Neurobiology of Learning and Memory 180 (2021) 107423

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

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

Inhibition of PACAP/PAC1/VPAC2 signaling impairs the consolidation of social recognition memory and nitric oxide prevents this deficit

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ARTICLE INFO

Keywords:
PACAP
Nitric oxide
Hippocampus
Amygdala
Consolidation
Social recognition memory

ABSTRACT

Social recognition memory (SRM) forms the basis of social relationships of animals. It is essential for social interaction and adaptive behavior, reproduction and species survival. Evidence demonstrates that social deficits of psychiatric disorders such as autism and schizophrenia are caused by alterations in SRM processing by the hippocampus and amygdala. Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and its receptors PAC1, VPAC1 and VPAC2 are highly expressed in these regions. PACAP is a pleiotropic neuropeptide that modulates synaptic function and plasticity and is thought to be involved in social behavior. PACAP signaling also stimulates the nitric oxide (NO) production and targets outcomes to synapses. In the present work, we investigate the effect of the infusion of PACAP-38 (endogenous neuropeptide and potent stimulator of adenylyl cyclase), PACAP 6–38 (PAC1/VPAC2 receptors antagonist) and S-Nitroso-N-acetyl-DL-penicillamine (SNAP, NO donor) in the CA1 region of the hippocampus and in the basolateral amygdala (BLA) on the consolidation of SRM. For this, male Wistar rats with cannulae implanted in CA1 or in BLA were subjected to a social discrimination paradigm, which is based on the natural ability of rodents to investigate unfamiliar conspecifics more than familiar one. In the sample phase (acquisition), animals were exposed to a juvenile conspecific for 1 h. Immediately, 60 or 150 min after, animals received one of different pharmacological treatments. Twenty-four hours later, they were submitted to a 5 min retention test in the presence of the previously presented juvenile (familiar) and a novel juvenile. Animals that received infusions of PACAP 6–38 (40 pg/side) into CA1 immediately after the sample phase or into BLA immediately or 60 min after the sample phase were unable to recognize the familiar juvenile during the retention test. This impairment was abolished by the coinfusion of PACAP 6–38 plus SNAP (5 μg/side). These results show that the blockade of PACAP/PAC1/VPAC2 signaling in the CA1 and BLA during a restricted post-acquisition time window impairs the consolidation of SRM and that the SNAP is able to abolish this deficit. Findings like this could potentially be used in the future to influence studies of psychiatric disorders involving social behavior.

1. Introduction

Social Recognition Memory (SRM) refers to the ability to identify and recognize a conspecific (Ferguson et al., 2002). It forms the basis of social relationships of animals, since discrimination between familiar and novel conspecifics is essential for the choice of appropriate behaviors, social interaction, reproduction and survival (Gabor et al., 2012; Garrido Zinn et al., 2016; Gheusi et al., 1994; van der Kooij & Sandi, 2012).

Previous studies have shown that SRM requires the participation of several brain regions such as amygdala, hippocampus, medial prefrontal cortex and anterior cingulate cortex (Kogan et al., 2000; Suzuki et al.,

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https://doi.org/10.1016/j.nlm.2021.107423
Received 14 December 2019; Received in revised form 18 February 2021; Accepted 3 March 2021
Available online 9 March 2021
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2011; Tanimizu et al., 2017). Indeed, it has been demonstrated that SRM can be modulated by the β-noradrenergic, D1/D5-dopaminergic and H2-histaminergic receptors in the hippocampus and basolateral amygdala (BLA) (Garrido Zinn et al., 2016). Besides these, other neurotransmitters and also neuropeptides have been shown to play an important role in SRM (Bielsky & Young, 2004; Griffin & Taylor, 1995; Loiseau et al., 2008; Marino et al., 2005; Meyer-Lindenberg, 2008; Millan et al., 2007; Ross & Young, 2009).

