. Thus, we studied whether ACPA-induced memory consolidation impairment could be affected by intra-BLA microinjections of serotonin 5-HT₄ receptor agonist and/or antagonist.

2. Results

2.1. *Effect of RS67333 or RS23597 microinjection into the BLA on context- and tone-dependent memory consolidation*

Fig. 1A-D shows the effect of post-training intra-BLA microinjection of RS67333 or RS23597 on context-dependent memory consolidation. One-way ANOVA showed that RS67333 [$F(3, 28) = 21.75, P < 0.001$] but not RS23597 [$F(3, 28) = 2.99, P > 0.05$] impaired context-dependent fear memory consolidation. In addition, one-way ANOVA illustrated that RS67333 produced a significant difference in latency [$F(3, 28) = 35.01, P < 0.001$] and % rearing [$F(3, 28) = 3.71, P < 0.05$] but not % grooming [$F(3, 28) = 1.92, P > 0.05$]. Similar analysis showed that RS23597 induced a significant difference in latency [$F(3, 28) = 8.69, P < 0.001$] and % grooming [$F(3, 28) = 16.69, P < 0.001$] but not % rearing [$F(3, 28) = 0.49, P > 0.05$]. As determined by Tukey's post-hoc comparisons, RS67333 (0.025 and 0.05 $\mu\text{g}/\text{mouse}$) impaired context-dependent memory consolidation and decreased latency to the freezing, while RS23597 (0.025 $\mu\text{g}/\text{mouse}$) or RS23597 (0.025 and 0.05 $\mu\text{g}/\text{mouse}$) increased % grooming or latency to the freezing respectively.

Fig. 1E-H shows the effect of post-training intra-BLA microinjection of RS67333 or RS23597 on tone-dependent memory consolidation. One-way ANOVA indicated that RS67333 [$F(3, 28) = 34.05, P < 0.001$] but not RS23597 [$F(3, 28) = 1.57, P > 0.05$] impaired tone-dependent fear memory consolidation. In addition, one-way ANOVA revealed that RS67333 produced a significant difference in latency [$F(3, 28) = 38.64, P < 0.001$] and % rearing [$F(3, 28) = 5.12, P < 0.01$] but not % grooming [$F(3, 28) = 1.78, P > 0.05$]. Similar analysis showed that RS23597 induced a significant difference in latency [$F(3, 28) = 6.45, P < 0.01$] and % rearing [$F(3, 28) = 5.39, P < 0.01$] but not % grooming [$F(3, 28) = 0.45, P > 0.05$]. As determined by Tukey's post-hoc comparisons, RS67333 (0.025 and 0.05 $\mu\text{g}/\text{mouse}$) impaired

tone-dependent memory consolidation and increased latency to the freezing. This drug at the dose of 0.025 µg/mouse increased % rearing. Moreover, RS23597 (0.025 and 0.05 µg/mouse) increased latency to the freezing.

[Fig.1 somewhere near here]

2.2. Effect of saline, a subthreshold dose of serotonin 5-HT₄ receptor agonist or antagonist microinjection into the BLA on context-dependent memory consolidation impairment induced by ACPA

Fig. 2A-D (left panel) shows the effect of post-training intra-BLA infusion of saline on context-dependent memory consolidation impairment induced by ACPA. One-way ANOVA showed that ACPA [$F(4, 35) = 8.86, P < 0.001$] impaired context-dependent fear memory consolidation. In addition, one-way ANOVA illustrated a significant difference in latency to the freezing [$F(4, 35) = 12.90, P < 0.001$] and % grooming [$F(4, 35) = 38.88, P < 0.001$] but not % rearing [$F(4, 35) = 2.05, P < 0.05$]. As determined by Tukey's post-hoc comparisons, ACPA (0.5 mg/kg, i.p.) impaired or increased context-dependent memory consolidation or latency and % grooming, respectively.

