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Modulation of the consolidation and reconsolidation of fear memory by three different serotonin receptors in hippocampus

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ABSTRACT

The process of memory formation is complex and highly dynamic. During learning, the newly acquired information is found in a fragile and labile state. Through a process known as consolidation, which requires specific mechanisms such as protein synthesis, the memory trace is stored and stabilized. It is known that when a consolidated memory is recalled, it again becomes labile and sensitive to disruption. To be maintained, this memory must undergo an additional process of restabilization called reconsolidation, which requires another phase of protein synthesis. Memory consolidation has been studied for more than a century, while the molecular mechanisms underlying the memory reconsolidation are starting to be elucidated. For this, is essential compare the participation of important neurotransmitters and its receptors in both processes in brain regions that play a central role in the fear response learning. With focus on serotonin (5-HT), a well characterized neurotransmitter that has been strongly implicated in learning and memory, we investigated, in the CA1 region of the dorsal hippocampus, whether the latest discovered serotonergic receptors, 5-HT_{5A}, 5-HT_6 and 5-HT_7, are involved in the consolidation and reconsolidation of contextual fear conditioning (CFC) memory. For this, male rats with cannulae implanted in the CA1 region received immediately after the training or reactivation session, or 3 h post-reactivation of the CFC, infusions of agonists or antagonists of the 5-HT_{5A}, 5-HT_6 and 5-HT_7 receptors. After 24 h, animals were subjected to a 3-min retention test. The results indicated that in the CA1 region of the hippocampus the 5-HT_{5A}, 5-HT_6 and 5-HT_7 serotonin receptors participate in the reconsolidation of the CFC memory 3 h post-reactivation. Additionally, the results suggest that the 5-HT_6 and 5-HT_7 receptors also participate in the consolidation of the CFC memory.

1. Introduction

Recently acquired memories are labile; through posttraining consolidation (McGaugh, 2000), a process that requires protein synthesis and takes place mostly but not exclusively in the hippocampus (Izquierdo, Furini, & Myskiw, 2016; Izquierdo & Medina, 1997), memory traces are stored and stabilized in the course of 2–6 h. While this happens, cognition relies on parallel short-term memory systems (Izquierdo et al., 1998). A variety of processes in several brain regions over the next many hours or days sustain the trace from then on, which are called systems consolidation (Izquierdo et al., 2016) and may include what some call memory persistence (Bekinschtein et al., 2008).

In the first few hours or days after posttraining consolidation is over, when the conditioned stimuli (CS) is reiterated, memories may be recalled and become again susceptible to disruption and/or to change (Balderas, Moreno-Castilla, & Bermudez-Rattoni, 2013; de Carvalho Myskiw, Furini, Schmidt, Ferreira, & Izquierdo, 2015; Rodriguez-Ortiz, Balderas, Garcia-DeLaTorre, & Bermudez-Rattoni, 2012; Santoyo-Zedillo, Rodriguez-Ortiz, Chavez-Marchetta, Bermudez-Rattoni, & Balderas, 2014). In order to be maintained the recalled memory requires another process of restabilization called reconsolidation, which also requires a peak of protein synthesis in the hippocampus and basolateral amygdala (Alberini, 2011; Alberini, Milekic, & Tronel, 2006; Bucherelli, Baldi, Mariottini, Passani, & Blandina, 2006). It may or may not be related to systems consolidation (Einarsson, Pors, & Nader, 2015; Izquierdo et al., 2016). Like extinction (de Carvalho Myskiw et al., 2015),

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reconsolidation depends on the sheer unreinforced reiteration of the CS (Balderas et al., 2013; Rodriguez-Ortiz et al., 2012; Santoyo-Zedillo et al., 2014), not as was originally believed (Nader, 2015; Nader, Schafe, & Le Doux, 2000), on retrieval (Izquierdo et al., 2016).

The earlier studies on reconsolidation proposed that it simply recapitulates consolidation (Nader et al., 2000; Sara, 2000). This has been suggested to indeed be the case in a simple *Aplysia* model (Lee et al., 2012), but in higher animals it was repeatedly shown that both processes require different arrays of brain structures and substrates (Alberini, 2005; Bucherelli et al., 2006; Tronson & Taylor, 2007). So far, reconsolidation has been most studied in fear memories (Izquierdo et al., 2016), where it may have clinical relevance for the treatment of many mental disorders that involved dysfunctional memories (Baldi & Bucherelli, 2015; Schwabe, Nader, & Pruessner, 2014).

