Dopamine neurotransmission in the VTA regulates aversive memory formation and persistence

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ABSTRACT

Dopamine (DA) neurons in the ventral tegmental area (VTA) innervating several limbic and neocortical regions of the mammalian brain have long been implicated in motivation, rewarding and aversive behaviors, and memory processing. Recently, we demonstrated that somatodendritic release of DA in the VTA regulates the formation and maintenance of appetitive long-term memories (LTM). However, less is known about the impact of DA neurotransmission in the VTA on aversive LTM. Here, we describe the modulation of negative-valence memories by D1/D5-type DA (D1R)-receptor-mediated neurotransmission in the VTA. As aversive stimuli elicit both active and passive behavioral responses, we used two single-trial aversive training protocols: inhibitory avoidance task and conditioned place aversion. We bilaterally microinfused SCH23390, an antagonist of D1R, into the VTA immediately after training and found that DA neurotransmission in the VTA modulates LTM consolidation and persistence of aversive experiences. Together with previous findings demonstrating that D1R-mediated DA neurotransmission in the medial prefrontal cortex and hippocampus is involved in the formation and persistence of LTM for aversive events, our present results indicate that memory processing of environmental stimuli with negative-valence depends on the integration of information mediated by D1R activation in both the VTA region and in selected downstream target areas.

1. Introduction

Dopaminergic neurons arise from the ventral tegmental area (VTA) and substantia nigra compacta (SNc) and are part of the key circuits that regulate rewarded behaviors [1,2] together with other important brain structures such as the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC). VTA neurons release dopamine (DA) not only onto their target areas but also in the VTA region itself. This process is known as 'somatodendritic release of DA' and can modify the firing of DA neurons and therefore, the release of DA in target structures, inducing thereby behavioral changes [2,3]. Several studies suggest that different functions of the VTA are mediated by diverse subpopulations of VTA DA neurons that are associated with distinct neuronal networks [4]. It has been previously reported that these neurons show differences in their afferent but also efferent connectivity [5]. Much attention has been focused on VTA DA neurons given their importance for the neuroplasticity associated with rewarding behaviors [6,7] and their projections target on NAc [8–10]. However, the role of DA neurons in the control of aversive learning and memory is less understood. Previous studies have shown that aversive or stressful events activate VTA DA neurons and cause DA release in the mPFC [11,12]. DA activation of the mPFC can generate a long-lasting aversive response [13] influencing behavioral responses to aversive stimuli [14]. In addition, a sparse dopaminergic projection originating mainly from the midbrain and including the VTA, projects directly to the hippocampus (Hip) modulating aversive memory processing [15–18]. In the present study, we uncover the role of DA signaling in the VTA for the formation and persistence of two different aversive long-term memories (LTM) induced by two different tasks, inhibitory avoidance (IA) and conditioned place aversion (CPA). In addition, we show the role of hippocampal D1R signaling 6 h after conditioning with the aversive agent LiCl in a CPA task. These findings highlight the important role of somatodendritic release of DA in the VTA on aversive memory processing and endorse the idea that providing proper control of DA signaling is essential for the

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physiological control of many adaptive behaviors. Further studies will be necessary to characterize the molecular mechanisms underlying the interaction of these two structures during the processing of aversive memories.

2. Materials and methods

2.1. Animals

A total of 142 Wistar male rats were used in these studies. Groups of 3 rats (weighting around 200 g upon arrival at the laboratory) were housed in an animal vivarium maintained on a 12 h direct light-dark cycle (lights on at 7:00 h) at a constant temperature of 21 °C. Experimental procedures followed the guidelines approved by the Animal Care and Use Committee of the University of Buenos Aires (CICUAL).

2.2. Drugs

Dopamine D1R antagonist SCH 23,390 hydrochloride (1.5 μg/μl, Sigma-Aldrich, Germany) and Lithium Chloride (LiCl, 150 mg/ml/kg, Cicarelli, Argentina) were dissolved in sterile 0.9% physiological saline. The doses used were determined based on previous studies showing their effect on learning or behavioral performance [19–21].