Pharmacological and genetic manipulations suggest that Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is strongly implicated in the social behavior modulation (Donahue et al., 2016; Hattori et al., 2012; Ishihama et al., 2010; Takuma et al., 2014). PACAP is a pleiotropic neuropeptide belonging to the vasoactive intestinal polypeptide (VIP)/glucagon/secretin family that modulates synaptic function and plasticity through three G-protein-coupled (GPCR) receptors (Cabezas-Llobet et al., 2018; Jayakar et al., 2014; Kondo et al., 1997; Roberto et al., 2001; Starr & Margiotta, 2017). The VPAC1/VPAC2 receptors have comparable affinity for PACAP and VIP (Arimura, 1998; Hashimoto et al., 2006; Hattori et al., 2012; Joo et al., 2004; Pantaloni et al., 1996; Roberto & Brunelli, 2000; Spengler et al., 1993; Vaudry et al., 2009; Yang et al., 2010), whereas the affinity of PAC1 receptor for PACAP is much higher than that for VIP (Harmar et al., 1998; Hirabayashi et al., 2018; Iemolo et al., 2016; Miyata et al., 1989, 1990; Pedersen et al., 2019). In the brain, PACAP receptors are widely expressed in regions involved with learning and memory, such as hippocampus and amygdala (Hashimoto et al., 1996; Hirabayashi et al., 2018; Joo et al., 2004; Sheward et al., 1995; Shioda et al., 1997; Usdin et al., 1994; Vaudry et al., 2009; Zhou et al., 2002).

We have previously demonstrated that PACAP/PAC1/VPAC2 signaling in the CA1 region of hippocampus and basolateral amygdala modulates the consolidation and extinction of the contextual fear conditioning memory through N-methyl-D-aspartate glutamate receptors (NMDAR) (Schmidt et al., 2015). The activation of NMDAR produces several effects, including the induction of nitric oxide (NO) production (Bredt & Snyder, 1989; Garthwaite, 2018; Neitz et al., 2014; Qiu & Knöpfel, 2007; Regehr et al., 2009; Zorumski & Izumi, 1998). The NO is a gaseous neurotransmitter that plays an important role in synaptic transmission, behavior and memory (Akar et al., 2014; Böhme et al., 1993; Jüch et al., 2009; Weitzdoerfer et al., 2004). Studies have shown that PACAP signaling leads to activation of neuronal nitric oxide synthase enzyme and to a consequent increase of NO production, which acts as a retrograde messenger to enhance presynaptic acetylcholine release (Jayakar et al., 2014; Pugh et al., 2010).

As well as PACAP, it has been suggested that NO also participates in the pathophysiology of neuropsychiatric disorders in which the social recognition is impaired, such as autism and schizophrenia (Henningsson et al., 2015; Tanda et al., 2009; Trevlopoulou et al., 2016; Wass et al., 2009). Therefore, in this work we investigate the participation of PACAP/PAC1/VPAC2 signaling in the CA1 region of the dorsal hippocampus and in the basolateral amygdala on the consolidation of SRM, as well as the relationship between PACAP and NO in both brain regions on this memory.

2. Materials and methods

2.1. Animals

Adult (3-month old) and juvenile (22–30 postnatal days) male Wistar rats (CrlCembe:WI) purchased from the Centro de Modelos Biologicos Experimentais (CeMBE) of the Pontifical Catholic University of Rio Grande do Sul (PUCRS) were used. They 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 the experimental procedures were approved and performed in accordance with guidelines of the Animal Committee on Ethics for the care and use of laboratory animals of PUCRS, in compliance with USA National Institutes of Health Guide for the care and use of laboratory animals. The sample size (n) for each experimental group/condition is indicated in the figure legends and was based on our previous experiments (Canto de Souza et al., 2017; Ferreira et al., 2019; Garrido Zinn et al., 2016; Schiavi et al., 2019).

2.2. Surgery

Under deep anesthesia (75 mg/kg ketamine plus 10 mg/kg xylazine; intraperitoneally), the adult animals were implanted bilaterally with stainless steel 22-gauge guide cannulae through stereotaxic procedures. The tips of the cannulae were aimed 1 mm above the CA1 region of the dorsal hippocampus (CA1; anterior, −4.2 mm; lateral, ±3.0 mm; ventral, −1.8 mm; from Bregma) or the basolateral amygdala (BLA; anterior, −2.4 mm; lateral, ±5.1 mm; ventral, −7.5 mm; from Bregma) according to Paxinos and Watson (1986). All the animals were allowed 7 days to recover from surgery prior to experimental 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. Social discrimination paradigm

The social recognition memory was assessed by the social discrimination paradigm as previously described (Cavalcante et al., 2017; Garrido Zinn et al., 2016). The apparatus used was an open-field arena with a frontal glass wall (60 × 40 × 50 cm) placed in a dimly illuminated room. Two identical transparent acrylic cylinders (9 cm diameter × 13 cm high) were positioned inside the arena near to the corners. The cylindrical cages had small holes (1 cm diameter spaced by 1 cm diameter) on the wall, allowing the passage of odors (olfactory cues) while preventing the direct interaction between adults and juveniles.

