Comparison of 5-HT_3 or 5-HT_4 agents function into the prelimbic area on passive avoidance memory consolidation

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ABSTRACT

Growing evidence suggests that serotonin plays an important role in learning and memory and all its receptors might be implicated in this process. The present study aimed at investigating and comparing the possible involvement of 5-HT_3 and 5-HT_4 receptor (R) agonists/antagonists upon consolidation of inhibitory avoidance memory in the pre-limbic (PL) area. Bilateral intra-PL microinjections of m-CPBG (m-Chlorophenylbiguanide hydrochloride: a selective 5-HT_3R agonist; 0.1 μg/rat), Y-25130 (a selective 5-HT_3R antagonist; 0.1 μg/rat), RS67333 (a potent and highly selective 5-HT_4R partial agonist; 0.5 μg/rat) and RS23597-190 (a selective 5-HT_4R antagonist; 0.5 μg/rat) were performed immediately after training. The step-through inhibitory avoidance (IA) task was used to memory assessment in adult male Sprague-Dawley rats. Our data revealed that the post-training intra-PL microinjection of m-CPBG relative to saline and Y-25130 decreased inhibitory avoidance memory consolidation in the PL area. On the contrary, RS67333 increased IA memory consolidation in comparison to saline and RS23597-190. In addition, there was also a significant difference between the effects of m-CPBG and RS67333 on IA memory consolidation in the PL area. M-CPBG induced reduction of IA memory consolidation, while RS67333 increased it. However, Y-25130 compared to RS23597-190 did not show any significant difference. All above interventions did not alter locomotor activity. This study indicated that local direct agonist activation of 5-HT_3Rs induced the reduction of IA memory consolidation, opposed to the local direct agonist activation of 5-HT_4Rs, which mediated enhancement of IA memory consolidation. It can be proposed that 5-HT_3Rs in comparison to 5-HT4Rs may be inversely involved in modulation of IA memory consolidation in the PL.

Keywords: m-Chlorophenylbiguanide hydrochloride (m-CPBG); Y-25130, RS67333; RS23597-190; Pre-limbic area; Inhibitory avoidance memory

INTRODUCTION

The serotonergic system is widely distributed in the central nervous system (CNS) and plays an important role in various cognitive and non-cognitive behavioral and physiological processes including appetite, food intake, pain perception, mood, activity rhythm, sleep, learning and memory, stress, anxiety and behavioral inhibition or emotional states [1, 2]. The role of serotonin (5-HT) in learning and memory has been of great interest in neuroscience as it plays a critical role in mediating short- and long-term memory. Studies have revealed that 5-HT pathways, 5-HT reuptake site/transporter complex and 5-HT receptors (5-HTRs) indicate regional distribution

in regions of the brain that are associated with learning and memory [2-4]. The 5-HT mediates its effects through seven distinct families of 5-HTRs with different functional properties. Except 5-HT_3R as a ligand-gated ion channel, the others belong to the G-protein coupled receptor superfamily [5].

In the CNS, the 5-HT_3Rs are expressed in the spinal cord, medulla oblongata, hindbrain, the forebrain (such as cerebral cortex, hippocampus and amygdala), striatum, nucleus accumbens and substantia nigra [6, 7]. It has been demonstrated that activation and blockade of 5-HT_3Rs may be involved in learning impairment and enhancement, respectively [8, 9]. 5-HT_3R antagonists seem to have procognitive effects and are able to improve memory acquisition in Y-maze and passive avoidance tasks [10] and spatial learning in a two-platform spatial discrimination task [11]. In contrast, 5-HT_3R agonists have opposite effects and cause memory deficits [9, 12]. 5-HT_4Rs belong to G-protein coupled 7-transmembrane domain receptor superfamily [13], coupled with Gs protein subunit [14], which facilitate the release of various neurotransmitters through blockage of potassium channels and subsequent mobilization of calcium ions [15]. Wide and heterogeneous regional distributions of 5-HT_4Rs have been reported in the brain limbic areas, representing the critical role of 5-HT_4Rs in memory function [16-19]. The importance of 5-HT_4Rs in memory processes is further emphasized through various studiesmanifesting that 5-HT_4 agonists improve learning and memory, either alone [20-23] or in combination with the other pharmacological agents [20]. Based on the studies, it seems that these receptors mediate various responses on different stages of memory formation dependent on the method of injection and memory assessment [9, 24, 25]. Presence of 5-HT_3R [26] and 5-HT_4R [27] has been reported in the pre-limbic (PL) area. PL cortex is a subdivision of medial prefrontal cortex (mPFC) [28], serving as an important role in modulation of aversive memory [29]. So, this study aimed at comparing the possible role of intra-PL micro-infusions of 5-HT_3 and 5-HT_4 receptor agonists/antagonists immediately after training, upon consolidation of aversive memory, using the inhibitory (passive) avoidance task.