Fig. 2A-D (middle panel) shows the effect of post-training intra-BLA microinjection of RS67333 on context-dependent memory consolidation impairment induced by ACPA. Two-way ANOVA indicated a significant difference between the effects of ACPA alone and those of ACPA plus RS67333 on the latency [intra-groups: $F(4, 70) = 21.42, P < 0.001$; inter-groups: $F(1, 70) = 25.46, P < 0.001$; inter-intra groups interaction: $F(4, 70) = 4.73, P < 0.01$], % grooming [intra-groups: $F(4, 70) = 18.41, P < 0.001$; inter-groups: $F(1, 70) = 1.06, P > 0.05$; inter-intra groups interaction: $F(4, 70) = 35.41, P < 0.001$] and % rearing [intra-groups: $F(4, 70) = 7.02, P < 0.001$; inter-groups: $F(1, 70) = 16.48, P < 0.001$; inter-intra groups interaction: $F(4, 70) = 4.61, P < 0.01$] but not on % freezing [intra-groups: $F(4, 70) = 20.35, P < 0.001$; inter-

groups: $F(1, 70) = 3.63$, $P > 0.05$; inter-intra groups interaction: $F(4, 70) = 1.77$, $P > 0.05$]. Further post hoc analysis illustrated that a subthreshold dose of RS67333 increased (0.01 mg/kg) or decreased (0.5 mg/kg) ACPA response on % grooming compared to their respective groups. Moreover, administration of RS67333 (0.025 μ g/mouse) into the BLA decreased ACPA (0.1 mg/kg) response on %rearing compared to saline/ACPA (0.1 mg/kg) group.

In addition, Fig. 2A-D (right panel) shows the effect of post-training intra-BLA microinjection of RS23597 on context-dependent memory consolidation impairment induced by ACPA. Two-way ANOVA indicated a significant difference between the effects of ACPA alone and those of ACPA plus RS23597 on the % freezing [intra-groups: $F(4, 70) = 10.02$, $P < 0.001$; inter-groups: $F(1, 70) = 5.92$, $P < 0.05$; inter-intra groups interaction: $F(4, 70) = 5.58$, $P < 0.01$], and % grooming [intra-groups: $F(4, 70) = 20.82$, $P < 0.001$; inter-groups: $F(1, 70) = 38.83$, $P < 0.001$; inter-intra groups interaction: $F(4, 70) = 20.12$, $P < 0.001$], but not on latency [intra-groups: $F(4, 70) = 16.30$, $P < 0.001$; inter-groups: $F(1, 70) = 27.33$, $P < 0.001$; inter-intra groups interaction: $F(4, 70) = 1.82$, $P > 0.05$] and % rearing [intra-groups: $F(4, 70) = 7.01$, $P < 0.001$; inter-groups: $F(1, 70) = 8.31$, $P < 0.01$; inter-intra groups interaction: $F(4, 70) = 1.77$, $P > 0.05$]. Further analysis illustrated that a subthreshold dose of RS67333 decreased ACPA (0.5 mg/kg) response on % grooming, while RS23597 increased ACPA (0.01 and 0.1 mg/kg) response on latency to the freezing compared with their respective groups.

[Fig. 2 somewhere near here]

2.3. Effect of saline, a subthreshold dose of serotonin 5-HT₄ receptor agonist or antagonist microinjection into the BLA on tone-dependent memory consolidation impairment induced by ACPA

Fig. 3A-D (left panel) shows the effect of post-training microinjection of saline on tone-dependent memory consolidation impairment induced by ACPA. One-way ANOVA indicated

that ACPA [$F(4, 35) = 12.32, P < 0.001$] impaired tone-dependent fear memory consolidation. In addition, one-way ANOVA revealed a significant difference in % grooming [$F(4, 35) = 4.92, P < 0.01$], % rearing [$F(4, 35) = 13.39, P < 0.001$] but not latency to the freezing [$F(4, 35) = 1.05, P > 0.05$]. As determined by Tukey's post-hoc comparisons, ACPA (0.05, 0.1 and 0.5 mg/kg, i.p.) impaired tone-dependent memory consolidation while the lower or the higher dose of the drug decreased % rearing.