The adult animals were subjected to a daily session of 20 min of habituation to the experimental apparatus for 4 consecutive days. The empty cylinder cages were kept inside the arena during the habituation session. The juveniles were habituated to being inside the cylindrical cages for 20 min 24 h before the sample phase. The sample phase (acquisition) was performed 24 h after the last habituation session. In this phase, the adults were individually placed in the center of the arena and allowed for 1 h to freely explore an unfamiliar juvenile placed in one of the cylinders (randomly selected and counterbalanced for each group) and an empty cylinder. The retention test occurred 24 h later, in which the adult animals were placed again in the open field with the previously presented juvenile (familiar) and a novel one placed in the cylinder that had been empty during the sample phase. The two juveniles were from different home cages to prevent the redundancy of olfactory cues and the second juvenile had no prior contact with the adult. After 5 min of free exploration, the adult animal returned to their home cages. The arena and the cylinders were cleaned with 70% v/v ethanol before and after each use. A schematic illustration of the behavioral paradigm used to study the SRM is shown in Fig. 1. Social exploratory behavior was defined as sniffing and touching the cylinder cages. The exploration time of each juvenile (familiar and novel) was measured during the retention test by a trained observer. Random allocation of animals to treatment groups and blinding of investigators assessing outcomes were adopted to reduce selection and detection bias. Total exploration time (time spent exploring the novel juvenile + time spent exploring the familiar juvenile) was evaluated in all groups as a control for eventual effects of the treatments on locomotion activity.

2.4. Pharmacological treatments

Microinjections were carried out intra-CA1 (1.0 μl/side) or intra-BLA (0.5 μl/side) immediately, 60 min or 150 min after the sample phase. The animals were gently restrained by hand, and the infusion needle (30 gauge) was fitted tightly into the guides, protruding 1 mm from the tip of the guide cannulae so as to reach the desired structures. The injection

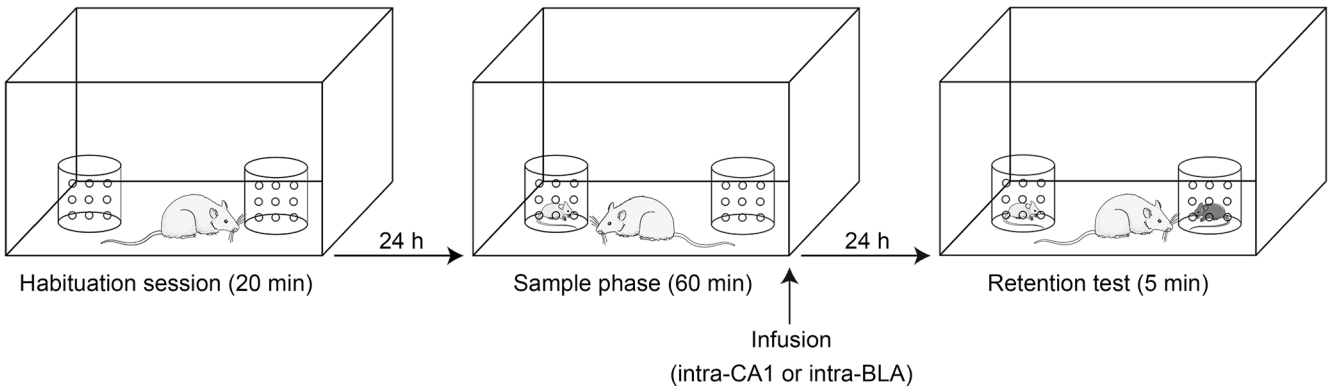

Fig. 1. Schematic illustration of the behavioral paradigm of social discrimination (adapted from Garrido Zinn et al., 2016).

needle was connected to a 10 µl Hamilton microsyringe and the infusions were performed at a rate of 0.5 µl/30 s. At the end of the microinfusion, the infusion needle was left in place 1 min, to allow the solution to diffuse away from the cannula tip, then carefully withdrawn and placed on the other side.

The drugs and the doses used were the endogenous neuropeptide and potent stimulator of adenylyl cyclase PACAP-38 (Sigma-Aldrich; St Louis, MO, USA), 40 pg/side (Schmidt et al., 2015); the PAC1/VPAC2 receptors antagonist PACAP 6–38 (Tocris), 40 pg/side (Sacchetti et al., 2001; Schmidt et al., 2015) and the nitrous oxide donor S-Nitrosospread-N-acetyl-DL-penicillamine, SNAP (Calbiochem), 5 ug/side (Furini et al., 2010; Zinn et al., 2009). All drugs were freshly dissolved in sterile saline 0.9%.