Participation of the main modulatory neurotransmitters of the brain [norepinephrine (NA), dopamine (DA), histamine (HIS), acetylcholine (ACh) and, to an extent, serotonin (5-HT)] in most aspects of memory is well documented (Izquierdo et al., 2016), except for reconsolidation, where the data are scanty and scattered. There is a consensus that reconsolidation depends on β-noradrenergic receptors (Bos, Beckers, & Kindt, 2014; Daniel, Kioko, & Federico, 2016; Villain et al., 2016; Zaichenko, Markevich, & Grigoryan, 2016) probably in the hippocampus and amygdala; one recent experiment suggests a parallel reliance on α2 receptors (Gazarini, Stern, Piornedo, Takahashi, & Bertoglio, 2014). Intra-hippocampal infusion of the α7-nicotinic receptor agonist choline enhances reconsolidation (Blake, Boccia, Krawczyk, & Baratti, 2013), whereas that of the antagonist methyllycaconitine inhibits it (Blake, Krawczyk, Baratti, & Boccia, 2014). Dependence on hippocampal dopamine D1 and D2 receptors (Daniel et al., 2016; Merlo et al., 2015) has also been suggested. There have also been suggestions that reconsolidation might depend on serotonin 5-HT_{1A} receptors (Ogren et al., 2008).

Here we focus on whether the more recently described serotoninergic receptors, 5-HT_{5A}, 5-HT_6 and 5-HT_7, are involved in the consolidation and reconsolidation of contextual fear conditioning memory, in the CA1 region of the dorsal hippocampus.

2. Materials and methods

2.1. Animals

Male *Wistar* rats (CrlCembe:WI; 3 months old, 300–330 g) purchased from Centro de Modelos Biologicos Experimentais of the Pontifical Catholic University of Rio Grande do Sul (our regular provider) were used. Animals were housed four to a cage and kept with free access to food and water, under a 12-h light/dark cycle (lights on at 7:00 a.m.). The temperature of the animals' room was maintained at 22–24 °C. All procedures were approved by the Animal Committee on Ethics in the Care and Use of Laboratory Animals of the Pontifical Catholic University of Rio Grande do Sul, in compliance with National Institutes of Health guidelines for the care and use of laboratory animals.

2.2. Surgery

Animals were anesthetized with intraperitoneal injections of ketamine (75 mg/kg) and xylazine (10 mg/kg) and a 22-gauge guide cannulae were bilaterally implanted, 1 mm above of the dorsal CA1 area of the hippocampus (anterior, −4.2 mm; lateral, ±3.0 mm; ventral, −1.8 mm) (Paxinos & Watson, 1986). Cannulae were fixed to the skull with dental cement. Animals were allowed 7 days to recover from surgery prior to behavioral procedures. Animals were handled once daily for 3 consecutive days and all behavioral procedures were conducted between 8:00 and 11:00 a.m.

2.3. Contextual fear conditioning apparatus

Contextual fear conditioning (CFC) was performed in a conditioning chamber with aluminium walls (35 × 35 × 35 cm) and a clear front lid (Panlab®, Barcelona, Spain). The floor of the chamber was made of parallel stainless-steel grid bars and connected to a shock source for the delivery of foot shock. The conditioning chamber was placed inside a sound-attenuating box with a ventilating fan. The chamber was cleaned with 70% ethanol before and after each use. The percentage of the time that the animals spent freezing in the apparatus was measured automatically by a counter connected to photocells. Freezing behavior (no visible movement except for respiration) was used as an index of fear learning (de Carvalho Myskiw, Benetti, & Izquierdo, 2013; Fiorenza, Rosa, Izquierdo, & Myskiw, 2012).

2.4. Behavioral procedures

2.4.1. Consolidation of contextual fear conditioning

On the training day, animals were placed in the conditioning chamber and after 2 min three electrical foot shocks (0.5 mA, 2 s) were delivered at a 30 s interval. Animals were removed from the conditioning chamber 30 s after the last foot shock and placed back in their home cages. After 24 h, animals were placed in the same apparatus for a 3-min retention test, with no foot shocks (Fiorenza et al., 2012).

