Neurobiology of Learning and Memory 138 (2017) 226–237

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Evidence of VTA and LC control of protein synthesis required for the behavioral tagging process

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ARTICLE INFO

Article history:
Received 28 March 2016
Revised 27 May 2016
Accepted 6 June 2016
Available online 9 June 2016

Keywords:
Memory
Ventral tegmental area
Locus coereleus
Behavioral tagging
Dopamine
Noradrenaline

ABSTRACT

Several works have shown that the formation of different long-term memories relies on a behavioral tagging process. In other words, to establish a lasting memory, at least two parallel processes must occur: the setting of a learning tag (triggered during learning) that defines where a memory could be stored, and the synthesis of proteins, that once captured at tagged sites will effectively allow the consolidation process to occur. This work focused in studying which brain structures are responsible of controlling the synthesis of those proteins at the brain areas where memory is being stored. It combines electrical activation of the ventral tegmental area (VTA) and/or the locus coeruleus (LC), with local pharmacological interventions and weak and strong behavioral trainings in the inhibitory avoidance and spatial object recognition tasks in rats. The results presented here strongly support the idea that the VTA is a brain structure responsible for regulating the consolidation of memories acting through the D1/D5 dopaminergic receptors of the hippocampus to control the synthesis of new proteins required for this process. Moreover, they provide evidence that the LC may be a second structure with a similar role, acting independently and complementary to the VTA, through the β-adrenergic receptors of the hippocampus.

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1. Introduction

Recent evidence shows that the formation of different long-term memories (LTMs) relies on a tagging and captures process (for review see: Moncada, Ballarini, Martinez, & Viola, 2015; Redondo & Morris, 2011). This means that, to establish a lasting memory, at least two parallel and complementary processes should occur: the setting of a mark (the learning tag –LT–) induced by the learning, that will determine that this information is plausible to be stored and where to do it; and the synthesis of Plasticity Related Proteins (PRPs) that once captured at the tagged sites will allow memory consolidation (Moncada & Viola, 2007). This process, named behavioral tagging (BT), was observed underlying the formation of aversive, non-aversive, contextual, spatial, taste-recognition, hippocampus and cortex-dependent long-term memories, as well as long-term extinction of the contextual fear memory (Almaguer-Melian et al., 2012; Ballarini, Moncada, Martinez, Alen, & Viola, 2009; Cassini et al., 2013; de Carvalho Myskiw, Benetti, & Izquierdo, 2013; Lu et al., 2011; Moncada & Viola, 2007; Wang, Wilcoxen, Nomoto, & Wu, 2007); transforming it into a strong candidate for a general mechanism of LTM formation. Thus, understanding the mechanisms and structures responsible for setting the LTs as well as identifying the mechanisms and structures that regulate the synthesis of PRPs, results essential for a deeper comprehension of how learning and memory processing occurs.

Recent studies have shown that the setting of a LT, particularly for the inhibitory avoidance (IA) task, depends on the functionality of the NMDA and TrkB receptors and CAMKIIα and PKA activities, and it is independent of the synthesis of PRPs (Lu et al., 2011; Moncada, Ballarini, Martinez, Frey, & Viola, 2011). This results are consistent with those obtained in functional plasticity model of synaptic tagging and capture, where the same kinases and receptors were shown to be specifically involved in the setting of the synaptic tag for long-term potentiation (Li et al., 2012; Lu et al., 2011; Redondo et al., 2010; Sajikumar, Navakkode, & Frey, 2007; Sajikumar, Navakkode, Sacktor, & Frey, 2005).

Unlike the knowledge of the tagging process that is fairly established, the control mechanisms of PRPs synthesis remain more

Abbreviations: BT, behavioral tagging; DH, dorsal hippocampus; IA, inhibitory avoidance; LC, locus coeruleus; LT, learning tag; OF, open field; PRP, Plasticity Related Protein; SOR, Spatial Object Recognition; VTA, ventral tegmental area.

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http://dx.doi.org/10.1016/j.nlm.2016.06.003
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uncertain. At electrophysiological level, it has been shown that LTP reinforcement through a synaptic tagging process requires the functionality of D1/D5 dopaminergic and β-adrenergic receptors during the PRPs synthesis process (O'Carroll and Morris, 2004; Sajikumar & Frey, 2004). Indeed they are also required to reinforce LTP in vivo by the association of behavioral tasks with weak electrophysiological stimulations (Korz & Frey, 2007; Li, Cullen, Anwyl, & Rowan, 2003).

At the behavioral level, PRPs are critical players in memory consolidation (McGaugh, 2000) and they can be attributed to the learning, if it is strong enough, or by an associated event experienced within a critical time window (Moncada et al., 2015). Different novel experiences, such as the exploration of novel arenas or novel objects and tasting novel flavors, have been used to provide the PRPs required for the consolidation of hippocampus- or cortex-dependent tasks such as the inhibitory avoidance (IA), spatial object recognition (SOR), contextual fear conditioning, taste aversion and event arena (Ballarini et al., 2009; Dong et al., 2012; Lu et al., 2011; Moncada & Viola, 2007; Wang, Redondo, & Morris, 2010). Indeed, contextual fear and water maze tasks could also promote the hippocampus dependent SOR memory (Cassini et al., 2013). However, not only behavioral treatments have been effective in providing PRPs for memory consolidation, but also the systemic injection of the dopaminergic and adrenergic agonists SKF3393 and dobutamine have been shown induce the synthesis of those PRPs required to consolidate the IA memory in the hippocampus (Moncada et al., 2011). In particular, hippocampal D1/D5 dopaminergic receptors are essential for the promotion of IA and 'Schemas' memories as well as long-term extinction of fear memories by exposure to novelty (Menezes et al., 2015; Moncada & Viola, 2007; Wang et al., 2010). Indeed, it has been shown that D1/D5 dopaminergic as well as β-adrenergic receptors are necessary to induce the synthesis of PRPs required for promotion and consolidation of IA memory; moreover, NMDA receptors are involved in this process as well (Moncada et al., 2011). Also, catecholaminergic receptors have been involved in the consolidation and modulation of several learning tasks, dependent on different brain structures (Furini, Myskiw, Schmidt, Marcondes, & Izquierdo, 2014; Izquierdo et al., 2006; Lisman & Grace, 2005; Roozendaal & McGaugh, 2011). Since the hippocampus requires the functionality of dopaminergic and β-adrenergic receptors to trigger the synthesis of PRPs, the control of these essential resources required for memory consolidation may be regulated by upstream brain structures. This work focuses in identifying them. Indeed, as the ventral tegmental area (VTA-) and the locus coeruleus (LC) are the main sources of dopamine and noradrenaline in the brain and have direct efferences to the hippocampus (Lisman & Grace, 2005; Sara, 2009), this research is based in the hypothesis that these structures are responsible for controlling the synthesis of the PRPs there where memory is meant to be stored.

This study shows that the electrical activation of the VTA associated with weak trainings in the IA or SOR tasks within a critical time window, is able to promote the formation of LTMs that otherwise would not exist. This promoting effect of IA- and SOR-LTM depends on D1/D5 receptors functionality in the hippocampus and can be blocked by the infusion of a protein synthesis inhibitor in this structure. Moreover the amnesia caused by antagonizing D1/D5-receptors or by inhibiting the synthesis of proteins in the hippocampus during strong training sessions was overcome by stimulation of the VTA, allowing the consolidation of LTMs by PRPs provided as a result of VTA activation rather than the strong training experience. As a whole, these results suggest that the VTA is actually one of the brain structures responsible for controlling the synthesis of PRPs required for memory consolidation. In addition, this work also provides evidence that the LC may be a second structure with a similar role, acting both in parallel as well as complementary to the VTA.

2. Materials and methods

2.1. Subjects

Male Wistar rats (weight: 280–300 g, 3 month old aprox.) from our own breeding colony in Magdeburg were used. Rats were housed in groups of five per cage, with water and food ad libitum, at a constant temperature of 23 °C and under a 12 h light/dark cycle (lights on: 6 a.m.). Behavioral procedures were conducted during the light phase.