Fig. 3A-D (middle panel) shows the effect of post-training intra-BLA microinjection of RS67333 on tone-dependent memory consolidation impairment induced by ACPA. Two-way ANOVA showed a significant difference between the effects of ACPA alone and those of ACPA plus RS67333 on latency [intra-groups: $F(4, 70) = 9.22, P < 0.001$; inter-groups: $F(1, 70) = 72.59, P < 0.001$; inter-intra groups interaction: $F(4, 70) = 5.20, P < 0.01$] and % rearing [intra-groups: $F(4, 70) = 15.34, P < 0.001$; inter-groups: $F(1, 70) = 30.12, P < 0.001$; inter-intra groups interaction: $F(4, 70) = 5.11, P < 0.01$] but not on % freezing [intra-groups: $F(4, 70) = 16.88, P < 0.001$; inter-groups: $F(1, 70) = 13.17, P < 0.01$; inter-intra groups interaction: $F(4, 70) = 1.23, P > 0.05$], and % grooming [intra-groups: $F(4, 70) = 9.90, P < 0.001$; inter-groups: $F(1, 70) = 2.97, P > 0.05$; inter-intra groups interaction: $F(4, 70) = 0.75, P > 0.05$]. Further post hoc analysis illustrated that a subthreshold dose of RS67333 increased or decreased ACPA response on latency to the freezing or ACPA (0.05 and 0.1 mg/kg) response on % rearing compared with their respective groups.

In addition, Fig. 3A-D (right panel) shows the effect of post-training intra-BLA microinjection of RS23597 on tone-dependent memory consolidation impairment induced by ACPA. Two-way ANOVA showed a significant difference between the effects of ACPA alone and those of ACPA plus RS23597 on the % freezing [intra-groups: $F(4, 70) = 11.64, P < 0.001$; inter-groups: $F(1, 70) = 0.08, P > 0.05$; inter-intra groups interaction: $F(4, 70) = 16.74, P < 0.001$], latency [intra-groups: $F(4, 70) = 8.17, P < 0.001$; inter-groups: $F(1, 70) = 19.00, P < 0.001$; inter-intra groups interaction: $F(4, 70) = 5.12, P < 0.01$] and % rearing [intra-groups: F

(4, 70) = 12.14, $P < 0.001$; inter-groups: $F(1, 70) = 15.86$, $P < 0.001$; inter-intra groups interaction: $F(4, 70) = 4.88$, $P < 0.01$], but not % grooming [intra-groups: $F(4, 70) = 7.93$, $P < 0.001$; inter-groups: $F(1, 70) = 0.42$, $P > 0.05$; inter-intra groups interaction: $F(4, 70) = 1.31$, $P > 0.05$]. Further post hoc analysis illustrated that intra-BLA microinjection of a subthreshold dose of RS23597 decreased or increased ACPA response at doses 0.01 or 0.05 mg/kg, respectively. Moreover, a subthreshold dose of RS67333 increased ACPA (0.1 or 0.5 mg/kg) response on latency to the freezing compared with their respective groups.

[Fig. 3 somewhere near here]

3. Discussion

The objective of the present investigation was to evaluate the role of BLA serotonin 5-HT₄ receptors in fear memory responses induced by the activation of cannabinoid CB₁ receptors in mice. In so doing, we examined (1) freezing behavior as a defensive behavior expressed by rodents in fearful situations, which is defined by immobility in a stereotyped crouching position (Blanchard and Blanchard, 1969; Nasehi et al., 2015a), (2) latency to the freezing which is the delay time to the first freezing behavior (Nasehi et al., 2015a), (3) Self-grooming behavior whose occurrence is influenced by emotional factors and is observed during (and following) exposure to different types of stressful situations/stimuli (Estanislau et al., 2013) and, (4) rearing behavior which is determined by emotionality and also reflects reaction to novelty (Borta and Schwarting, 2005). Since freezing behavior is the main factor to evaluate fear memory, we will discuss this factor more comprehensively. The obtained data revealed that post-training intraperitoneal administration of ACPA, as a cannabinoid CB₁ receptor agonist, produced a marked decrease in freezing time percentage in the contextual and tone fear conditions in comparison to the saline-treated (control) animals. In other words, the drug impaired contextual (hippocampus-dependent) and tone (hippocampus-independent) fear memory consolidations. The higher dose of the drug increased % grooming and latency to the