2.4.2. Reconsolidation of contextual fear conditioning

The training session of contextual fear conditioning was performed as described above, and after 24 h animals were placed in the same conditioning chamber for a 3-min reactivation session of CFC, in the absence of the foot shocks. After 24 h, animals were placed again in the same apparatus for a 3-min retention test, again with no foot shocks.

2.5. Pharmacological treatments

Microinjections into the CA1 region of the hippocampus were carried out less than 1 min after the contextual fear conditioning training or either 1 min or 3 h after the contextual fear conditioning reactivation session. Animals were gently restrained by hand and the injection needle (30 gauge) was fitted tightly into the guide, extending 1 mm from the tip of the guide cannula. The injection needle was connected to a 10 μl Hamilton microsyringe and the infusions were performed at a rate of 1.0 μl/60 s. The microinfusion volume used was 1.0 μl per side into the CA1 region of the dorsal hippocampus. At the end of the microinfusion, the injection needle was left in place for an additional 60 s to minimize backflow, then carefully withdrawn and placed on the other side, and the procedure repeated.

The drugs and the doses used were the protein synthesis inhibitor anisomycin, 80 μg/side (de Carvalho Myskiw et al., 2013; Furini et al., 2015); the 5-HT_{5A} receptor antagonist, SB-699551, 10 μg/side (Xu, Zhao, Huo, Du, & Tang, 2013); the 5-HT_6 receptor antagonist, SB-271046, 10 μg/side (Loiseau, Dekeyne, & Millan, 2008); the 5-HT_6 receptor agonist, WAY-208466, 0.04 μg/side (Loiseau et al., 2008); the 5-HT_7 receptor antagonist, SB-269970, 5 μg/side (Liy-Salmeron & Meneses, 2007) and; the 5-HT_7 receptor agonist, AS-19, 5 μg/side (Eriksson et al., 2012), all purchased from Sigma-Aldrich or Tocris. All drugs were freshly dissolved in dimethyl sulfoxide 10% or in sterile saline 0.9% (Veh).

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2.6. Cannulae placements

Correct cannulae placements were verified as follows. Two to four days after the end of the last behavioral procedure, animals were infused with a 4% methylene blue solution over 30 s into the CA1 region of the dorsal hippocampus (1.0 µl/side) at the coordinates mentioned above. Thirty min later, animals were sacrificed by excess anesthesia and the brains were removed and kept in 10% formalin. The extension of the spread of the dye was considered to represent an estimate of the amount of the drug infused. Cannula placement was considered correct when the spread was $\leqslant$1 mm from the intended infusion site; this occurred in 98% of the animals (de Carvalho Myskiw et al., 2013).

2.7. Statistical analysis

Data are presented as means ± standard errors, and were analyzed statistically by one-way ANOVA followed by Newman-Keuls Test using Graphpad Prism® software. P values less than 0.05 were considered statistically significant.

3. Results

Initially, with the purpose of verify the existence of reconsolidation process in our protocol, animals were trained in CFC, and 24 h later subjected to a 3-min reactivation session. Immediately or 3 h post-reactivation animals received bilateral intra-CA1 infusions of the Veh or protein synthesis inhibitor, anisomycin (80 µg/side). The anisomycin-treated animals showed, immediately (Fig. 1A) or 3 h (Fig. 1B) post-reactivation, an impairment of CFC memory, a pattern previously reported, that indicates the existence of reconsolidation (Debiec, LeDoux, & Nader, 2002; Nader et al., 2000).

Once the protocol to study reconsolidation was established, in order to evaluate the participation of the serotonergic receptors in the consolidation and reconsolidation of CFC memory, animals received after CFC training or after reactivation session, bilateral intra-CA1 infusions of the Veh, antagonists or agonists of the 5-HT_{5A}, 5-HT_6 and 5-HT_7 receptors. Twenty-four hours later, animals were subjected to a 3-min retention test (de Carvalho Myskiw et al., 2013; Fiorenza et al., 2012).

3.1. Participation of the 5-HT_{5A} receptors in the consolidation and reconsolidation of the contextual fear conditioning memory

As shown in Fig. 2, animals that received the antagonist of the 5-HT_{5A} receptors, SB-699551 (10 µg/side), into the CA1 region of the hippocampus immediately after the CFC training, exhibited similar levels of freezing than the Veh group during the retention test. Likewise, animals that received infusions of veh or SB-699551 (10 µg/side) into the CA1 region of the hippocampus immediately after reactivation (Fig. 3A) exhibited similar levels of freezing during the reactivation session and retention test. In contrast, those that received SB-699551 (10 µg/side) intra-CA1 3 h after the reactivation exhibited lower levels of freezing during the retention test when compared with the Veh group (Fig. 3B).