The experimental protocols for this study followed the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

SCH-23390, Propranolol, Muscimol and anisomycin were purchased from Sigma. SCH23390 (2 μg/side), Propranolol (5 μg/side) and Anisomycin (80 μg/side, dissolved in HCl, diluted in saline and adjusted to pH 7.4 with NaOH) were locally infused in the dorsal hippocampus (DH) (CA1 or DG; 0.8 μl/side), Muscimol (50 ng/side) was infused in the VTA (0.4 μl/side) or LC (0.2 μl/side).

2.3. Surgery and drug infusion

Cannula implantation, drug infusion and histological examination of cannula placements were performed as described previously (Moncada & Viola, 2007). Antero-Posterior (AP) and Lateral (L) coordinates were taken using Bregma as reference, except when stated different. Ventral (V) coordinate was reached taking Dura as reference. Briefly, guide cannuli were stereotaxically placed 0.5 mm above the pyramidal cell layer of the CA1 region of the DH (AP −4.0 mm, L ± 3.0 mm, V 3.0 mm), 0.5 mm above the granular cell layer of the DG at the DH (A −4.0 mm, L ± 1.8 mm, V 3.6 mm), 0.5 mm above the VTA (AP −5.6 mm, L ± 2.3 mm, V 7.3 mm, arm inclination 10°) or 0.5 mm above the LC (A −3.0 to −3.1 mm from lambda, L ± 1 mm, V 5.2 mm, head inclination 20°) of deeply anesthetized rats, using the coordinates of the atlas of Paxinos and Watson (1997) as guide (Fig. 1). Rats were allowed to rest and recover for at least one week before any procedural manipulation. To infuse the drugs, a 30-gauge infusion needle with its tip protruding 0.5 mm beyond that of the guide was used. Only data from animals with correct cannula/electrodes implants (>95% of the rats) were included in the analyses.

2.3.1. Electrode implantation

Tungsten stimulation electrodes were bilaterally implanted into the VTA (A −5.6 mm, L ± 2.3 mm, V 7.8 mm, arm inclination 10°) the LC (A −3.0 to −3.1 mm from lambda, L ± 1 mm, V 5.7 mm, head inclination 20°) or both (Fig. 1).

2.4. Behavioral apparatus and procedures

To avoid unnecessary emotional stress, all rats were handled daily for 3 min during 3 days before any behavioral procedure. Then animals were randomly assigned to each experimental group/condition.

The open field (OF) apparatus was previously described (Moncada & Viola, 2007). A novel environment exploration consisted of a 5 min OF session.

The inhibitory avoidance (IA) paradigm was described previously (Moncada & Viola, 2007). In training session rats received a weak foot-shock (wIA: 0.23 mA, 2 s) or a strong foot-shock (sIA: 0.5 mA, 3 s). LTM was evaluated in a test session 24 h after later, by comparing the step-down latency in the training and test ses-

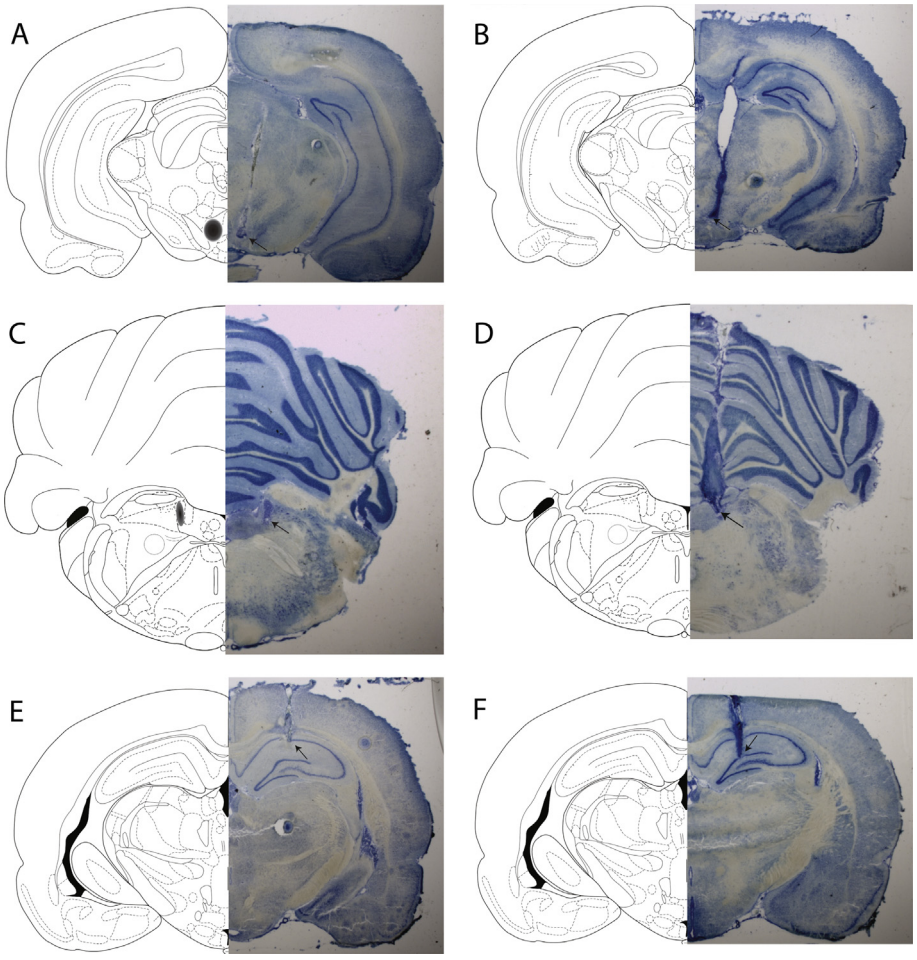


Fig. 1. Schemas (left) and sample photographs (right) showing electrode and cannula placement. (A) Placement of electrodes in VTA. Electrodes were considered as correctly implanted when observed within the degraded pattern in left side of the image, including and AP depth of approximately ±0.3 mm AP. (B) Placement of cannula in VTA. (C) Placement of electrodes in LC. Electrodes were considered as correctly implanted when observed within the degraded pattern in left side of the image, including and AP depth of approximately ±0.35 mm AP. (D) Placement of cannula in LC. (E) Placement of cannula in the CA1 region of DH. (F) Placement of cannula in the DG of the DH.

sions. As training performance was always equivalent for the different experimental groups, the training (TR) group was always composed by pooling a random and representative set of animals to reach a total of them equal to the number of animals in the larger experimental group. This avoids the artificial increase in the degrees of freedom that would result by adding all the trained animals to this group.

The Spatial Object Recognition apparatus was a 60 cm wide × 40 cm length × 50 cm height acrylic box with different visual clues. For habituation to the context, animals explored the arena without objects for 20 min once a day during 2 days for a weak SOR training (wSOR). Habituation lasted 30 min for strong trainings (sSOR). On the training day, two identical objects were included in the arena in two adjacent corners, 10 cm from lateral walls. In the training session, animals were left to explore the arena for 4 min (wSOR) or 8 min (sSOR) and exploration time for each of the objects was measured. During the test session, one of the objects was switched to a new position and animals were allowed to explore for 2 min. Animals expressed SOR memory when they spent more time exploring the object in the novel position. The time of exploration to each object was recorded and expressed as a percentage of the total exploration time to both objects.

In order to reduce the use of animals, as a general rule rats were used twice, once for experiments involving the IA task and once for experiments involving the SOR task. Animals were allowed to rest between 1 and 2 weeks before starting the second experimental round. IA and SOR behavior and performance was equivalent either they had performed the task in the first or second round of experiments. A total of 445 animals were used. 217 had electrodes implanted in the VTA. 61 had electrodes in the LC. 18 had electrodes in VTA and LC. 149 were not implanted with electrodes.

2.5. Electrophysiological manipulations

All electrical stimulations were performed in a Plexiglas recording box of 40 cm side and 55 cm high with a floor of steel bars. For three days animals were submitted to these recording boxes during 30 min in order to make them familiar and avoid any spatial novelty during the stimulation in the experimental day (Ballarini et al., 2009; Moncada & Viola, 2007). On day 4, electrical activation of the VTA, the LC or both structures was performed by applying 3 bursts of 10 biphasic constant current impulses (0.1 ms per polarity, of 0.4 mA at f = 200 Hz) with a 10 s inter burst interval.