MATERIALS AND METHODS

Animals

In this study, adult male Sprague-Dawley rats (250-290 g, purchased from the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences, Shiraz, Iran) were used. Animals (four rats/cage) were maintained in the animal room under a 12/12-h light/dark cycle (lights on 07:00 hours) and controlled temperature of 22±2 °C. Animals had free access to food and water and were adapted to the laboratory conditions for at least one week prior to the surgery. Animal handling was limited to the time of home cage cleaning (each 48 h), weighing, and drugs administration only. The experiments were conducted between 9:00 am and 14:00 pm. Each experimental group included eight animals and each animal was used once only. The experiments were done in accordance with the guide for the care and use of laboratory animals (N.R.C., 2011) [30].

Stereotaxic surgery

The animals were anesthetized by intra-peritoneal injection of ketamine hydrochloride 10% (70 mg/kg) plus xylazine 2% (7 mg/kg), and positioned in a stereotaxic frame (Stoelting Co., Wood Dale, Illinois, USA). Then, using the rat brain atlas by Paxinos and Watson [31] and to aim the PL cortex (AP = +3.4 mm, ML = ±0.9 mm, and DV = −3 mm; Figure), two stainless steel guide-cannulas (22-gauge) with a length of 10.0 mm were bilaterally implanted and fixed to the skull bone. Animals were allowed to recover from the surgery for one week.

Behavioral Tests

Inhibitory (passive) avoidance task / measurement of memory

The step-through inhibitory avoidance (IA) task was performed as previously described [32, 33]. In this task, animals learn to avoid and suppress their natural tendency to prefer specific places because of an aversive experience. A learning box consisted of two compartments separated by a sliding door (7 cm × 9 cm). A light compartment (20 cm × 20 cm × 30 cm) was made of white opaque resin, and a dark compartment (20 cm × 20 cm × 30 cm) was made of black opaque resin. Stainless steel grids were placed on

the dark compartment floor to deliver a foot shock. Intermittent mild electric shocks (Intensity =0.7 mA, Frequency=50 Hz, Duration=3 s) were delivered to the grid floor by an isolated stimulator (Borj Sanat Co., Tehran, Iran).

All animals were allowed to habituate to the experimental room, at least half an hour prior to experiments. Then, animals were individually placed in the bright compartment and allowed to explore for 15 s. Then, the sliding door was opened and once the animal crossed with all four paws to the dark compartment, the sliding door was closed and the latency to enter the dark compartment was recorded and the animal immediately was returned into its home cage. Animals that waited for more than 120 s to cross to the dark compartment were excluded. After 30 min during PA training, the habituation trial was repeated for each animal except that an unavoidable foot shock was immediately delivered from the grid floor, as soon as the animal crossed to the dark compartment. After 15 s, the animal was removed from the apparatus and retested 2 min later in the same way as in the previous trials. If the rat did not enter the dark compartment within 120 s, a successful learning (IA response) was recorded; otherwise, the animal received the shock again, as soon as it crossed to the dark compartment. Each animal was allowed to train up to three times. Animals that could not acquire successful learning were excluded. After successful acquisition, the animal immediately received post-training injection of the drugs. After 24 h, during retention session, the animal was gently placed in the light compartment once more, and the step-through latency or latency to enter the dark compartment (with a cut off time of 300s) was recorded which was considered as an index of the IA memory consolidation. Post-training drug administration used in order to assess the impact of drugs on emotional memory consolidation.

Locomotion test

Locomotor activity was assessed by an animex activity meter apparatus (Type DS, Farad electronics, Sweden), immediately after the retention trial session. Animals were individually placed on the measurement platform, and allowed to freely explore for a duration of 5 min; the number of animal movements was automatically recorded.

Drugs

The drugs used in this study included: m-Chlorophenylbiguanide hydrochloride (m-CPBG; a prototypical potent and selective 5-HT_3 serotonin receptor agonist, 0.1 µg/rat), Y-25130 hydrochloride (a selective and potent 5-HT_3 serotonin receptor antagonist, 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 5-HT_4 partial agonist, 0.5 µg/rat) and RS 23597-190 hydrochloride (3-(piperidin-1-yl) propyl 4-amino-5-chloro-2-methoxybenzoate hydrochloride; a high affinity, selective competitive antagonist at 5-HT_4Rs, 0.5 µg/rat). They all were obtained from Tocris (Tocris Bioscience United Kingdom). Ketamine hydrochloride / xylazine (Alfasan Chemical Co, Woerden, Holland) was used for inducing anesthesia. The infusion time and doses of the drugs used in this study were chosen according to the published work in scientific literature [32] and unpublished studies at our laboratory.