These results indicate that the antagonist of the 5-HT_{5A} receptors does not modulate the consolidation of CFC memory in the CA1 region of the dorsal hippocampus, but does modulate the reconsolidation of the CFC memory 3 h post-reactivation, impairing the original memory.

3.2. Participation of the 5-HT_6 receptors in the consolidation and reconsolidation of the contextual fear conditioning memory

As demonstrated in Fig. 4, animals that received the agonist of the 5-HT_6 receptors, WAY-208466 (0.04 µg/side), intra-CA1 immediately after the CFC training exhibited similar levels of freezing as the Veh group during the retention test. In turn, animals that

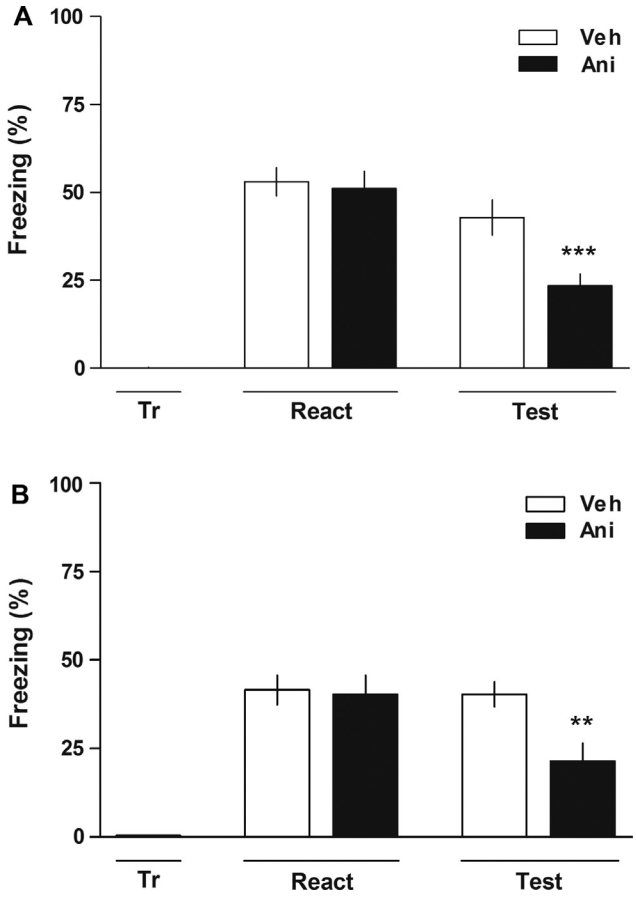

Fig. 1. Effect of the protein synthesis inhibitor, anisomycin, in the CA1 region of the hippocampus on the reconsolidation of contextual fear conditioning. Immediately (A) or 3 h (B) post-reactivation of contextual fear conditioning, animals received bilateral intra-CA1 infusions of Veh or anisomycin (Ani; 80 µg/side) and 24 h later they were subjected to a 3-min retention test. Data are presented as mean ± SEM of the percentage of time spent freezing. **p < 0.01 and ***p < 0.001 for Veh vs. Ani on the retention test. Newman-Keuls test after one-way ANOVA; n = 11–12 animals per group.

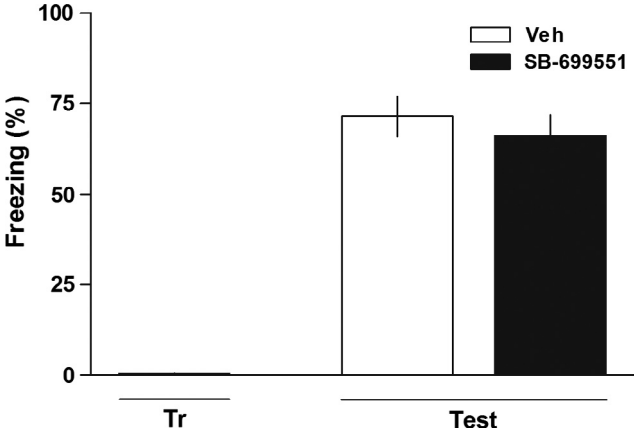

Fig. 2. Participation of the 5-HT_{5A} receptors in the CA1 region of the hippocampus on the consolidation of contextual fear conditioning. Immediately after the contextual fear conditioning, animals received bilateral intra-CA1 infusions of Veh or SB-699551 (10 µg/side) and 24 h later they were subjected to a 3-min retention test. Data are presented as mean ± SEM of the percentage of time spent freezing. Newman-Keuls test after one-way ANOVA; n = 11–12 animals per group.