2.6. Data analysis

Newman-Keuls multiple comparison tests after one-way analysis of variance (ANOVA) was applied using GraphPad Prism 5 (GraphPad Software Inc. San Diego, CA).

3. Results

As it was introduced above, there is an extensive background suggesting that LTM formation relies on a BT process whose regulation involves the catecholaminergic systems. Indeed, we have previously shown that the exploration to a novel arena 1 h before a weak IA training (wIA) that induces only short forms of memory, is capable of promoting IA-LTM consolidation through a protein synthesis-dependent mechanism that relies on adrenergic and dopaminergic pathways (Moncada et al., 2011; Moncada & Viola, 2007). This new work is based on the hypothesis that structures like the VTA and LC (main sources of dopamine and noradrenaline) are responsible for regulating the synthesis of PRPs there where memory is meant to be store during the BT process. Thus, to start testing this hypothesis we analyzed whether activating the VTA was able to promote IA-LTM when associated with a wIA training.

To perform this, stimulation electrodes were implanted in the VTA of rats and this structure was activated by a tetanic stimulation, 60 min before subjecting them to a wIA training. Animals from a control group were allowed to freely explore the familiar recording box during the same duration used for the tetanization but without being stimulated. A third group of animals, used as positive control, was subjected to explore a novel open field (OF) during 5 min (instead of activating the VTA) 60 min before the wIA. As can be seen in Fig. 2A, the activation of the VTA promoted the consolidation of a conspicuous IA-LTM, otherwise inexistent in control animals (only trained with a wIA), which were unable to remember this task the next day ($p < 0.001$). Interestingly, those animals subjected to the novel OF instead of the simulation protocol, showed a better performance during the test session than those of the VTA group ($p < 0.001$). These results suggest that VTA could be one of the brain regions activated by novel OF exposure, but other mechanisms could be also recruited in order to promote a better IA-LTM.

The time course of the promoting effect was then analyzed by stimulating different groups of animals at different time points before or after a wIA. It was observed that VTA activation promoted IA-LTM when performed 60 min before or 30 or 60 min after training ($p < 0.001$), but not at 120 min around it, suggesting more effectiveness when it occurs closer to the learning session (Fig. 2B). Given that stimulation of the VTA after the wIA training also promoted IA_LTM, it is unlikely to relate the promoting effect to a sensitization process or a change in training conditions triggered by the stimulations. Indeed, this critical time window of efficacy is consistent with the idea that the VTA promotes memory through a BT process, in which a LT set by the wIA training must coexist with the PRPs, synthesized before or after, to allow memory consolidation.

Since VTA is one of the main dopamine sources of the brain (Lisman & Grace, 2005) and taking into consideration that it extends dopaminergic projections to the hippocampal region (Gasbarri, Packard, Campana, & Pacitti, 1994; Gasbarri, Verney, Innocenzi, Campana, & Pacitti, 1994; Scatton, Simon, Le Moal, & Bischoff, 1980), one of the main structures that processes the IA task (Bekinschtein et al., 2007; Izquierdo et al., 2006), it was then

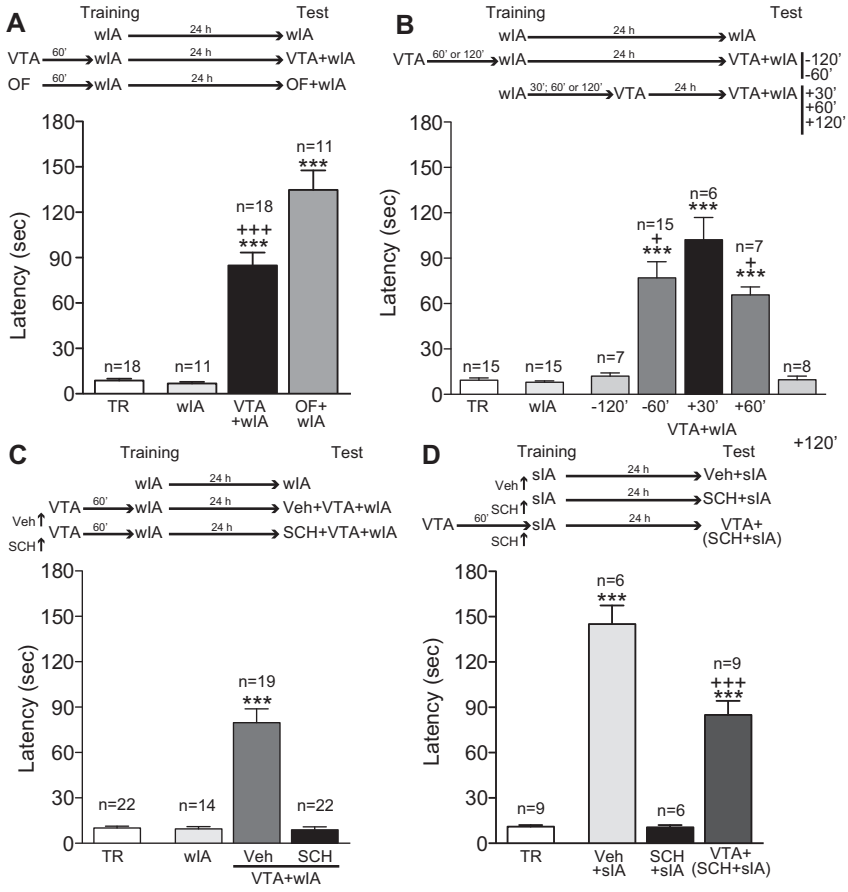


Fig. 2. Electrical VTA activation promotes IA-LTM through hippocampus D1/D5-dopaminergic receptors within a restricted time window. Figures show mean ± SEM of step down latencies during training (TR) and the test sessions. Top: experimental design showing moment of stimulation, drug infusion and training protocol (weak: wIA; Strong: sIA). (A) VTA stimulation promotes IA-LTM of less magnitude than OF exploration. *** $p < 0.001$ vs TR and wIA, +++ $p < 0.001$ vs OF+wIA ($F = 66.30$, $df = 57$). (B) VTA stimulation promotes IA-LTM within a critical time window. ***$p < 0.001$ vs TR, wIA; VTA+wIA: −120′ and +120′; + $p < 0.05$ vs +VTA+wIA: +30′ ($F = 30.36$; $df = 72$). (C) SCH infusion impairs VTA promoted IA-LTM. *** $p < 0.001$ vs. all groups ($F = 59.21$; $df = 74$). (D) VTA stimulation prevents SCH induced amnesia. *** $p < 0.001$ vs TR and SCH+sIA, +++ $P < 0.001$ vs Veh +sIA ($F = 68.85$; $df = 29$).

studied whether the promoting effect on memory formation could rely on the dopaminergic action housing from this brain structure. To evaluate this, besides the electrodes placed in the VTA, rats were also implanted with guide cannuli in the CA1 region of the dorsal hippocampus (DH). After recovery, they were infused either with the D1/D5-dopaminregic receptors antagonist SCH23390 (SCH) or with vehicle solution (Veh), 13 min after the VTA was stimulated and after other 60 min the animals were subjected to a wIA training. Fig. 2C shows that IA-LTM promoted by a VTA stimulation performed 60 min before a wIA training, was specifically impaired in those animals infused with SCH ($p < 0.001$), but not in those infused with Veh.

Previous studies showed that in the context of a strong IA training (sIA), which usually induces LTM formation, the infusion of SCH impairs the consolidation of a lasting memory or its persistence (Furini et al., 2014; Moncada et al., 2011; Rossato, Bevilaqua, Izquierdo, Medina, & Cammarota, 2009). Thus, to further understand how the VTA could provide the resources required to consolidate the IA memory, a fourth experiment analyzed whether the pre-activation of this structure, was able to overcome the amnestic effect of SCH infusions in the context of a sIA training. Fig. 2D shows that SCH infusion 13 min before a sIA training impaired IA-LTM formation ($p < 0.001$). However a pre-activation of the VTA 60 min before training overcame the amnestic effect of SCH infusion ($p < 0.001$). This result puts into evidence that SCH impairs IA-LTM by affecting resources required for memory consolidation, which can be provided by previous activation of the VTA, but without affecting the setting of the IA-learning tag.