Intra-PL microinjection procedures

For a bilateral infusion of the drugs into the PL area, the animals were restrained gently by hand; the stylets were removed from the guide cannulae and substituted by infusion needles (27-gauge). The injection needles were advanced until their tips reached 1 mm below the tip of the guide cannulae. Then, solutions were manually injected in a total volume of 0.6 µl/rat (0.3 µl in each side) over a 60 s period, using a 2-µl Hamilton glass syringe. A polyethylene tube was interposed between the upper end of dental needles and the micro-syringes. The displacement of an air bubble inside the polyethylene tube was used to monitor the drug flow. To facilitate the diffusion of the drugs and to allow proper infusion, needles were removed 60 s after the completion of the injection [25, 34].

Drug treatments

Animals were randomly assigned to five groups (n=8). One group received saline (0.6 µl/rat) and the other four groups received m-CPBG (0.5 µg/rat), Y-25130 (0.5 µg/rat), RS67333 (0.5 µg/rat) or RS23597-190 (0.5 µg/rat). The drugs were dissolved in 0.9% sterile saline. Bilateral intra-PL microinjection of the drugs in a volume of 0.6µl/rat (0.3µl/side) was performed immediately after a training session. 24 hours following the drug administration, behavioral tests (passive avoidance & locomotor activity) were performed in all groups.

Histological study

To verify the correct cannulae placements according to the atlas of Paxinos and Watson [31], 1% methylene blue dye solution was injected (0.3-µl/side), followed by coronal sectioning on the vibroslice after the completion of the experiments. If the cannulae placement was outside the PL region, the data of the rat was excluded from the analysis.

Statistical analysis

The normal distribution of data was assessed by the Kolmogorov– Smirnov test. One-way ANOVA and Tukey's post-hoc tests were used for data analysis. To compare the effects of 5-HT3R and 5-HT4R agonists/antagonists on IA memory consolidation in the PL area, first the percentage change of control for each rat (treatment/mean control×100) was calculated and then the difference in mean of percentage change of control between groups were analyzed by one way ANOVA. Tukey's post-hoc analysis, following a significant F value, was performed to evaluate the paired-group comparisons. SPSS software ver. 16 was used for all statistical analysis and $P < 0.05$ was considered as a significant level.

RESULTS

Effect of post-training intra-PL administration of 5-HT3 and 5-HT4 receptor agonists / antagonists on the inhibitory avoidance memory consolidation

One-way ANOVA demonstrated that post-training intra-PL administration of the drugs significantly altered memory consolidation [$F(4, 35) = 20.262$, $P < 0.0005$, (Fig. 1A)] but did not affect locomotor activity [$F(4, 35) = 0.637$, $P = 0.639$, (Fig. 1B)]. Moreover, tukey post hoc analysis showed that sole intra-PL microinjection of m-CPBG impaired IA memory consolidation, whereas RS67333 increased it, compared with the saline control group. In addition, mere Y-25130 and RS23597-190 did not affect the IA memory consolidation. Moreover, a comparison between the two receptors in one-way ANOVA demonstrated that post-training intra-PL administration of the drugs significantly altered memory consolidation [$F(3,28) = 30.657$, $P < 0.0005$, (Fig. 2A)] but did not affect locomotor activity [$F(3,28) = 0.100$, $P = 0.959$, (Fig. 2B)]. Tukey's post hoc analysis showed that 5-HT_3R agonist (m-CPBG) relative to 5-HT_3R antagonist (Y-25130) decreased IA memory consolidation in the PL area. Moreover, 5-HT_4R agonist (RS67333) significantly increased IA memory consolidation in comparison with 5-HT_4R antagonist (RS23597-190). Tukey's post hoc analysis also demonstrated that there was also a significant difference between the effects of m-CPBG and RS67333 on IA memory consolidation in the PL area. In addition, Y-25130 compared to RS23597-190 did not show any significant difference. In summary, the results indicated that 5-HT3R agonist acts opposed to the 5-HT_4R agonist on IA memory consolidation in the PL area and reduces memory consolidation.