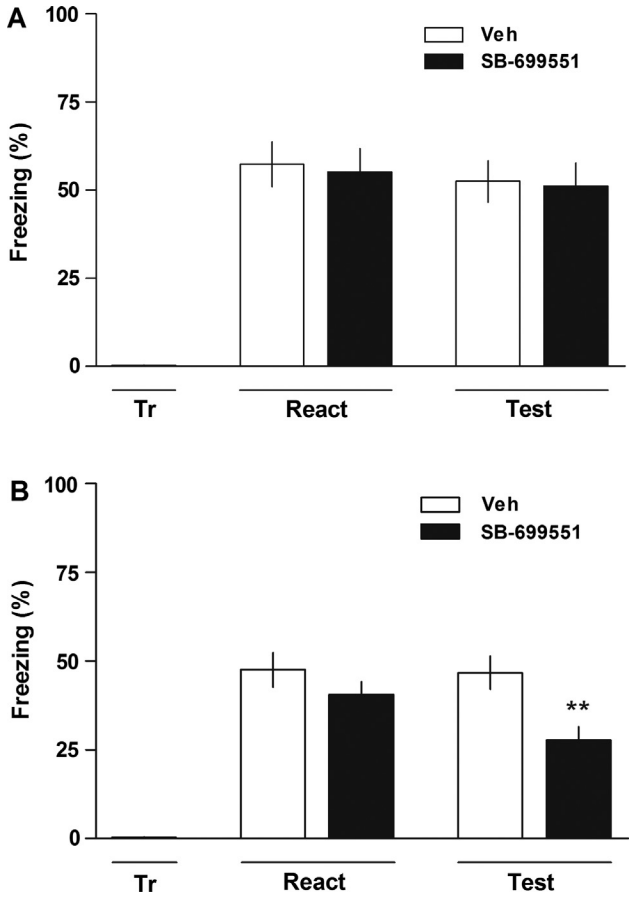

Fig. 3. Participation of the 5-HT_{5A} receptors in the CA1 region of the hippocampus on the reconsolidation of contextual fear conditioning. Immediately (A) or 3 h (B) post-reactivation of the contextual fear conditioning, animals received bilateral intra-CA1 infusions of Veh or SB-699551 (10 μg/side) and 24 h later they were subjected to a 3-min retention test. Data are presented as mean ± SEM of the percentage of time spent freezing. $^{**}p < 0.01$ for SB-699551 vs. Veh group on the retention test. Newman-Keuls test after one-way ANOVA; n = 11–12 animals per group.

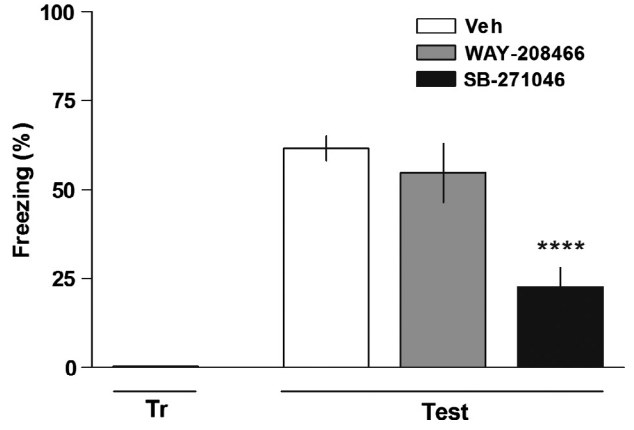

Fig. 4. Participation of the 5-HT_6 receptors in the CA1 region of the hippocampus on the consolidation of contextual fear conditioning. Immediately after the contextual fear conditioning, animals received bilateral intra-CA1 infusions of Veh, WAY-208466 (0.04 μg/side) or SB-271046 (10 μg/side) and 24 h later they were subjected to a 3-min retention test. Data are presented as mean ± SEM of the percentage of time spent freezing. **** $p < 0.0001$ for SB-271046 vs. Veh group. Newman-Keuls test after one-way ANOVA; n = 11–12 animals per group.