2.5. Histology

Correct cannulae placements were verified two days after 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) or into the BLA (0.5 µl/side) at the coordinates mentioned above. Thirty min later, animals were sacrificed and the brains were removed and kept in 10% formalin. The spread of the dye was taken to represent an estimate of the spread of the drug. Placements were considered correct when the spread was ≤ 1 mm^3 from the intended infusion sites (Rosa, Myskiw, Furini, Sapiras, & Izquierdo, 2013). Only data from animals with correct cannulae implants were analyzed (Fig. 2).

2.6. Statistical analysis

Experimental data were converted in percentage of exploration time and expressed as means ± standard error (SEM). One-sample *t*-test analysis was performed to assess differences to the theoretical mean of 50%. Two-way ANOVA followed by Bonferroni's Multiple Comparison Test was performed to assess differences in percentages of exploration time for the novel juveniles. Unpaired *t*-test or one-way ANOVA followed by Bonferroni's Multiple Comparison Test was performed to

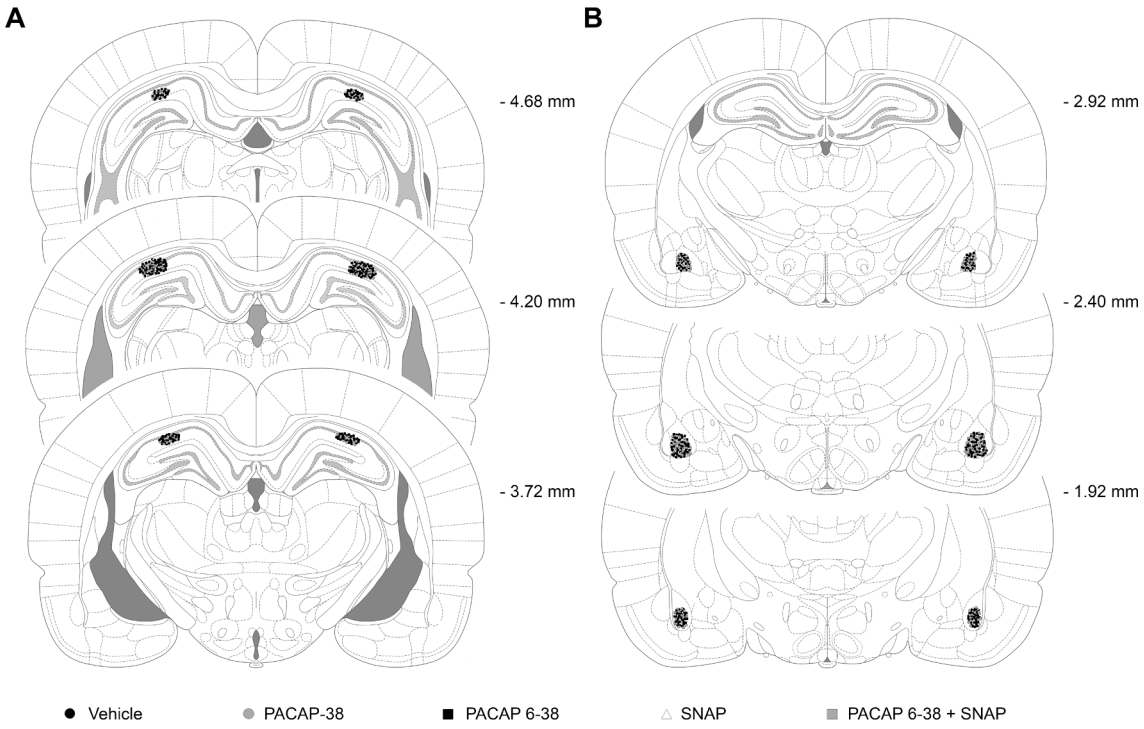

Fig. 2. Schematic representation of cannulae placement. Histological reconstruction of coronal section of the rat brain showing the injection sites (black spots) in the CA1 region of the dorsal hippocampus (A) in planes A − 4.68 mm, − 4.20 mm and − 3.72 mm, and in the basolateral amygdala (B) in planes A − 2.92 mm, − 2.40 mm and − 1.92 mm of the atlas by Paxinos and Watson. Numbers represent distance in millimeters from bregma.