freezing in the context-dependent memory retention test, while the lower and higher doses of the drug decreased % rearing in the tone-dependent memory retention. It should be considered that in order to exclude the possible influences of the drugs on acquisition or any sensory, motor, or motivational processes, the drugs were injected immediately after training (McGaugh, 1966). There are reports both in agreement and disagreement with our present results. It seems that systemic post-training injection of cannabinoid receptor agonists impair (Mackowiak et al., 2009; Yim et al., 2008), while their antagonists improve (Wise et al., 2008; Wolff and Leander, 2003) memory consolidation. In agreement with our findings, some other studies have indicated that exogenous cannabinoid administration may be associated with impaired functioning in a variety of learning paradigms including spatial (Robinson et al., 2010; Wise et al., 2009), fear conditioning (Pamplona and Takahashi, 2006), and object recognition (Clarke et al., 2008; Yousefi et al., 2013) tasks. It has been suggested that the activation of CB1 receptors disturbs consolidation of fear memory in contextual fear conditioning, possibly by affecting polysialylated neural cell adhesion molecule (PSA-NCAM) expression in the dentate gyrus, which plays a key role in synaptic rearrangement during the formation of memory traces (Mackowiak et al., 2009). There is evidence that the activation of BLA CB1 receptors reduce fear consolidation (Kuhnert et al., 2013). We know that CB1 receptors are highly expressed in brain regions involved in the regulation of memory formation and emotional response such as the hippocampus, amygdala and prefrontal cortex (Herkenham et al., 1991). As a result, the observed impairments followed by systemic administration of ACPA may be induced via activation of CB1 receptors in all brain regions.

Our results illustrated that intra-BLA microinjection of a subthreshold dose of RS67333 did not alter context- and tone-dependent fear memory consolidation impairment induced by ACPA, while sole infusion of the drug into the BLA impaired both fear memories. Moreover, intra-BLA microinjection of the lower dose of RS23597 did not change context-dependent memory consolidation impairment induced by ACPA, while the drug potentiated or reversed

tone-dependent memory deficit induced by ACPA at doses of 0.01 or 0.05 mg/kg respectively. Meanwhile, sole infusion of RS23597 into the BLA produced significant difference in neither fear memories compared with the control group. Two higher doses of the RS23597 increased latency to the freezing at both conditions, while two higher doses of the RS67333 decreased or increased latency in the context or tone fear conditioning respectively. The middle dose of RS23597 increased %grooming in the context-dependent fear conditioning, while the middle dose of RS67333 increased %rearing in the tone-dependent fear conditioning. In the context-dependent fear memory conditioning, subthreshold dose of RS67333 or RS23597 decreased ACPA response on % grooming at the higher dose, while subthreshold dose of RS67333 increased ACPA response on % grooming at the lower dose. Subthreshold dose of RS67333 decreased ACPA response on the % rearing at the dose of 0.1 mg/kg. Finally, subthreshold dose of RS23597 increased ACPA response on latency to the freezing at the doses of 0.01 and 0.1 mg/kg. In the tone-dependent fear memory conditioning, subthreshold dose of RS67333 decreased ACPA response on % rearing at the doses of 0.05 and 0.1 mg/kg. Finally, subthreshold dose of RS67333 or RS23597 increased ACPA response on latency to the freezing at all doses or 0.1 and 0.5 mg/kg respectively. Interestingly, it has been reported that a pre-training injection of BIMU-1 or BIMU-8, mixed 5-HT₄ agonists/5-HT₃ antagonists for investigating the behavioral role of the 5-HT₄ receptors enhanced acquisition in the autoshaping task, but post-training administration of these drugs impaired consolidation (Meneses and Hong, 1997). In addition, post-training administration of the 5-HT₄ receptor antagonist SDZ-205557, and GR-125487 or PCA pretreatment does not appear to affect learning itself, but is able to reverse the effect of the 5-HT₄ receptor agonist. Ample evidence suggests that pre- or post-synaptic activation by 5-HT₄ receptor agonists can increase releases from cholinergic, GABAergic (dentate gyrus), dopaminergic, and serotonergic neurons (Bockaert et al., 2006). An increased serotonin levels followed by conditioned fear stress was reported to induce memorization of conditioned fear (Yokoyama et al., 2005). It is expected that foot shock stress