received the antagonist of the 5-HT_6 receptors, SB-271046 (10 μg/side), into the CA1 region of the hippocampus, after the CFC training exhibited lower levels of freezing during the retention test when compared with the animals that received Veh. These results suggest that the antagonist of the 5-HT_6 receptors, SB-271046, impairs the consolidation of CFC memory in the CA1 region of the dorsal hippocampus.

Animals that received WAY-208466 (0.04 μg/side) or SB-271046 (10 μg/side) into the CA1 region of the hippocampus, immediately post-reactivation (Fig. 5A), exhibited similar levels of freezing in both reactivation session and retention test. On the other hand, animals that received WAY-208466 (0.04 μg/side) 3 h post-reactivation (Fig. 5B) showed lower levels of freezing during the retention test when compared with the animals that received Veh.

These results suggest that the antagonist of the 5-HT_6 receptors modulates the consolidation of CFC memory in the CA1 region and the agonist modulates the reconsolidation of CFC memory in the CA1 region 3 h post-reactivation, impairing the original memory.

3.3. Participation of the 5-HT_7 receptors in the consolidation and reconsolidation of the contextual fear conditioning memory

As shown in Fig. 6, animals that received the agonist of the 5-HT_7 receptors, AS-19 (5 μg/side), intra-CA1 immediately after the CFC training exhibited similar levels of freezing than the Veh group during the retention test. Interestingly, animals that received the antagonist of the 5-HT_7 receptors, SB-269970 (5 μg/side), into the CA1 region after the CFC training exhibited higher levels of freezing during the retention test when compared with the animals that received Veh. Similarly, animals that received SB-269970

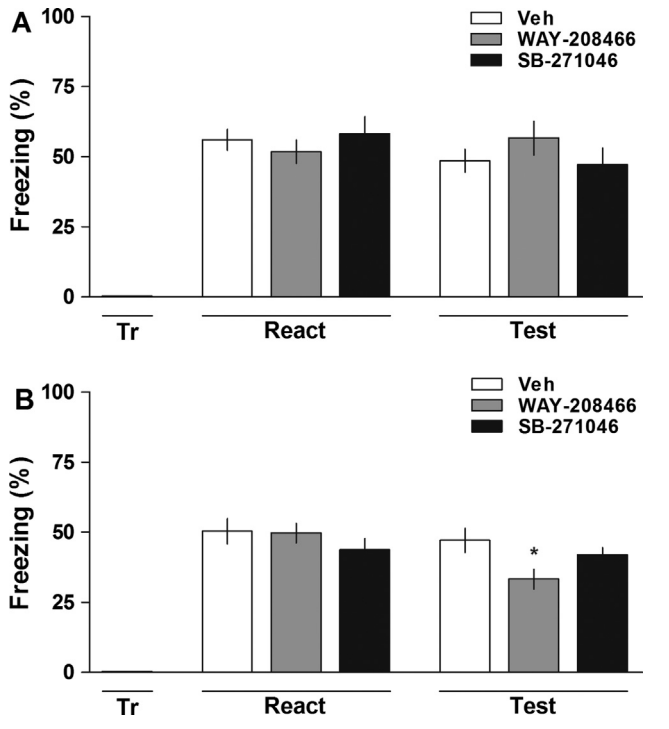

Fig. 5. Participation of the 5-HT_6 receptors in the CA1 region of the hippocampus on the reconsolidation of contextual fear conditioning. Immediately (A) or 3 h (B) post-reactivation of the contextual fear conditioning, animals received bilateral intra-CA1 infusions of Veh, WAY-208466 (0.04 μg/side) or SB-271046 (10 μg/side) and 24 h later they were subjected to a 3-min retention test. Data are presented as mean ± SEM of the percentage of time spent freezing. $^{*}p < 0.05$ for WAY-208466 vs. Veh group. Newman-Keuls test after one-way ANOVA; n = 11–12 animals per group.