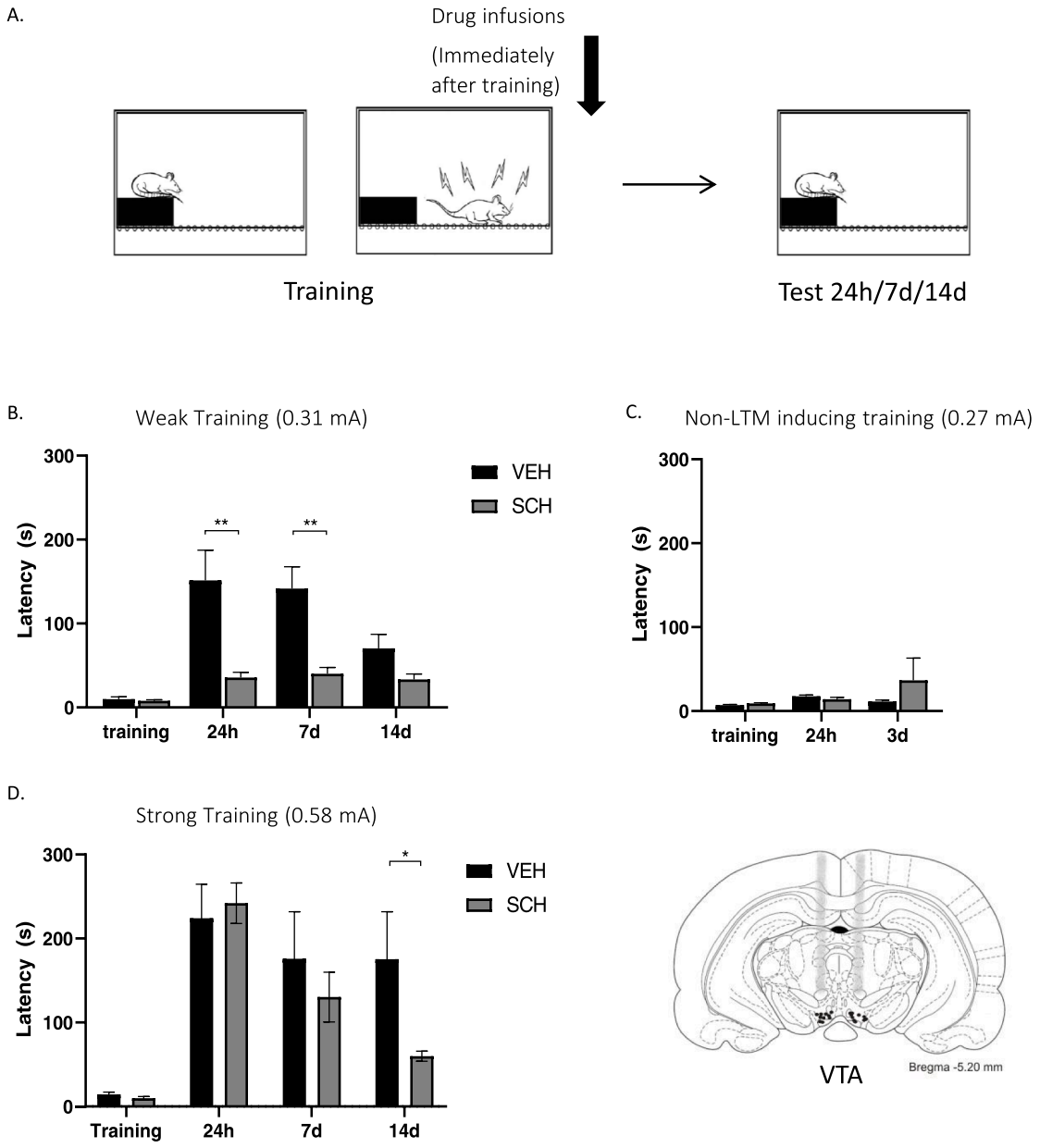

Fig. 1. Inhibition of the D1/D5 receptors impairs aversive LTM in a training strength dependent manner. (A) Schema of the behavioral protocol used. (B) Dopaminergic blockade in the VTA immediately after a 0.31 mA IA training impairs LTM when measured at 24 h and 7 days later. Lower memory expression was found 14 days later for both groups being not significantly different (Tukey *post-hoc* analysis after two-way repeated measures ANOVA$_{(2,26)}$, $F_{(time*infusion)} = 1.810$, $p = 0.18$; $F_{(time)} = 2.23$, $p = 0.12$; $F_{(infusion)} = 27.70$, $p < 0.001$). (C) A 0.27 mA footshock does not generate aversive LTM. (D) Dopaminergic blockade in the VTA immediately after a 0.58 mA IA training does not impair memory formation. Animals showed LTM at 24 h but memory persistence decayed faster when dopaminergic receptors were blocked after training compared to the control group (Tukey *post-hoc* after two-way repeated measures ANOVA $_{(2,20)}$, $F_{(interaction)} = 4.977$, $p = 0.0176$; $F_{(time)} = 16.59$, $p < 0.001$; $F_{(infusion)} = 0.771$, $p = 0.40$). Tukey, *$p < 0.05$; **$p < 0.01$.