increases serotonin release. Thus, if RS23597 blocks postsynaptic 5-HT₄ receptors located on GABAergic interneurons in the BLA, serotonin may, via the activation of other receptors, induce a depolarization in GABAergic neurons. As a result, RS23597, at applied doses, can not impair conditioned fear memory. Moreover, the general effect of peripheral administration of ACPA is a reduction in serotonin release. We suggest that the effect of RS23597 via blockade of postsynaptic 5-HT₄ receptors is associated with decreased serotonin level induced by ACPA; the resultant response is an impairment in fear memory consolidation. On the other hand, the previous data indicated that blockade of serotonin signaling can reverse cannabinoid agonist effects and responses following deletion of CB₁ receptors from cortical glutamatergic neurons (Haring et al., 2013). Several studies have shown that cannabinoids induce biphasic modulatory effects in the regulation of various behaviors. It may be suggested that microinjection of RS23597 followed by ACPA at the higher dose desensitizes cannabinoid receptors because of the overdose of cannabinoid concentration level. In this way, RS23597 can restore memory impairment induced by ACPA at the higher dose. In conclusion, the results indicated that the blockade of BLA serotonin 5-HT₄ receptors modulates the ACPA effect on tone fear conditioning.

4. Experimental procedure

4.1. Subjects

We used male NMRI mice (Institute for Cognitive Science Studies; Tehran, Iran) that weighed 28-33 g at the start of the surgery. Animals were housed in a colony, maintained at 22±2°C with a 12-h light/12-h dark cycle (lights on at 07:00 h) and had free access to food and water inside the standard polypropylene cages. The experiments were carried out during the light phase of the cycle. Animal handling was limited to the time of home-cage cleaning (each 48 h), weighing, and drugs administration only.

4.2. Ethical statement

The study was conducted in compliance with the international laws on animal experimentation and approved by the committee of Ethics of Tehran University of Medical Sciences, Tehran, Iran. All procedures were carried out in accordance with the guidelines for animal care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 2010).

4.3. Stereotaxic operations

Double guide cannulae (22 gauge) were implanted under aseptic conditions during deep anesthesia [50 mg/kg ketamine-5 mg/kg xylazine, injected intraperitoneally (i.p.)] as described previously (Chegini et al., 2014). The double guide cannulae were implanted 1 mm above the BLA according to the stereotaxic coordinates; -1.5 mm caudal, ± 2.8 mm mediolateral, and 4.5 mm dorsoventral to the bregma (Paxinos, 2001). The cannulae were anchored to the skull by means of dental cement. The drugs microinjections were made through guide cannulae by stainless steel stylets (27 gauge), 1 mm longer than the guide cannulae.

4.4. Drugs and injections

ACPA, Arachidonylcyclopropylamide (a potent and selective CB1 receptor agonist), RS67333 hydrochloride (a potent and highly selective 5-HT4 partial agonist), and RS23597-190 hydrochloride (a high affinity, selective competitive antagonist 5-HT4 receptors) were purchased from Tocris (Tocris, Cookson Ltd., UK). The drugs were dissolved in sterile 0.9% saline just before the experiments, while ACPA was stored at -20°C as a prepared solution dissolved in anhydrous ethanol at a concentration of 5 mg/ml and then was diluted to the

required volume using sterile 0.9% saline before related experiments. The highest concentration of ethanol contained in a dose was used as the control group.