In summary, this series of experiments suggests that VTA activation provides dopamine to the DH and its action through D1/D5 receptors is necessary to consolidate an IA-LTM within a critical time window.

Then, to directly address if the VTA is responsible for controlling the synthesis of PRPs required in the hippocampus to consolidate a hippocampus-dependent LTM, the protein synthesis inhibitor anisomycin (Ani) was infused in the CA1 region of DH 13 min in advance of activating the VTA. Ani infusion completely impaired the promoting effect of VTA stimulation, regardless of whether the VTA was activated 60 min before or 30 min after learning (Fig. 3A, $p < 0.001$). To further analyze this issue it was then

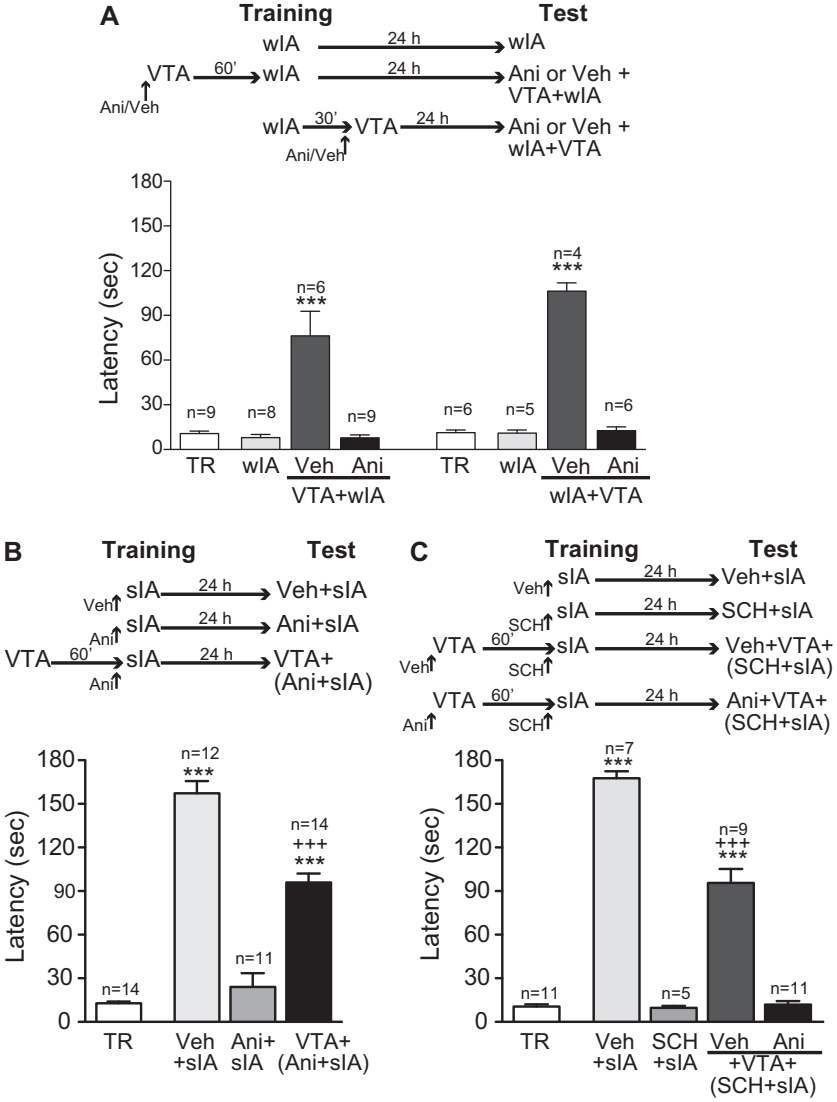


Fig. 3. VTA activation promoted IA-LTM relays on dopaminergic receptors functionality and the synthesis of PRPs in the hippocampus. Figures show mean ± SEM of step down latencies during training (TR) and the test sessions. Top: experimental design showing moment of stimulation, drug infusion and training protocol (weak: wIA; Strong: sIA). (A) Ani infusion in DH impairs VTA promoting effect over IA-LTM when stimulated either before (Left) or after (Right) wIA training. *** $p < 0.001$ vs all groups (Left: $F = 23.22$; $df = 31$. Right: $F = 218$; $df = 20$). (B) VTA stimulation prevents Ani induced amnesia. *** $p < 0.001$ vs TR and Ani+sIA, +++ $P < 0.001$ vs Veh+sIA ($F = 100.9$; $df 50$). (C) VTA preventive effect over SCH induced amnesia is blocked by Ani infusion in DH. *** $p < 0.001$ vs TR, SCH+sIA and Ani+VTA+(SCH+sIA); +++ $p < 0.001$ vs Veh+sIA ($F = 175.3$; $df = 42$).

evaluated whether the pre-activation of the VTA could overcome the amnesia provoked by inhibiting protein synthesis in the hippocampus during a sIA training. Thus, the VTA was activated, after 47 min Ani was infused in the DH and 13 min later rats were subjected to a sIA. Other two groups of animals were infused with either Ani or Veh and trained in the sIA but not stimulated. Confirming previous data, Ani infusion completely blocked the IA-LTM 24 h later ($p < 0.001$). However this amnestic effect was partially prevented in those rats whose VTA was activated 60 min before training (Fig. 3B, $p < 0.001$). To further explore the possibility that VTA activation allows the consolidation of IA memory through a dopamine release- and protein synthesis-dependent mechanism, it was then checked whether the preventive effect of stimulating the VTA on SCH induced amnesia (Fig. 2D) was a protein synthesis-dependent phenomenon. To achieve this, Ani or Veh was infused into the DH 13 min before a VTA activation, which was performed 60 min in advance of a sIA training in presence of SCH. As shown in the Fig. 3C, when Ani was infused, VTA activation was incapable of preventing the amnestic effect of peri-training SCH administration into the DH ($p > 0.05$). In summary, VTA activation was able to promote the consolidation of IA-LTM through a mechanism dependent on dopamine release in the hippocampus acting through the activation of D1/D5 receptors and the induction of the newly synthesized PRPs.

In the previous experiments, performance during the IA-LTM promoted by VTA stimulation was always lower to that observed in the IA-LTM promoted by a novel OF or the one triggered by a sIA training. These data suggest that other brain structures, beyond the VTA, may be also responsible for controlling hippocampal protein synthesis required during the consolidation of this memory. Indeed, novel OF exploration is able to promote IA-LTM through a mechanism that also depends on the β-adrenergic receptors of the dentate gyrus (DG) (Moncada et al., 2011). Moreover, the LTM induced by a strong IA-training also relies on the functionality of these receptors (Moncada et al., 2011). Since the locus coeruleus (LC) is the main hippocampus' noradrenaline source (Jones & Moore, 1977; Sara, 2009), it was then analyzed whether this structure is also responsible for regulating hippocampal PRPs synthesis using the adrenergic system. Thus, stimulation electrodes were implanted into the LC and this structure was activated 60 min before a wIA training. Similar to what occurred with the VTA activation, stimulating the LC resulted in the promotion of an IA-LTM ($p < 0.001$), which also resulted to be of a lower level than that observed in the animals that explored a novel OF instead of being stimulated (Fig. 4A, $p < 0.001$). The infusion of the β-adrenergic receptor antagonist propranolol (Prop) into the DG of the DH 13 min before LC activation completely impaired the promoting effect of LC stimulation (Fig. 4B left, $p < 0.001$). A similar result