2.3. Surgical and intracerebral infusion procedures

Each rat was anesthetized with a mix of ketamine (85 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally (i.p.) and placed in a stereotaxic frame. The skull was exposed and aligned (flat skull, lambda and Bregma at the same elevation degree) and 22-G guide cannulae (measuring 1 cm length) for intracerebral infusions were bilaterally implanted aiming at different structures. The stereotaxic coordinates used for the different structures were as follows: for VTA: AP −5.3 mm/*L* ± 1 mm/DV - 7.2 mm; For Hippocampus: AP −3.90 mm/*L* ± 3.00 mm/DV - 3.00 mm from Bregma [22]. Cannulae were fixed to the skull with acrylic cement. After surgery, animals were injected with a single dose of meloxicam (0.2 mg/kg) as analgesic and were left in their home cage during 1 week for recovery. For intracerebral infusions, 30-G needles connected to Hamilton syringes were used (1.2 cm length for the VTA, 1.1 cm length for the HP). The infusions were always bilateral with 0.5 μl for the VTA and 1 μl for the Hippocampus (injection rate: 1 μl/30 s). The needle was left in place for an additional minute after infusion to allow diffusion and to prevent reflux. At the end of each experiment, the placement of cannulae was verified by infusions of 0.5 μl for VTA and of 1 μl for Hip of 4% methylene blue in saline. Animals were decapitated after 15 min and histological localization of the infusion site was established. The extension of the dye infused was taken as indicative of the presumable diffusion of the drugs previously given to each animal. Infusions spread with a radius ranging from 1 to 1.2 mm^3 depending on the volume infused [19,23]. Animals with both cannulae in the correct place were included in the study (Schemes in Figs. 1 and 2). For simplicity, animals showing the same cannulae placement were indicated only once in the scheme. Four animals were excluded from the analysis following this criterion.

2.4. Behavioral paradigm

2.4.1. Inhibitory avoidance (IA)

A single-trial conditioning was used in this task. During training (Fig. 1A), rats were placed on a 5 cm high, 9 cm wide platform placed on the left of a 47 × 25 × 30 cm^3 opaque acrylic box, with a grid floor [18,24]. As they stepped down onto the grid with all four paws, they received a 3-s scrambled foot shock depending on the group: around 0.6 mA, 0.31 mA or 0.27 mA. Latency to step down was measured in