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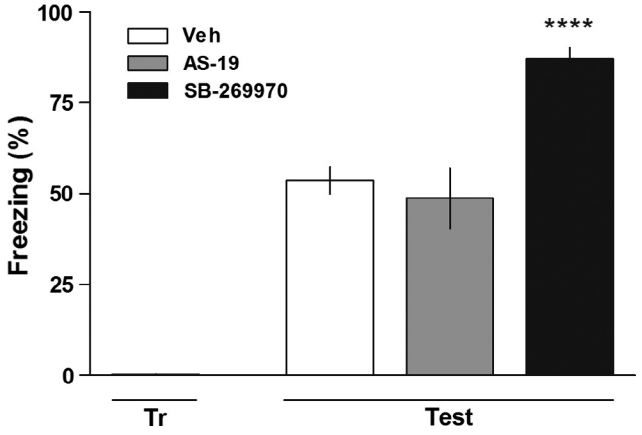

Fig. 6. Participation of the 5-HT$_7$ receptors in the CA1 region of the hippocampus on the consolidation of contextual fear conditioning. Immediately after the contextual fear conditioning, animals received bilateral intra-CA1 infusions of Veh, AS-19 (5 μg/side) or SB-269970 (5 μg/side) and 24 h later they were subjected to a 3-min retention test. Data are presented as mean ± SEM of the percentage of time spent freezing. ****$p < 0.0001$ for SB-269970 vs. Veh group on the retention test. Newman-Keuls test after one-way ANOVA; n = 11–12 animals per group.

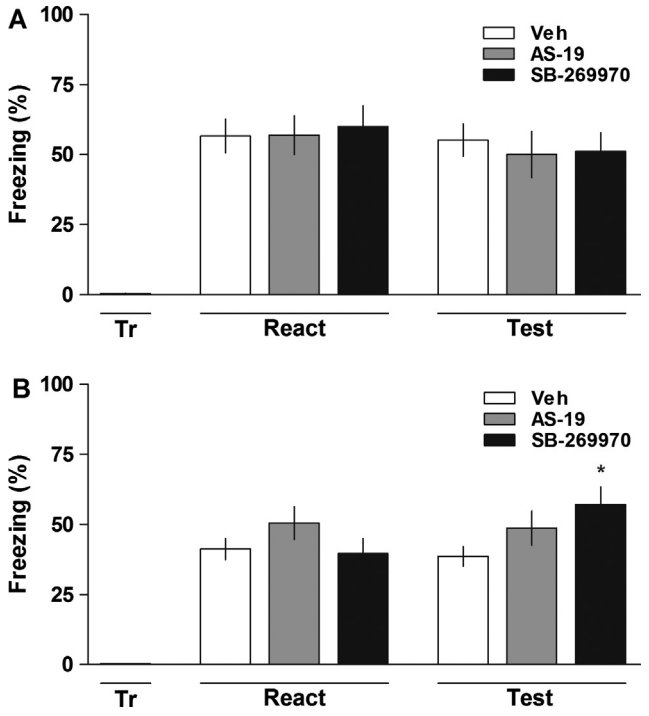

Fig. 7. Participation of the 5-HT$_7$ receptors in the CA1 region of the hippocampus on the reconsolidation of contextual fear conditioning. Immediately (A) or 3 h (B) post-reactivation of the CFC, the animals received bilateral intra-CA1 infusions of Veh, AS-19 (5 μg/side) or SB-269970 (5 μg/side) and 24 h later they were subjected to a 3-min retention test. Data are presented as mean ± SEM of the percentage of time spent freezing. *$p < 0.05$ for SB-269970 vs. Veh group on the retention test. Newman-Keuls test after one-way ANOVA; n = 11–12 animals per group.

(5 μg/side) into the CA1 region of the hippocampus 3 h post-reactivation (Fig. 7B), but not immediately after it (Fig. 7A), exhibited higher levels of freezing during the retention test when compared with the Veh group.

Together, these results indicate that the antagonist of the 5-HT$_7$ receptors, SB-269970, facilitates both consolidation and reconsolidation of CFC memory, which is the opposite of what 5-HT$_{5A}$ and 5-HT$_6$ receptors do (Figs. 3 and 5).

4. Discussion

The memory stabilization processes are essential for the formation and maintenance of memories. Despite of some similarities between consolidation and reconsolidation, the time course of the two processes is different and the brain structures and molecular mechanisms involved may be not necessarily coincident (Alberini, 2005; Bucherelli et al., 2006; Tronson & Taylor, 2007).