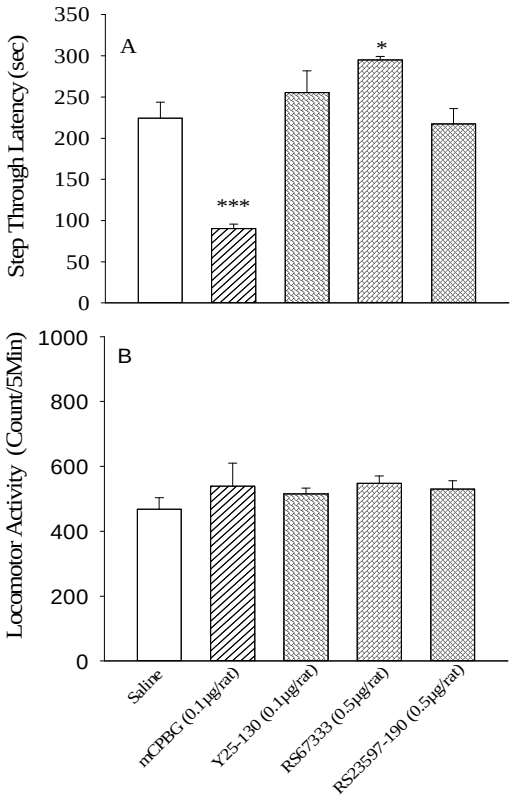

Figure.1. Effects of the post-training intra-PL microinjections of m-CPBG, Y-25130, RS67333 and RS23597-190 on IA memory consolidation (A) and locomotor activity (B) in rats. Five groups of animals were used. one group received saline (0.6 µl/rat) and the other Four groups received m-CPBG (0.1 µg/rat) or Y-25130 (0.1 µg/rat) or RS67333 (0.5 µg/rat) or RS23597-190 (0.5 µg/rat) immediately after training. Step through latency and locomotor activity were examined in all groups, after 24h. Each bar represents mean ± SEM. ***P<0.001 and +P<0.05, different from the saline control group.

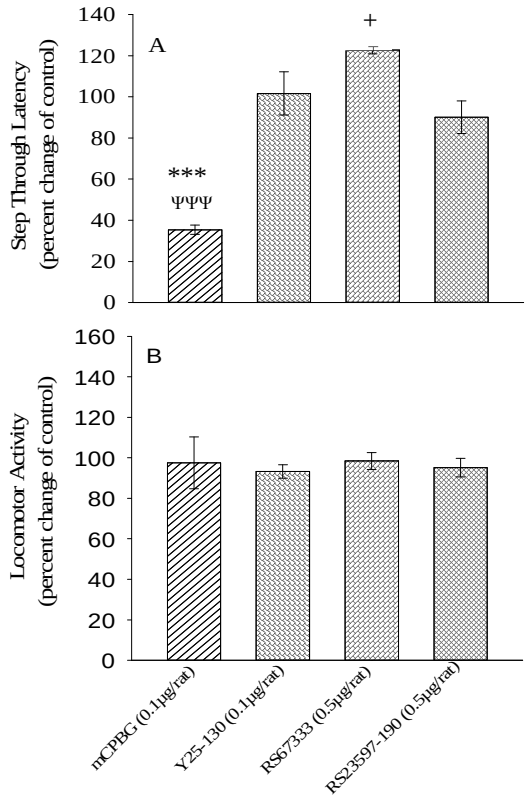

Figure.2. Comparison of 5-HT_3 or 5-HT_4 agonists and antagonists function on IA memory consolidation (A) and locomotor activity (B) in rats
Bars represent mean of percent change of control ± S.E.M. ***P<0.001, different from Y-25130 treatment group. +P<0.05, different from RS623597-190 treatment group. ΨΨΨP<0.001, different from RS67333 treatment group.

DISCUSSION

Our results revealed that there was a significant difference between the effects of the 5-HT_3 and 5-HT_4 receptor agonists on the inhibitory avoidance memory consolidation in the PL area, while their antagonists did not have significant difference. The present results indicated that m-CPBG (5-HT_3R agonist) in comparison to saline, Y-25130 (5-HT_3R antagonist) and RS67333 (5-HT4R agonist) reduced IA memory consolidation in the PL cortex. In this regard, in previously published work we had shown that the post training intra-PL microinjection of mCPBG at a dose of 0.1 µg/rat decreased IA memory consolidation [32], whereas Y-25130 did not alter memory consolidation. Also, consistent with these results, it has been reported that administration of m-CPBG impaired learning and memory process and induced amnesia in the passive avoidance [4, 35], autoshaping [9] and associative learning [36] tasks.