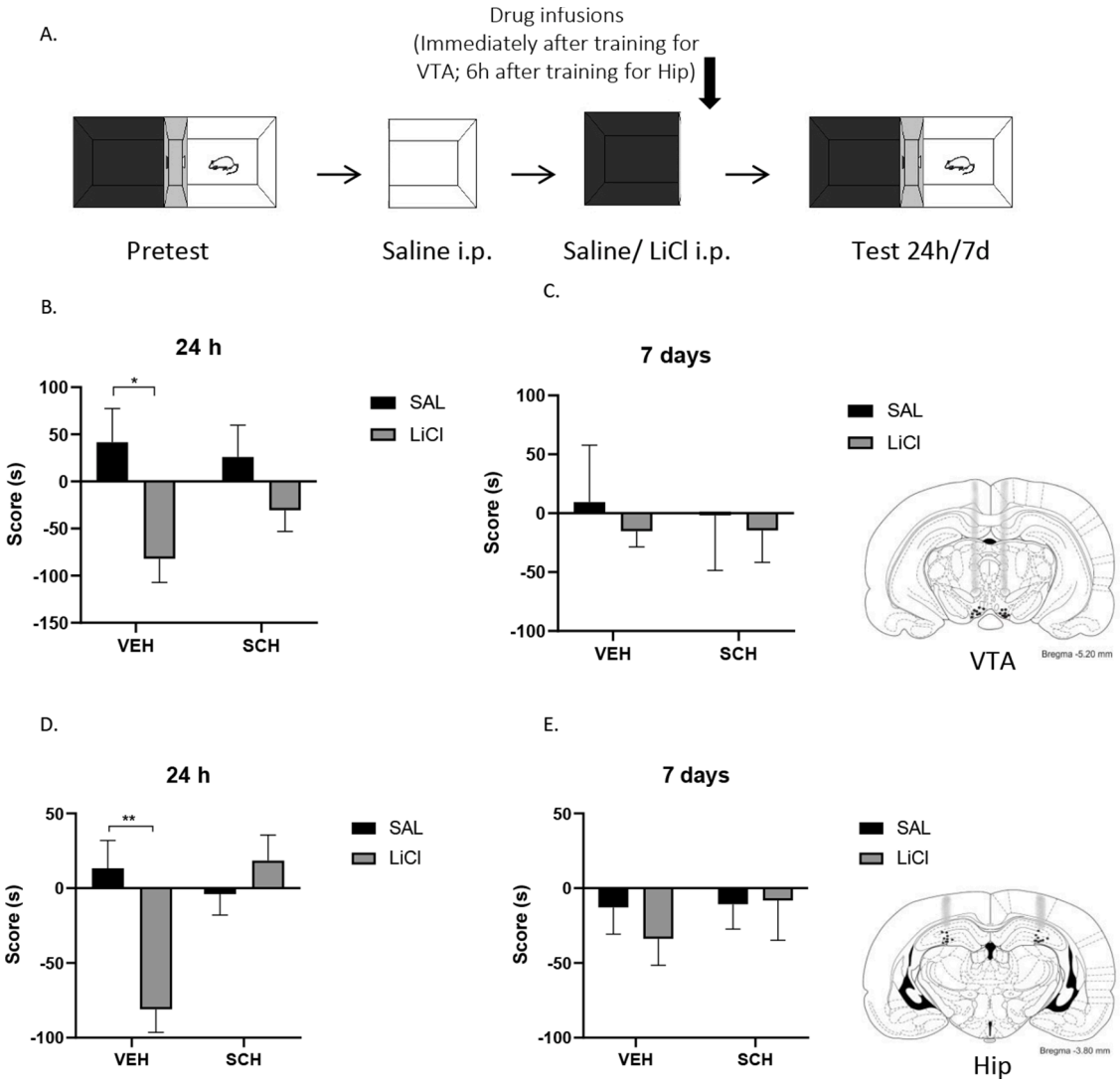

Fig. 2. Inhibition of D1/D5 receptors impairs long-term aversive conditioning in a temporal and anatomical dependent manner. (A) Schema of the protocol used. (B-C) Dopaminergic blockade in the VTA immediately after LiCl conditioning impairs LTM at 24 h without altering memory durability (For 24 h: Tukey *post-hoc* after two-way $ANOVA_{(1,36)}$, $F_{(interaction)} = 1.355$, $p = 0.25$; $F_{(conditioned\ drug)} = 9.736$, $p = 0.0036$, $F_{(infusion)} = 0.38$, $p = 0.54$). (D-E) Dopaminergic blockade in the Hip 6 h after LiCl conditioning impairs LTM at 24 h but does not alter memory durability (For 24 h: Tukey *post-hoc* analysis after two-way $ANOVA_{(1,30)}$, $F_{(interaction)} = 12.36$, $p = 0.0014$, $F_{(conditioned\ drug)} = 4.69$, $p = 0.03$, $F_{(infusion)} = 6.10$, $p = 0.019$). Tukey, $^{*}p < 0.05$,$^{**}p < 0.01$.

seconds. Immediately after training, rats were infused with vehicle or D1/D5 dopaminergic receptor's antagonist SCH23390 into the VTA. Rats were tested at 24 h, 7 days and 14 days after training. During test, no foot-shock was given.