With focus on the more recently described serotonergic receptors, we investigated whether the 5-HT$_{5A}$, 5-HT$_6$ and 5-HT$_7$ receptors of the CA1 region of the hippocampus are involved in the consolidation and reconsolidation of contextual fear conditioning (CFC) memory. The present findings show that hippocampal the 5-HT$_{5A}$, 5-HT$_6$ and 5-HT$_7$ serotonin receptors modulate the reconsolidation of CFC memory, and in addition 5-HT$_6$ and 5-HT$_7$ also modulate the consolidation of this memory.

Here we demonstrated that 5-HT$_{5A}$ receptors seem not to be involved with the consolidation of CFC. On the other hand, we found that this receptor participates in the reconsolidation process impairing the original memory. While in associative learning task, the systemic administration of 5-HT$_{5A}$ antagonists decreased conditioned responses during short-term and long-term-memory (Gonzalez, Chávez-Pascacio, & Meneses, 2013), here we verified an effect only on the reconsolidation process. These differences on the effect can be explained by the different doses and routes of administration.

Considering that there are no selective 5-HT$_{5A}$ receptors agonists, further studies with selective 5-HT$_{5A}$ compounds and using other behavioral tasks are necessary.

There is general agreement that 5-HT$_6$ receptor antagonists produce promnesic and/or antiamnesic effects on different memories (Meneses, 2001; Quiedeville et al., 2014; Ramírez, 2013; Woods, Clarke, Layfield, & Fone, 2012; Woolley, Bentley, Sleight, Marsden, & Fone, 2001; Woolley, Marsden, Sleight, & Fone, 2003). However, some discrepant results have been reported (Gravius et al., 2011; Lindner et al., 2003; Russell & Dias, 2002). This differences can be probably related to the use of different antagonists, experimental conditions and instruments for measuring behavior (Meneses, 2013). Here we show that the blockade of 5-HT$_6$ receptors impair the consolidation of CFC memory while the activation of this receptor modulates and participates of the reconsolidation process impairing the original memory.

Studies described that the activation of 5-HT$_6$ receptors can reduce the long-term potentiation, a physiological model for studying memory, and this effect can be blocked by a selective antagonist of these receptors. Also, the activation of the 5-HT$_6$ receptors can increase the release of GABA into the CA1 region of the hippocampus (West, Marcy, Marino, & Schaffhauser, 2009), what could explain the results observed on the reconsolidation of CFC memory.

The findings on that came as a surprise were those on 5-HT$_7$ receptor which modulates both consolidation and reconsolidation positively, i.e. in an opposite way to that shown here for 5-HT$_{5A}$ and 5-HT$_6$.

There are several possible explanations for this. One is that 5-HT$_7$ receptors may interact functionally with other 5-HT receptors, and change the valence of their behavioral actions. Stiedl, Pappa, Konradsson-Geuken, & Ögren, 2015 described interactions between 5-HT$_7$ and 5-HT$_{1A}$ receptors, including 5-HT$_{1A}$ autoreceptors, which may alter the function of both. Meneses & Gasbarri, 2016 comment on the proposal by Guseva, Wirth, and Ponimaskin (2014) that indeed 5-HT$_7$ and 5-HT$_{1A}$ receptors may form heterodimers, which leads to a wide spectrum of possibilities. Of course, two related possibilities that might deserve examination are: (i) that the serotonin-releasing fibers that end upon

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hippocampal neurons containing 5-HT_7 receptors are separate from those that release serotonin upon the other receptors; (ii) that the hippocampal cells with 5-HT_7 receptors are different from those that contain the other 5-HT receptors. This would require a histochemical re-study of the distribution of serotonergic neurons within the raphe nucleus and of their endings in the hippocampus and perhaps other brain regions as well. Similar dichotomies have been recently proposed for the role of histaminergic synapses on hippocampal neurons in the regulation of retrieval in the hippocampus, where H1 receptors have stimulant effects opposite to H2 effects which are inhibitory (Izquierdo et al., 2016).

Thus, a new role for the serotonergic 5-HT_{5A}, 5-HT_6 and 5-HT_7 receptors on memory process has been described here, representing a new perspective with regard to their functions in process related to memory mechanisms as well as their therapeutic applications, providing new evidences in memory research that encourage new lines of investigation.

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