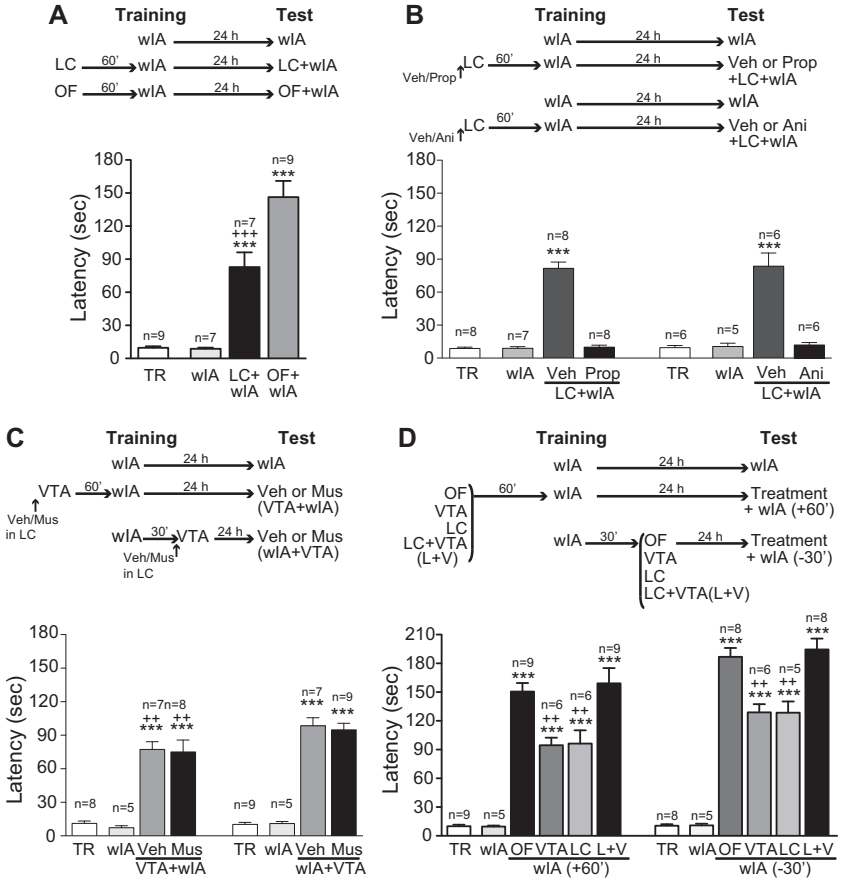


Fig. 4. LC and VTA promote IA-LTM formation through independent and complementary mechanisms. Figures show mean ± SEM of step down latencies during training (TR) and the test sessions. Top: experimental design showing moment of stimulation, drug infusion and weak training protocol (wIA). (A) LC stimulation promotes IA-LTM of less magnitude than OF exploration. *** $p < 0.001$ vs TR and wIA, +++ $p < 0.001$ vs OF+wIA ($F = 41.23$; $df = 31$). (B) LC induced IA-LTM depends on β-adrenergic receptors functionality (Left) and protein synthesis (Right) in DH. *** $p < 0.001$ vs all groups (Left: $F = 129.9$; $df = 30$. Right: $F = 31.51$; $df = 22$). (C) LC inhibition with Muscimol (Mus) dos not impairs IA-LTM promoted by a stimulation of the VTA performed before or after a wIA training. *** $p < 0.001$ vs TR ($n = 8$) and wIA (Left: $F = 28.34$; $df = 27$. Right: $F = 99.78$; $df = 29$). (D) Co-activation of the VTA and the LC promotes IA-LTM comparable to that induced by OF exploration, with better performance than that observed in animals in which only one structure was stimulated *** $p < 0.001$ vs TR and wIA, ++ $p < 0.01$ vs OF+wIA and LC+VTA+wIA (both at +60 or −30 min) (Left: $F = 39.91$; $df = 43$. Right: $F = 92.99$; $df = 39$).

was observed when Ani was infused in this region before LC stimulation (Fig. 4B right, $p < 0.001$). Therefore, LC stimulation promoted IA-LTM through a mechanism dependent on noradrenaline release and synthesis of PRPs in the DH. Taking into consideration the similarity of these results to those obtained by the stimulation of the VTA, and that different works have shown the existence of connections between these structures (Ornstein et al., 1987; Sara, 2009; Weinshenker & Schroeder, 2007), it was important to address whether they promoted memory through connected or independent pathways. To answer this, animals were subjected to a wIA and 60 min before or 30 min after a wIA training, the VTA was stimulated whereas the LC was inactivated by the local infusion of muscimol (Mus). Interestingly, VTA activation was equally capable of promoting an IA-LTM in animals infused either with Mus or Veh, ruling out a down stream participation of the LC in this process (Fig. 4C). In the same way, the activation of the LC 30 min after wIA training in animals infused with Mus in the VTA, resulted in a promotion of IA-LTM comparable to that observed in animals infused with Veh (Training: 8.55 ± 1.81, $n = 8$; wIA: 9.95 ± 2.15, $n = 5$; wIA+LC+Veh in VTA: 100.1 ± 14.68 $n = 6^{***}$ and wIA+LC+Mus in VTA: 100.2 ± 9.43, $n = 8^{***}$; ***: $p < 0.001$ vs Training and wIA). Thus, VTA and LC activation promote IA-LTM independent of each other inducing the synthesis of PRPs in the hippocampus. However, IA-LTM induced either by a sIA training or promoted by an OF exploration is both: (1) more conspicuous and (2) dependent on dopaminergic and adrenergic receptors of the hippocampus. For that reason, it was then analyzed whether the simultaneous activity of VTA and LC resulted in a better IA-LTM. To do this, one or both structures were stimulated, either 60 min before or 30 min after a wIA. As can be seen (Fig. 4D) co-stimulation of these structures resulted in an IA-LTM significantly higher to that obtained by the sole stimulation of one of them ($p < 0.01$). In addition, the memory promoted by the co-stimulation was comparable to that observed in rats whose IA-LTM was promoted by the exploration of a novel OF (Fig. 4D), suggesting that in this case the better IA-LTM was a result of recruiting the activity of both neurotransmitter systems instead of one.

As a whole, the previous results show that VTA activation can promote IA-LTM through a mechanism that depends on the functionality of D1/D5 dopaminergic receptors in the hippocampus. Indeed its activation prior to a strong training can prevent the amnestic effect of blocking hippocampal D1/D5 receptors during a sIA training. Moreover, these promoting and preventive effects of VTA stimulation depended on the ability of this structure to trigger the synthesis of new PRPs in the hippocampus; putting into evidence that VTA activation allows the consolidation of lasting memories by inducing the synthesis of PRPs, at least in this structure. In addition, it was shown that LC activation was also capable to promote IA-LTM through a protein synthesis-dependent mechanism. The results demonstrated that the effect of LC activation was not only independent of VTA functionality but also complementary, suggesting that both structures may be responsible for controlling the synthesis of those PRPs required in the hippocampus to allow IA memory consolidation.

Dopaminergic and adrenergic receptors are involved in the formation and modulation of different memories (Furini et al., 2014, 2010; Izquierdo et al., 2006; Lisman & Grace, 2005; Roozendaal & McGaugh, 2011). Therefore it is reasonable to think that VTA and LC activation may indeed be master keys that regulate the synthesis of PRPs required for the consolidation of several types of memories. To further investigate this possibility, the previous series of experiments was repeated using a different hippocampus-dependent learning task: the spatial version of the object recognition task (SOR). In contrast to the IA, which is a fear driven operant-like conditioning (Izquierdo et al., 2006), the SOR is a spatial task in which animals learn the location of two identical objects in a particular environment (the training arena) (Dix & Aggleton, 1999; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002).

Similar to what was observed for the IA task, the electrical activation of the VTA 60 min before a weak SOR training session (wSOR) was capable of promoting the formation of an otherwise inexistent SOR-LTM ($p < 0.001$); also the expression of this memory was lower compared to that observed in the SOR-LTM promoted by an OF exposure ($p < 0.01$) (Fig. 5A). This promoting effect over SOR-LTM was restricted to a larger time window, which extended to 2 h around the wSOR training ($p < 0.001$) and was ineffective three hours around it (Fig. 5B). Moreover, this VTA-dependent promoting effect also relied on the functionality of hippocampal D1/D5-dopaminergic receptors during the activation of the structure (Fig. 5C, $p < 0.001$). Likewise, while antagonizing this receptors with SCH in DH 13 min before a strong SOR training (sSOR) blocked the SOR-LTM ($p < 0.001$), this amnestic effect could be overcome if the VTA was activated 60 min before the training ($p < 0.001$), when the antagonist was not yet infused (Fig. 5D).