2.4.2. Conditioned place aversion (CPA

A single-trial conditioning was also used in this task (Fig. 2A). Place conditioning experiments were carried out using a three-compartment apparatus [19]; the central compartment was a short-connecting passageway between two other compartments. One of them had black walls, white-square patterns, and a grid floor, whereas the other one had white walls, black-line pattern, and a perforated floor. All experiments were independent and carried out with different groups of animals. Mainly, the protocol consisted of three phases (Fig. 2A): a pretest in which the animals were allowed to explore the entire apparatus for 15 min and the preference for each compartment was determined by measuring the time spent in each compartment, a conditioning phase performed 24 h after the pretest in which animals were maintained in one of the compartments (white compartment) immediately after saline i.p. injections and, on the next day, confined in the other compartment (black compartment) immediately after saline/LiCl i.p injections. Animals thus learned the white compartment as safe and the black compartment as safe or as aversive due to the unpleasant consequences of LiCl injections. Immediately or 6 h after removing them from this compartment, animals were infused with SCH23390 into the VTA or the Hip according to the experiment. The LiCl dose used was enough to produce a significant discomfort in the animals for the aim of this study [25,26]. An increase in the dose or in the number of injections applied would have resulted in extremely painful or stressful situations for the animals. This methodological limitation was the main reason for not to carrying out a stronger CPA training. Moreover, a strong multi-trial training involves several conditioning sessions which could provoke a long-lasting aversive state that could lead to retrieval interference within the consolidation of the memory trace during the subsequent training sessions. Results were analyzed using the score corresponding to the time spent in the compartment that was followed by a drug infusion in the brain minus the time spent in that compartment on the pretest. Exploration time was measured by a blind subject seating near the apparatus but not visible to the rats. Intracerebral injections were performed on a separate table, distant from the CPA apparatus. No animal was conditioned at that moment. Finally, the test phase was carried out 24 h or 7 days after conditioning, allowing the animals to explore freely the entire apparatus for 15 min during which the time spent in each compartment was also measured. During the pretest phase, animals that spent less than 90 s in any of the compartments were excluded. One animal out of 86 was excluded from the analysis based on this criterion.

2.5. Statistical analysis

For the IA, the latency spent before stepping down of the platform is presented in seconds (s). For the CPA task, a score in seconds (s) was calculated as the time spent in the LiCl-associated compartment minus the time spent in the to-be LiCl-associated compartment during the pretest. Results were presented as mean ± SEM. Data were analyzed using two-way analysis of variance (ANOVA) or two-way repeated measures ANOVA. Conditioning drug and modulating drug were included as statistical factors within a 2 × 2 design for CPA. In cases of significant interaction, post hoc analyses were made using Tukey test when the p value of one or two factors was significant. In cases in which the interaction was non-significant, a main effect ANOVA was run. A result was considered significant when $p < 0.05$. All data were analyzed using Graphpad and InfoStat software.

3. Results

3.1. D1/D5 dopamine receptors in the VTA regulate IA long-term memory

DA neurotransmission in target structures like the mPFC and Hip is involved in aversive processes [18,27–29] and aversive or stressful events excite VTA DA neurons (see [12] for references). One-trial IA is a simple, brief training task that is rapidly acquired and induces an aversive LTM lasting several days. It is therefore suitable for investigating time-dependent mechanisms initiated by training without the possible interference of retrieval of the learned response that occurs in multi-trial tasks [18,24,30]. Since the activation of VTA D1R modulates the firing rate of the DA-releasing neurons [31], we infused the D1R antagonist SCH 23,390 (0.5 μl per side) immediately after a single IA trial (0.3 mA footshock, Fig. 1A) into the VTA and tested IA LTM at 1, 7 and 14 days after training (Fig. 1B). Blockade of VTA D1R immediately after IA training impaired LTM at 1 and 7 days. No differences in retention scores were found 14 days after training, given that the weak training protocol induced low retention performances at this prolonged delay (Tukey *post-hoc* analysis after two-way repeated measures $ANOVA_{(2,26)}$, $F_{(time*infusion)} = 1.810$, $p = 0.18$; $F_{(time)} = 2.23$, $p = 0.12$; $F_{(infusion)} = 27.70$, $p < 0.001$). The bilateral infusion of SCH 23,390 into the VTA had no effect when IA training did not induce LTM due to the use of a lower shock intensity (Two-way repeated measures $ANOVA_{(1, 33)}$, $F_{(time*infusion)} = 0.98$, $p = 0.32$, $F_{(time)} = 0.55$, $p = 0.46$, $F_{(infusion)} = 0.31$, $p = 0.58$) (Fig. 1C). The duration of IA LTM can be adjusted by modifying the foot shock strength at the moment of training. A strong foot-shock (0.6 mA) can induce a long-lasting aversive memory that can be expressed up to 14–28 days after training [18,32]. As shown in Fig. 1D, D1R blockade in the VTA did not impair aversive memory formation. LTM was not affected at tests carried out at 1 and 7 days, however, memory persistence was lower 14 days after training in animals that received the infusion of D1R antagonist with respect to the control group (Tukey *post-hoc* after two-way repeated measures $ANOVA_{(2,20)}$, $F_{(interaction)} = 4.977$, $p = 0.0176$; $F_{(time)} = 16.59$, $p < 0.001$; $F_{(infusion)} = 0.771$, $p = 0.40$).