ACPA (at the volume of 10 ml/kg) was administered intraperitoneally at the doses of 0.01, 0.05, 0.1 and 0.5 mg/kg, while 5-HT₄ receptor agents (at the volume of 0.3 µl/each side) were microinjected into the BLA at the doses of 0.025, 0.25 and 0.05 µg/mouse. The injection cannula was 1 mm longer than the guide cannulae to extend 1 mm beyond the tip of the guide cannulae into the center of the BLA. The injection cannula was attached with a polyethylene tube to a 1 µl Hamilton syringe. The injection cannula was left in place for an additional 60 s to facilitate diffusion of the drugs from the tip of the injection cannula.

4.5. *Fear conditioning experiments*

Fear conditioning was conducted using a protocol developed by Shoji and colleagues (Shoji et al., 2014) for assessing conditioned fear in mice. The fear conditioning apparatus consisted of a square chamber (55 cm × 53 cm × 67 cm) with acoustic walls and a light source, two sound generators, and video camera attached to the ceiling above the apparatus. During training, mice were placed in a square Plexiglas chamber (25 cm × 25 cm × 25 cm) with an electrifiable grid floor which constantly illuminated (24 w) the fear conditioning chamber; the mice were typically allowed to freely explore the chamber for 120 sec. Thereafter, a tone (conditioned stimulus, CS, 4 kHz, 35 dB) is presented for 30 sec, and a foot shock (unconditioned stimulus, US, 1 mA, 50 Hz) is given to the mice during the last 2 sec of the sound. Mice were removed from the fear conditioning box 30 s after shock termination in order to avoid aversive association with the handling procedure. Before entrance of each mouse into the task, the box was thoroughly cleaned using 70% ethanol solution.

In order to test context dependent retention, animals were placed in the same conditioning box 24 h after the first day for a period of 300s without CS and US presentations.

Tone dependent memory test was conducted one hour after the context dependent memory assessment. In this test, mice were placed into another testing chamber with very different properties in texture, bedding and shape, providing a novel context that was unrelated to the conditioning chamber. The procedure was repeated with CS presentation for 3 min. A video camera was used to record the animal behaviors. The observed behaviors were manually recorded as raw data. Freezing, defined as the lack of movement besides respiration and heartbeat, was recorded using a stopwatch. Latency to the freezing, grooming and rearing were scored as percentages of the time during which these behaviors were respectively expressed. Experiments were carried out by someone blinded to the experimental groups.

4.6. *Experimental design*

In each experiment, eight mice were used which were tested once only. ACPA was administered intraperitoneally immediately after training. Other drugs were injected intra-BLA bilaterally (0.3 µl/each side) 5 min after intraperitoneal administration of saline (1 ml/kg) or different doses of ACPA. Control groups received saline in a volume of 0.6 µl/mouse or 10 ml/kg. The test session was performed 24 h after the training; context test was followed by tone test with 1 hour interval.

4.6.1. *Experiment 1: post-training microinjection of RS67333 or RS23597 into the BLA on context- and tone-dependent memory consolidation*

Eight groups of mice received saline (0.6 µl/mouse, two groups), RS67333 (0.0025, 0.025 and 0.05 µg/mouse) or RS23597 (0.0025, 0.025 and 0.05 µg/mouse) intra-BLA, immediately after training. 24 hours after the training, animals were tested for both context- and tone-dependent memory retention.

4.6.2. Experiment 2: post-training microinjection of saline, a subthreshold dose of serotonin 5-HT₄ receptor agonist or antagonist into the BLA on ACPA-induced memory consolidation impairment

15 groups of mice were divided into three sets of five groups and received different doses of ACPA (0, 0.01, 0.05, 0.1 and 0.5 mg/kg, i.p.) plus saline (0.6 µl/mouse), RS67333 (0.0025 µg/mouse) or RS23597 (0.0025 µg/mouse) into the BLA, immediately after training. 24 hours after the training, animals were tested for both context- and tone-dependent memory retention.

4.7. Verification of cannulae placements

After all behavioral testing, animals were overdosed with chloroform. Mice received the intra-BLA injection of ink (0.3 µl/side; 1% solution of methylene blue). The brains were fixated in a 10% formalin solution (for 10 days) in order to determine the location of the cannulae based on Paxinos and Franklin's atlas (Paxinos, 2001). Data from animals with injection sites located outside the regions were not included in the analysis (Fig. 4).