Analyzing the protein synthesis dependency of the process, it was observed that Ani infusion into the CA1 region of the DH 13 min before VTA stimulation completely impaired its promoting effect on SOR-LTM (Fig. 6A, $p < 0.001$). Moreover, Ani infusion into the DH 13 min before a sSOR training completely impaired LTM formation ($p < 0.001$); however this amnestic effect was overcome by previous activation of the VTA (Fig. 6B, $p < 0.001$). Further evidence that this structure was required for hippocampal synthesis of PRPs necessary for SOR memory consolidation, came from the fact that the preventive effect of VTA activation over the SCH-induced (Fig. 5D) amnesia was completely blocked when the stimulation protocol was performed in the presence of Ani in the DH (Fig. 6C, $p < 0.001$). Thus, VTA activation induced SOR-LTM formation through a protein synthesis-dependent mechanism triggered by D1/D5 dopaminergic receptors activity in the hippocampus.

Similar to the observations of the VTA role in IA memory formation, SOR-LTM promoted by stimulation of the VTA resulted to be of less magnitude than that induced by an OF exposure or sSOR training. Therefore a door opened here to further analyze whether LC could be involved in PRPs synthesis regulation as well. Performing this, it was observed that the electrical activation of the LC was able to promote SOR-LTM in weakly trained animals (Fig. 7A, $p < 0.001$). Moreover, this promoting effect was also impaired by the infusion of either Prop or Ani into the DG (Fig. 7B, $p < 0.001$), suggesting that a protein synthesis dependent process is triggered by the release of noradrenaline in the hippocampus (among other possible structures). When inhibiting the LC, by Mus infusion in this structure, VTA stimulation, either 1 h before or after the wSOR training, still presented the ability to promote SOR-LTM (Fig. 7C, $p < 0.001$). Furthermore, LC stimulation also promoted SOR memory even if the VTA had been inactivated by the infusion of Mus (wSOR: Training = 51.87 ± 1.05, Test = 49.6 ± 0.64, $n = 5$; wSOR + LC + Veh in VTA: Training = 49.82 ± 2.75, Test = $64.72 \pm 0.85^{***}$, $n = 5$; wSOR + LC + Mus in VTA: Training = 49.25 ± 0.49, Test = $66.77 \pm 1.91^{***}$, $n = 7$. *** $p < 0.001$: vs Training and wSOR). As expected, co-stimulation of these structures resulted in a better performance during SOR-LTM test than that induced by the activation of VTA or LC alone ($p < 0.01$), being its levels comparable to those observed in the animals whose SOR-LTM was promoted by the exploration of a novel arena (Fig. 7D). Thus, VTA and LC activation controls hippocampus protein synthesis dependent SOR-LTM formation independently, but also complementary, to the activity of each other, suggesting that in this task both structures may be also responsible for controlling the PRPs synthesis process.

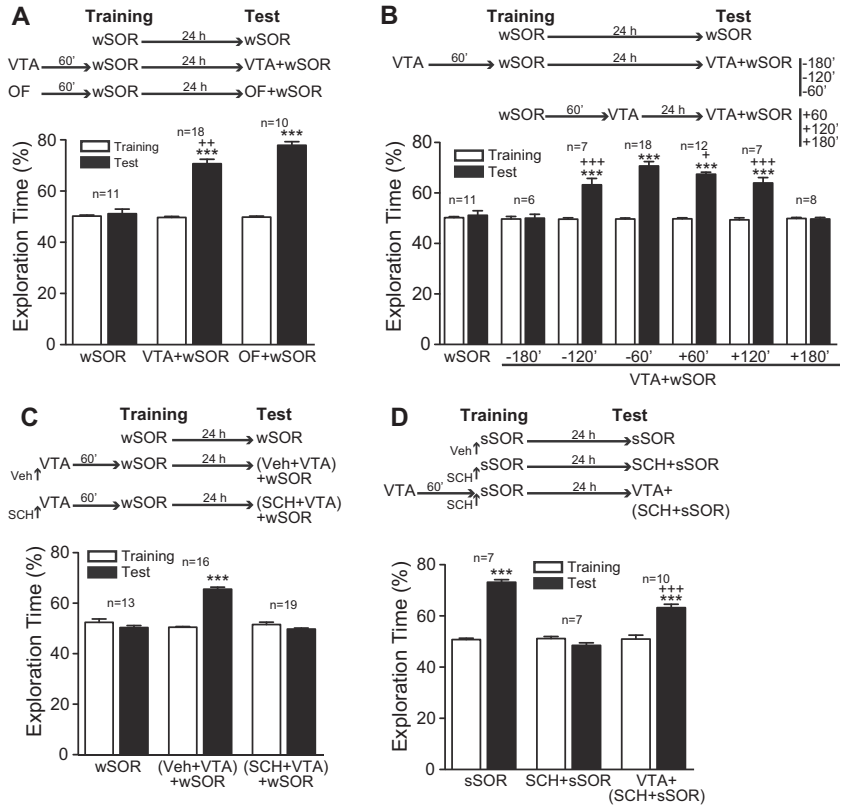


Fig. 5. Electrical VTA activation promotes SOR-LTM through hippocampus D1/D5-dopaminergic receptors within a restricted time window. Figure shows mean ± SEM of the exploration time (in percentage), for the object moved to the novel position, during training (white) and test (black) sessions. Top: experimental design showing the moment of stimulation, drug infusion and training protocol (weak: wSOR; Strong: sSOR). (A) VTA stimulation promotes SOR-LTM of less magnitude than OF exploration. *** $p < 0.001$ vs TR and wSOR, $^{++}$ $p < 0.01$ vs OF+wSOR ($F = 94.18$; $df = 77$). (B) VTA stimulation promotes SOR-LTM within a critical time window. *** $p < 0.001$ vs TR, wSOR; VTA+wSOR: −180′ and +180′; +, and $^{+++}$ $p < 0.05$ and $p > 0.001$ vs +VTA+wSOR: −60′ ($F = 45.13$; $df = 137$). (C) SCH infusion impairs VTA promoted SOR-LTM. *** $p < 0.001$ vs. all groups ($F = 61.71$; $df = 95$). (D) VTA stimulation prevents SCH induced amnesia. *** $p < 0.001$ vs TR and SCH+sSOR, $^{+++}$ $P < 0.001$ vs Veh+sSOR ($F = 60.19$; $df = 47$).