The present results also indicated that local application of 5-HT_4 receptor selective agonist (RS67333) in the PL area in comparison to saline and its selective antagonist (RS23597-190) increased IA memory consolidation. In line with our results, it has been reported that intraperitoneal infusions of RS67333 increases place and object recognition memory consolidation in a two-trial recognition test [20] and enhance both short and long term memory in the autoshaping learning task [9]. Likewise, Intra-nucleus accumbens (NAc) shell administration of RS67333 also increased memory consolidation in the elevated plus-maze (EPM) test [37] and its antagonist, RS23597-190, decreased memory consolidation. It has been shown that local intra-nucleus basalis magnocellularis (NBM) administration of RS67333 induced enhancement of the acquisition and the consolidation of the place recognition memory in a place recognition task, but the recall of memory was not affected by the 5-HT_4 agonist [24]. Although, much evidence has supported the facilitating role of RS67333 in memory processing, some studies report that RS67333 impairs context or tone fear memory consolidation in a fear conditioning apparatus [38], reduces memory acquisition in a single-trial step-down passive avoidance task [25] and induces emotional memory impairment in the EPM test-retest paradigm [39]. It seems that activation of 5-HT_4Rs exerts varying effects at different stages (acquisition, consolidation and retrieval) of memory formation, depending on the type of the drugs used, the site of administration (central vs. systemic), timing of the drug administration, and memory assessment.

Notably, the present results probably illustrate the existence of an inverse relationship between the effects of 5-HT_3 and 5-HT_4Rs on memory consolidation in the PL. It appears that different mechanisms are involved in the incidence of these differences. Evidence from invertebrates to human studies demonstrates that the serotonergic system involved in modulation of short and long term memory function and the opposite findings has also been reported [9]. Linkage to second messengers and anatomical, cellular and subcellular locations of receptors are at least two criteria that need to be considered in defining the role of a receptor in the memory process. It has been demonstrated that the 5-HTRs are either positively or negatively coupled to adenylate cyclase (AC) enzyme and influence cyclic adenosine monophosphate (cAMP) production. cAMP initiates activation of other molecules implicated in memory process by facilitation or the blockade of protein synthesis [2]. So, it is possible that the effects of 5-HT_4Rs in the enhancement of IA memory consolidation in the PL cortex are due to the followings:

1. 5-HT_4Rs is predominantly localized into limbic structures linked to memory and cognition. The 5-HT_4Rs expression has been reported in ~60% of pyramidal neurons of the prefrontal cortex (PFC) [40]. Lucas et. al. also demonstrated the presence of 5-HT_4Rs in the PL sub-region of mPFC, by in situ hybridization histochemistry [41]. 2. 5-HT_4Rs are linked to Gs proteins and their activation induced cAMP production [42].

3. 5-HT_4Rs mediated various neurotransmitter release (dopamine, serotonin, GABA and acetylcholine) and facilitate synaptic transmission which may influence memory development [43, 44].On the other hand, it is thought that the effects of 5-HT_3Rs on memory consolidation, at least in part, is also related to their structural and functional characteristics. The 5-HT_3R, as a ionotropic ion channel, is primarily present on presynaptic nerve terminals [45, 46], and is permeable to Na^+, K^+ and Ca^{++} ions [47]. Its selective agonist activation by conducting cations, most notably Ca^{2+}, leads to an excitatory response in neurons and is involved in mediation or modulation of other neurotransmitter release [1]. In non-primate mammals, the 5-HT_3Rs are expressed in the limbic areas in particular cingulate, prelimbic and infralimbic areas [1, 7]. Interestingly, the vast majority of 5-HT_3R-expressing cells in the prelimbic cortex are GABAergic interneurons, as assessed by in situ hybridization [26, 48]. It seems that local direct agonist activation of 5-HT_3Rs, which probably is located on inhibitory GABAergic interneurons, via the initiation of inhibitory postsynaptic potentials (IPSPs) in cortical pyramidal neurons [1, 49], negatively participate in the modulation of memory consolidation in the PL subregion of medial prefrontal cortex. However, further experiments are required to clarify the exact mechanisms involved. In conclusion, our results

revealed that local direct agonist activation of 5-HT_3Rs induced the reduction of IA memory consolidation, while local direct agonist activation of 5-HT_4Rs mediates the enhancement of IA memory consolidation. In summary, it can be proposed that 5-HT_3Rs in comparison to 5-HT_4Rs may be inversely involved in modulation of inhibitory avoidance memory consolidation in the pre-limbic area.

"The authors declare no conflict of interest"

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