3.2. D1/D5 dopamine receptors in the VTA regulate the formation of CPA LTM

In previous studies, we demonstrated that appetitive cocaine-associated memories are regulated at different time points by both VTA and Hip affecting different memory phases. While dopaminergic neurons in the VTA participate in both, memory formation and active forgetting processes of cocaine-associated conditioned place preference (CPP) [23], the Hip is activated at a later time point (12 h after cocaine-conditioning) regulating cocaine-memory persistence phase [19]. Therefore, to further investigate if DA neurotransmission in the VTA guides adaptive behavior elicited by aversive stimuli, we studied the effect of VTA D1R blockade on CPA memory formation and persistence. While LiCl-injected animals expressed an aversive memory 1 day after training (Fig. 1B, "VEH"), the bilateral infusion of SCH23390 into the VTA immediately after LiCl conditioning impaired LTM at the same period (Fig. 2B, "SCH". Tukey *post-hoc* after two-way $ANOVA_{(1,36)}$, $F_{(interaction)} = 1.355$, $p = 0.25$; $F_{(conditioned\ drug)} = 9.736$, $p = 0.0036$, $F_{(infusion)} = 0.38$, $p = 0.54$, $n = 9,12$ depending on the group). D1R blockade in the dorsal Hip 6 h after the conditioning phase also impaired long-term CPA memory when tested 24 h later (Fig. 2D. Tukey *post-hoc* analysis after two-way $ANOVA_{(1,30)}$, $F_{(interaction)} = 12.36$, $p = 0.0014$, $F_{(conditioned\ drug)} = 4.69$, $p = 0.03$, $F_{(infusion)} = 6.10$, $p = 0.019$, $n = 9$ per group) indicating participation of hippocampal DA neurotransmission in aversive memories several hours after training, as it occurs with cocaine-associated CPP [23] and IA training [18,27]. No differences were found between infused and conditioned animals 7 days after training (Fig. 2C, 2E).

4. Discussion

In the present study we studied the control of aversive-memory consolidation and persistence by D1R neurotransmission in the VTA and Hip during early and several hours after acquisition stages of memory processing, respectively. The present findings have important implications to understand how negative experiences can be stored and persist, and to determine which neural structures and brain circuits might be involved in the processing of traumatic memories, crucial for developing therapeutic interventions. Our present findings suggest two different time points that might be critical for this type of memory processing. Immediately after acquisition, VTA is necessary for the formation of conditioned place aversion memory and for the formation but also for the persistence of memories induced by another aversive task such as the IA task. In addition, and extending previous findings, we have shown that 6 h after a CPA acquisition the hippocampal D1R signaling is also required for aversive memory formation, coinciding with a peak of DA levels in the Hip [17]. Several years ago, a functional loop was described between these two structures where new information is processed and sent into long-term storage [18,33]. We propose that DA in the VTA is involved in aversive memory processing immediately after acquisition while DA signaling in the Hip is important to trigger LTM storage from 6 h after training.

The role of VTA DA neurons in aversion was first suggested when electrophysiological studies demonstrated that these neurons were activated by rewarding stimuli and inhibited by aversive events. Several years later it was shown that there are subpopulations of DA neurons in the VTA that are excited by aversive stimuli or cues that predict their appearance (see [34]). Many of the terminal fields of midbrain DA neurons in limbic and cortical regions of the brain participate in avoidance learning and memory, in adaptive passive or active behaviors critical for the survival of organisms [35–38]. Our present findings add new evidence to the already complex picture of DA neurotransmission and aversion. Our experiments do not allow us to establish the mechanisms by which the dopaminergic transmission in the VTA system controls aversive behaviors. The most parsimonious explanation is that the blockade of D1R in the VTA prevents the action of the so-called somatodendritic release of DA on that region [2,3,39]. Some DA released by collaterals of axons coming from DA neurons in the substantia nigra may also contribute to the effects observed in the present study. We also cannot rule out the possibility that D1R localized to astrocytes into the VTA might be involved in controlling the consolidation of aversive LTM. In fact, Gomez and coworkers (2019) [40] found that VTA astrocytes regulate real-time learned avoidance in mice and it was recently demonstrated that D1R are localized into accumbal astrocytes [41].