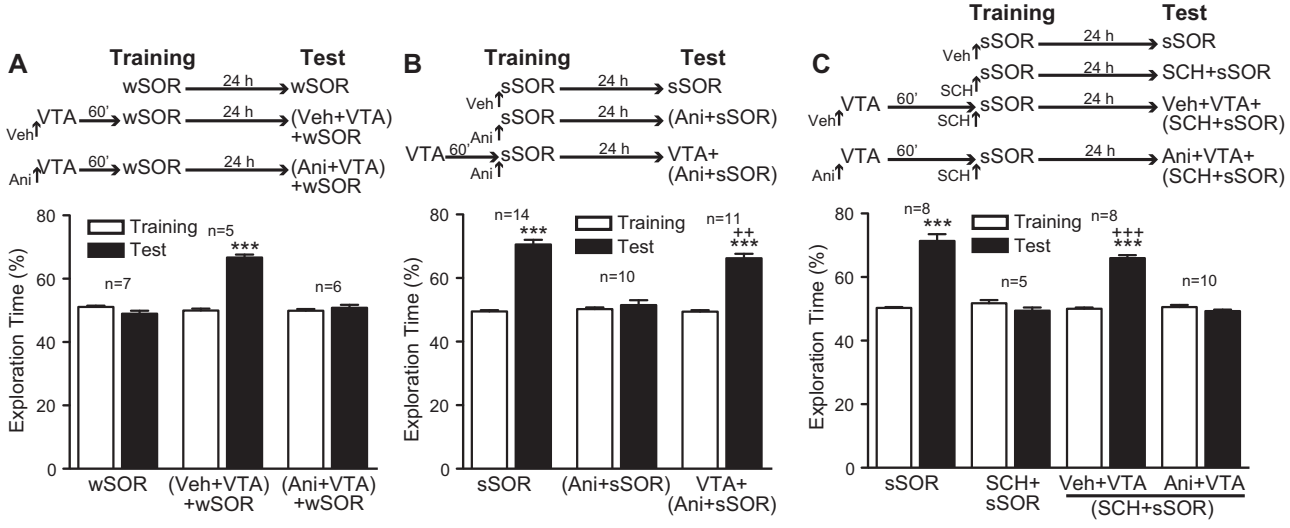
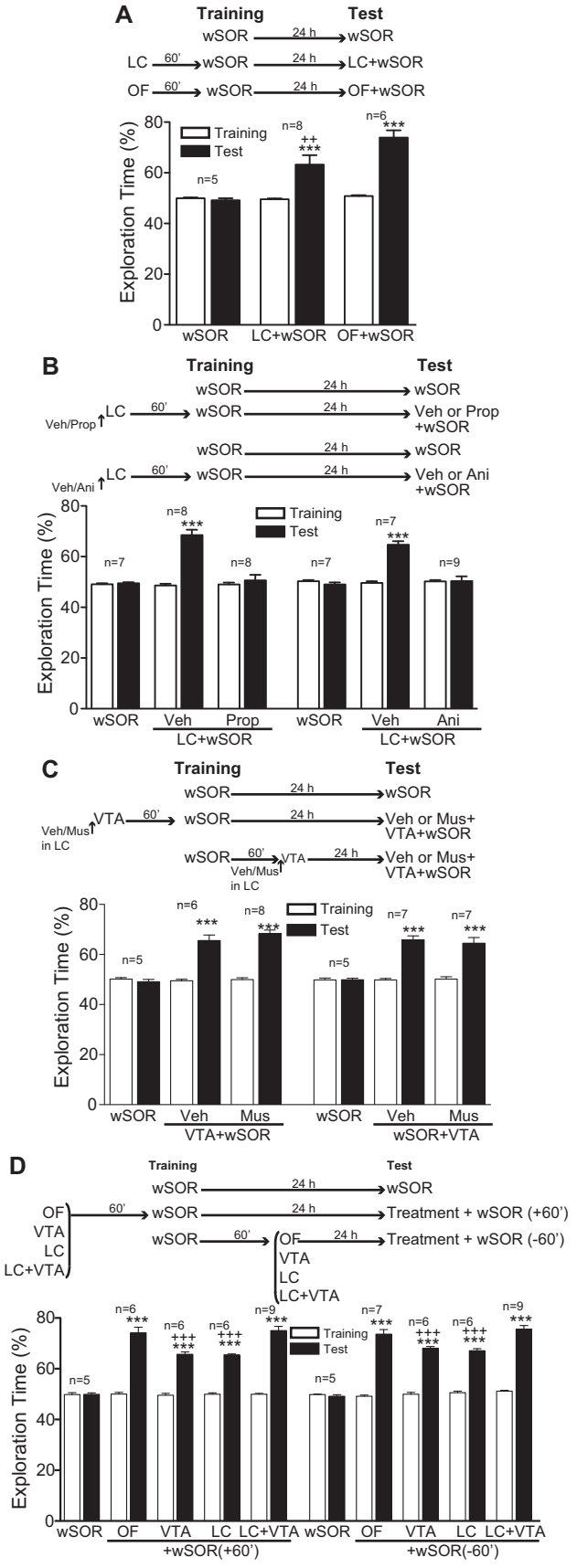


Fig. 6. VTA activation promoted SOR-LTM relays on dopaminergic receptors functionality and the synthesis of PRPs in the hippocampus. Figure shows mean ± SEM of the exploration time (in percentage), for the object moved to the novel position, during training (white) and test (black) sessions. Top: experimental design showing the moment of stimulation, drug infusion and training protocol (weak: wSOR; Strong: sSOR). (A) Ani infusion in DH impairs VTA stimulation to promote SOR-LTM. *** $p < 0.001$ vs all groups ($F = 73.73$; $df = 35$). (B) VTA stimulation prevents Ani induced amnesia. *** $p < 0.001$ vs TR and Ani+sSOR, $^{++}$ $p < 0.01$ vs Veh+sSOR ($F = 82.11$; $df = 69$). (C) VTA preventive effect over SCH induced amnesia is blocked by Ani infusion in DH. *** $p < 0.001$ vs TR, SCH+sSOR and Ani+VTA+(SCH+sSOR); $^{+++}$ $p < 0.001$ vs Veh+sSOR ($F = 71.04$; $df = 61$).



4. Discussion

The previous series of experiments performed either in the IA or SOR tasks, show that VTA and LC control protein synthesis-dependent memory consolidation in the hippocampus, through parallel and complementary mechanisms. The inadequacy of weak trainings to induce LTM for these hippocampus dependent learning tasks was overcome by the association of the weak training along with an electrical activation of VTA within a critical time window. This promoting effect of the VTA depended on the functionality of D1/D5 receptors and the synthesis of new proteins in the hippocampus. In addition, VTA activation was able to prevent the amnestic effect of local infusion of Ani or SCH in the dorsal hippocampus in the context of strong trainings, which normally induce LTM. This preventive effect was dependent on the synthesis of PRPs in the hippocampus triggered by the activation of the VTA. In the same way LC activation was able to promote IA- and SOR-LTM when it was associated to a weak training in both tasks, through a process dependent on the β-adrenergic receptors and protein synthesis in the hippocampus. Remarkably, VTA activation was able to promote these memories in a context were the LC activity was blocked by the local infusion of muscimol, and *vice-versa*. Thus, each of theses structures promoted IA- and SOR-LTM without acting over the other structure. Furthermore co-activation of VTA and LC induced, for both tasks, a "better" LTM than that observed after the sole activation of only one of them. In addition, these improved LTMs were comparable to those promoted by the exploration of a novel arena, which usually recruits the adrenergic and dopaminergic systems.

Different works have shown the requirement of D1/D5 dopaminergic receptors for the consolidation of memories, as well as for the induction of lasting synaptic changes in functional plasticity models (Frey, Matthies, Reymann, & Matthies, 1991; Furini et al., 2014; Lisman & Grace, 2005; Moncada et al., 2011; O'Carroll and Morris, 2004; Sajikumar & Frey, 2004; Smith, Starck, Roberts, & Schuman, 2005). Indeed β-adrenergic receptors have been involved in the formation of several LTMs as well (Gazarini, Stern, Carobrez, & Bertoglio, 2013; Ji, Wang, & Li, 2003; Ji, Zhang, & Li, 2003; McGaugh & Roozendaal, 2009; Sara, 2009). Moreover, later synaptic tagging experiments showed that the reinforcement of early into late-LTP by exposing animals to a spatial novelty was impaired by the infusion of SCH during the exploration of the novel environment (Li et al., 2003). Also, the functionality of these receptors was shown to be essential for the promotion of IA and event arena (schemas) LTM caused by the exploration of the novel arena, as well as for long-term extinction

Fig. 7. LC and VTA promote SOR-LTM formation through independent and complementary mechanisms. Figure shows mean ± SEM of the exploration time (in percentage), for the object moved to the novel position, during training (white) and test (black) sessions. Top: experimental design showing the moment of stimulation, drug infusion and weak training protocol (wSOR). (A) LC stimulation promotes SOR-LTM of less magnitude than OF exploration. *** $p < 0.001$ vs TR and wSOR, $^{++}$ $p < 0.01$ vs OF+wSOR ($F = 19.82$; $df = 37$). (B) LC induced SOR-LTM depends on β-adrenergic receptors functionality (Left) and protein synthesis (Right) in DH. *** $p < 0.001$ vs all groups (Left: $F = 31.05$; $df = 45$. Right: $F = 29.07$; $df = 45$). (C) LC inhibition with Muscimol (Mus) dos not impairs SOR-LTM promoted by a stimulation of the VTA performed before (left) or after (Right) a wSOR training. *** $p < 0.001$ vs TR and wSOR (Left: $F = 51.81$; $df = 37$. Right: $F = 33.13$; $df = 37$). (D) Coactivation of the VTA and the LC promotes a SOR-LTM comparable to that induced by OF exploration, with better performance than that observed in animals in which only one structure was stimulated *** $p < 0.001$ vs TR and wSOR, $^{++}$ $p < 0.01$ vs OF+wSOR and LC+VTA+wSOR (Left: $F = 99.47$; $df = 63$. Right: $F = 139.7$; $df = 65$).

of fear memories, throughout behavioral tagging mechanisms (Menezes et al., 2015; Moncada & Viola, 2007; Wang et al., 2010). This research steps forward, showing that the VTA is responsible for activating dopaminergic receptors in the hippocampus to induce the consolidation of lasting memories by promoting the synthesis of PRPs.