4.8. Statistical analysis

Data were expressed as mean $\pm$ SEM. Analysis of data was performed using one-way and two-way analyses of variance (ANOVA). In combination studies, two-way ANOVA was performed with five levels (0, 0.01, 0.05, 0.1 and 0.5 mg/kg) of ACPA as intra-group factors, and two levels, namely ACPA (0 mg/kg) and RS67333 or RS23597 as inter-group factors. Following a significant F-value, post-hoc analysis (Tukey-test) was performed for assessing specific inter-group differences. In all cases, a $P < 0.05$ was considered statistically significant. Calculations were performed using the SPSS (version 19.0) statistical package.

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Legends

Fig. 1: Effect of post-training microinjection of RS67333 or RS23597 into the BLA on context- or tone-dependent memory consolidation. Eight groups of animals received intra-BLA microinjection of saline (0.6 μ l/mouse), SR67333 (0.0025, 0.025 and 0.05 μ g/mouse), saline (0.6 μ l/mouse) or SR23597 (0.0025, 0.025 and 0.05 μ g/mouse) immediately after training. The test session was performed 24 h after the training; context test was followed by tone test after 1 hour. % freezing (A and E), latency to the freezing (B and F), % grooming (C and G) and % rearing (D and H). Values are expressed as mean $\pm$ S.E.M (n = 8 in each group). **P < 0.01 and ***P < 0.001 different from its saline control group; +P < 0.05, ++P < 0.01 and +++P < 0.001 different from its saline control group.

Fig. 2: Effect of post-training microinjection of saline, a subthreshold dose of serotonin 5-HT₄ receptor agonist or antagonist into the BLA on context-dependent memory consolidation impairment induced by ACPA. 15 groups of animals were divided into three sets of five groups. They received different doses of ACPA (0, 0.01, 0.05, 0.1 and 0.5 mg/kg) plus saline (0.6 μ l/mouse), SR67333 (0.0025 μ g/mouse) or SR23597 (0.0025 μ g/mouse) immediately after training. The test session was performed 24 h after the training; context test was followed by tone test after 1 hour. % freezing (A), latency to the freezing (B), % grooming (C) and % rearing (D). Values are expressed as mean $\pm$ S.E.M (n = 8 in each group). ***P < 0.001 different from saline control group; +++P < 0.001 different from the respective saline/ACPA group and ^{\$}P < 0.05 and ^{\$\$\$}P < 0.001 different from the respective saline/ACPA group.

Fig. 3: Effect of post-training microinjection of saline, a subthreshold dose of serotonin 5-HT₄ receptor agonist or antagonist into the BLA on tone-dependent memory consolidation impairment induced by ACPA. 15 groups of animals were divided into three sets of five groups.

They received different doses of ACPA (0, 0.01, 0.05, 0.1 and 0.5 mg/kg) plus saline (0.6 µl/mouse), SR67333 (0.0025 µg/mouse) or SR23597 (0.0025 µg/mouse) immediately after training. The test session was performed 24 h after the training; context test was followed by tone test after 1 hour. % freezing (A), latency to the freezing (B), % grooming (C) and % rearing (D). Values are expressed as mean $\pm$ S.E.M (n = 8 in each group). *P < 0.05, **P < 0.01 and ***P < 0.001 different from saline control group; +P < 0.05, ++P < 0.1 and +++P < 0.001 different from the respective saline/ACPA group and \$\$P < 0.01 and \$\$\$P < 0.001 different from the respective saline/ACPA group.

Fig. 4: Representative photomicrographs illustrating placement of cannula and needle tip in the basolateral amygdala (BLA). Arrow denotes location of the needle tip and the verified section adapted from the atlas of Paxinos and Franklin (Paxinos, 2001) is also included.

Figure 1

Fig. 1

Figure 2

Fig. 2

Figure 3

Fig. 3

Figure 4

Bregma: -1.58 mm

Fig. 4