The experiments carried out with strong and weak IA training protocols suggest that the regulation of IA memory formation and persistence by VTA D1R-mediated activation depends, at least in part, on the strength of the aversive stimulus. Due to a persistent malaise induced by strong CPA protocols, this idea could not be substantiated in a second aversive task. Regarding the methodology used in this study, we consider important to stress out that hippocampal lesions impair both the acquisition and expression of trace fear memory, possibly because the hippocampus is required for maintaining a memory trace of the CS during the trace interval [42,43], although other explanations have been proposed [44]. Importantly, it has been shown that when the hippocampus is lesioned prior to a classical fear conditioning training, animals often acquire normal levels of contextual fear, presumably because extra-hippocampal learning mechanisms can compensate. In addition, there is a minimum area that should be lesioned to properly alter a behavioral outcome which in this case is not goaled by the cannulae placement [45–47]. Importantly, both control and experimental groups have received the same surgeries and consequently we rule out the possibility that the behavioral observations made in this study could be due to lesions of brain structures along the cannulae placement.

Two different mechanisms may explain the amnesic effect of intra VTA SCH23390 infusion on IA and CPA LTM: 1- the blockade of D1R-mediated neurotransmission controls the release of glutamate from glutamatergic inputs to dopaminergic neurons that project to regions related to aversion; microdialysis data have shown that D1R agonists increase the extracellular level of glutamate in the VTA [31]. This increase was blocked by co-infusion of SCH23390. Mesocortical DA pathway is uniquely sensitive to aversive stimuli tuning mPFC neurons projecting to downstream brain regions for orchestrating aversive responses [14]. In this context, it is interesting to stress that a similar impairing effect on IA LTM persistence (Fig 1D) was observed after VTA infusion of AP5, the specific antagonist of NMDA receptors [18]; and 2- D1R is located to presynaptic terminals of GABA afferents to the VTA [48]; the blockade of D1R modifies the release of GABA and thus inhibits DA neurons involved in the reward circuit [40,49,50]. These two mechanisms are not mutually exclusive.

Given that animals were tested more than once in the IA experiments, it could be thought that memory performance could be down-regulated because of memory extinction. However, several published articles also suggest that an extinction protocol after a strong training for an IA task requires at least 5–6 non-reinforced tests during subsequent days to induce effective extinction. In our study, memory was already altered at the second non-reinforced test (performed 7 days after training), thus ruling out pure extinction accounts as it would be unusual to find out extinction effects after only one test.

D1R family is composed by D1 and D5 subtypes of DA receptors. It has been shown that D5 subtypes in the VTA is localized to cell bodies of DA neurons [51,52]. Since the D1R antagonist SCH23390 does not distinguish between D1 and D5 subtypes, the effects observed in the present study may also involve the inhibition of D5 subtype receptors on DA neurons.

5. Conclusion

In conclusion, we propose that VTA DA neurons are involved in aversive memory processing from the initial moments after acquisition of new information, while the Hip DA neurons are important to trigger LTM storage mechanisms 6 h after the acquisition phase. These findings have important implications for understanding how negative experiences can be stored and persist. They are crucial to design therapeutics interventions related to brain areas and circuits involved in the processing of aversive or traumatic memories.

Author contributions

All the experiments were designed by Jorge H. Medina and Fernando Castillo Díaz, and were carried out by Fernando Castillo Díaz, Juliana F. Dalto and Magdalena Pereyra. The manuscript was written by Fernando Castillo Díaz and Jorge H. Medina with corrections and contributions by Juliana F. Dalto and Magdalena Pereyra. All authors read and approved the final manuscript.

Ethics approval

Experimental procedures in this study followed ethical guidelines and were approved by the Animal Care and Use Committees of the University of Buenos Aires (CICUAL).

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability

The datasets generated and analyzed during the current study are not

publicly available in a repository but can made available upon reasonable request to the corresponding author.

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