Furthermore, these results are consistent with recent findings from Shivarama Shetty, Gopinadhan and Sajikumar (2016) that used an experimental design based on the synaptic tagging and capture framework in hippocampus slices. They showed that D1/D5 receptors activation by SKF led to LTP reinforcement through a tagging and capture process; that the process occurred through MAPK signaling; and that the levels of dopaminergic activation and MAPK activity had direct impact in the processing of information from multiple inputs at cellular level. A sufficiently high activation level triggered a state of cooperative processing leading to late associativity phenomena. At the other end of the rope, low activation switched the processing to a competence mode that impaired the induction of late changes in any activated input. Finally, in between them, medium activation allowed strong tags to keep their lasting potentiation while weakly tagged inputs presented only early forms of plasticity (Shivarama Shetty et al., 2016). The work puts into evidence that VTA stimulation may be promoting LTM formation through a mechanism that requires MAPK activation. Moreover, as in previous works we have shown that OF exploration promotes IA-LTM through a tagging and capture process that requires D1/D5 receptors activation (Moncada & Viola, 2007) and that this promotion occurs in detriment of the LTM of habituation trace (Martinez, Alen, Ballarini, Moncada, & Viola, 2012), it is likely to think that this competition may be avoided and changed to a fully cooperative state if further dopaminergic activation is induced, for example by direct VTA stimulation.

Using an object recognition task, in which the 'memory of the object' rather than the 'memory to their location' was evaluated, Rossato et al. (2013) have recently shown that the consolidation of this memory requires D1/D5 receptors activity in the amygdala and medial prefrontal cortex but not in the hippocampus. In contrast to object in place memory, the involvement of the hippocampus in the formation of object recognition memory is less consistent (Balderas, Rodriguez-Ortiz, & Bermudez-Rattoni, 2015; Barker, Bird, Alexander, & Warburton, 2007; Barker & Warburton, 2011; Rossato et al., 2007; Winters, Forwood, Cowell, Saksida, & Bussey, 2004). However, it results interesting that in the case of the 'memory of the object' the dopaminergic system appears to be required outside the hippocampus where this memory is usually processed (Rossato et al., 2013). Thus, different aspects of object recognition memory seem to be consolidated in different brain structures using the same receptors systems. This supports the idea that the VTA activity could be controlling the storage of memories in different structures through the release of dopamine and the synthesis of PRPs.

Recent works performed in mice cortex and hippocampus, indicate that dopamine receptors distribution does not necessarily match with the topographic organization of dopaminergic terminals from VTA neurons, and show that LC terminals can be partially responsible of the dopaminergic transmission (Nomura et al., 2014; Smith & Greene, 2012). The present work demonstrates that the electrical activation of the VTA and the LC induces the formation of LTMs that depend on the activity of hippocampal D1/D5 dopaminergic and β-adrenergic receptors, respectively. In addition, their effect was independent on the functionality of the other structure at the moment of the activation and moreover, the co-stimulation of the VTA and the LC was capable of inducing better IA- and SOR-LTM than that observed by the sole activation of only one of them. Thus, while it was proposed that certain release of dopamine could be originated from LC stimulation, the present study demonstrated that a larger effect was observed when VTA is also activated. Therefore, if this were the case, not all dopaminergic receptors would be targeted by the dopamine released through the LC efferents. In the same manner, not all β-adrenergic receptors would be targeted from VTA efferents as well.

In addition to its role in the formation of memories, it has been shown that the dopaminergic system is also required for their persistence In that sense, it has been shown that a late phase of protein synthesis, occurring 12 h after training through a mechanism that depends on D1/D5 dopaminergic receptors and BDNF synthesis in the hippocampus, is required to allow the late persistence of IA-LTM (Bekinschtein et al., 2007; Rossato et al., 2009). Moreover, recent findings revealed that photostimulation of mice hippocampus dopaminergic inputs improved the later recall of neural representations revealing that midbrain dopaminergic neurons promote hippocampal network dynamics associated with memory persistence (McNamara, Tejero-Cantero, Trouche, Campo-Urriza, & Dupret, 2014). These results are consistent with the idea of the VTA as a novelty and salience detector (Lisman, Grace, & Duzel, 2011). Thus, VTA may determine not only if a specific event in its context is salient enough to trigger the release of dopamine and induce the synthesis of PRPs required to allow memory consolidation, but also to grant its persistence. Indeed, given the involvement of the LC activity in LTM formation, as well as the positive effect on memory persistence by noradrenaline infusion in the DH 12 h post training (Katche et al., 2010), it is likely that the balance of VTA and LC activation, through the release of dopamine and noradrenaline, is essential to determine whether a memory will be stored for short, long or persistent periods of time.

LC is the main source of noradrenaline of the brain (Moore & Bloom, 1979; Sara, 2009). This neurotransmitter, acting through the β-adrenergic receptors of the Amygdala, has been shown to play a central role in the modulation of LTMs (Cahill & McGaugh, 1996). While the infusion of adrenergic agonists in the amygdala is able to enhance LTM formation for MWM and IA, among other tasks, the inhibition of this structure do not impair the formation (McDonald & White, 1993; McDonald et al., 2010) or expression of these memories (Izquierdo et al., 1997; Packard, Cahill, & McGaugh, 1994; Parent, West, & McGaugh, 1994). Thus, leaving a preferential role for this structure as neuromodulator rather than final storage site of those memories (McIntyre, McGaugh, & Williams, 2012). On the other hand, we have shown than β-adrenergic receptors functionality in the hippocampus is required to consolidate IA-LTM (Moncada et al., 2011). Indeed the amnesia induced by propranolol infusion in this structure can be overcome by previous activation of β-adrenergic receptors in this structure, either pharmacologically (Moncada et al., 2011) or by electrical activation of the LC as shown here. These results show that the LC also influences directly on memory formation acting through β-adrenergic receptors of the hippocampus. Therefore, the modulator effects of the LC through the BLA pathway might act as a complementary process allowing the formation of enhanced LTMs.

In light of the present findings, it worth considering that other neuromodulator systems may be also involved in the BT process. In example, the serotoninergic and cholinergic systems also play important roles in memory and functional plasticity processes and their modulation. Indeed, depending the kind of memory evaluated and the subtype of activated receptors, they produce positive or negative effects on memory and act over the acquisition or the storage phase of the process (Giovannini, Lana, & Pepeu, 2015; Hasselmo, 2006; Stiedl, Pappa, Konradsson-Geuken, & Ogren, 2015; Zhang & Stackman, 2015). Thus, future experiments performing direct activation of the raphe nuclei or the medial septum (main sources serotonin and acetyl choline of the hippocampus and cortices – (Azmitia & Segal, 1978; Frotscher & Leranth, 1985)

could be used to unveil if they are involved or not regulating the core functions of the BT process.

In summary, this research shows that the VTA and LC, two major brain sources of catecholamines, regulate the formation of different hippocampus dependent LTMs. It provides the first evidence of independent and complementary mechanisms involving the activity of dopaminergic and nor-adrenergic systems in a target structure that regulate the synthesis of PRPs required during the BT process to allow the consolidation of lasting memories. This discovery opens a window to understand in which context, the association of a learning with another event capable of modifying the VTA and/or the LC function could lead to: the promotion of an otherwise inexistent LTM, to the blockade of a usually induced LTM, or to modulate a LTM. All this depending on the final impact in the PRPs synthesis resulting of the activation or inhibition of these brain structures.

Acknowledgments

The author thanks to Dr. Haydée Viola, and Dr. Pedro Beckinstein for their helpful comments and discussion of the manuscript; to Miss Silvia Wievig for her helpful technical advice and special thanks to Miss Jeanette Maiwald for her excellent technical assistance and constant help. This work was supported by LIN-Magdeburg, ANPCyT and CONICET.

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