analyze differences in the total exploration time between groups. GraphPad Prism® software was used to the statistical analyses. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of PACAP-38 in the CA1 region of hippocampus and basolateral amygdala on the consolidation of SRM

Immediately after the sample phase, animals received intra-CA1 (Fig. 3a and 3c) or intra-BLA (Fig. 3b and 3d) infusions of Veh or PACAP-38 (40 pg/side). One-sample *t*-test revealed that all groups were able to recognize the familiar juvenile on the retention test (Fig. 3a: Veh $t_{(8)} = 2.826$, $p = 0.0223$; PACAP-38 $t_{(6)} = 4.824$, $p = 0.0029$; Fig. 3b: Veh $t_{(8)} = 3.153$, $p = 0.0135$; PACAP-38 $t_{(7)} = 2.853$, $p = 0.0246$). Two-way ANOVA showed significant effect of juvenile (Fig. 3a: $F_{(1,28)} = 54.71$, $p < 0.0001$; Fig. 3b: $F_{(1,30)} = 68.43$, $p < 0.0001$), but no significant effect of treatment (Fig. 3a: $F_{(1,28)} = 0.00$, $p > 0.9999$; Fig. 3b: $F_{(1,30)} = 0.00$, $p > 0.9999$) or interaction between factors (Fig. 3a: $F_{(1,28)} = 0.1591$, $p = 0.6930$; Fig. 3b: $F_{(1,30)} = 0.9267$, $p = 0.3434$). Bonferroni's post-test showed no significant differences between Veh-N *vs.* PACAP-38-N groups on the retention test (Fig. 3a: $p > 0.05$, $n = 7–9$; Fig. 3b: $p > 0.05$, $n = 8–9$). Unpaired *t*-test revealed no differences between groups in the total exploration time during the retention test (Fig. 3c: $t_{(14)} = 0.6145$, $p = 0.5488$, $n = 7–9$ animals per group; Fig. 3d: $t_{(15)} = 1.496$, $p = 0.1554$, $n = 8–9$ animals per group), indicating that the pharmacological treatments did not affect motor skills or basal motivation to explore the juveniles. These results suggest that intra-CA1 or intra-BLA infusions of PACAP-38 immediately after the sample phase did not affect the consolidation of SRM.

3.2. Effect of PACAP 6–38 and SNAP in the CA1 region of hippocampus on the consolidation of SRM

Immediately (Fig. 4a and 4d), 60 min (Fig. 4b and 4e) or 150 min (Fig. 4c and 4f) after the sample phase, animals received intra-CA1 infusions of Veh, PACAP 6–38 (40 pg/side), SNAP (5 μg/side) or PACAP 6–38 + SNAP.

In the Fig. 4a, one-sample *t*-test revealed that the animals that received intra-CA1 infusions of Veh, SNAP or PACAP 6–38 + SNAP immediately after the sample phase were able to recognize the familiar juvenile on the retention test (Veh $t_{(6)} = 8.109$, $p = 0.0002$; SNAP $t_{(4)} = 3.845$, $p = 0.0184$; PACAP 6–38 + SNAP $t_{(6)} = 4.192$, $p = 0.0057$), while the animals that received PACAP 6–38 were not ($t_{(6)} = 0.2599$, $p = 0.8038$). Two-way ANOVA showed no effect of treatment ($F_{(3,44)} = 0.00$, $p > 0.9999$), but significant effect of juvenile ($F_{(1,44)} = 101.07$, $p < 0.0001$) and interaction between factors ($F_{(3,44)} = 10.80$, $p < 0.0001$). Bonferroni's multiple comparisons test showed significant differences between the following groups on the retention test: Veh-N *vs.* PACAP 6–38-N ($p < 0.01$, $n = 7$), PACAP 6–38-N *vs.* SNAP-N ($p < 0.05$, $n = 5–7$) and PACAP 6–38-N *vs.* PACAP 6–38 + SNAP-N ($p < 0.05$, $n = 7$). One-way ANOVA revealed no differences between groups in the total exploration time during the retention test (Fig. 4d: $F_{(3,22)} = 0.7056$, $p = 0.5589$, $n = 5–7$ animals per group).

On the other hand, when animals received intra-CA1 infusions of Veh, PACAP 6–38 60 min (Fig. 4b) or 150 min (Fig. 4c) after the sample phase, one-sample *t*-test revealed that all groups were able to recognize the familiar juvenile on the retention test (Fig. 4b: Veh $t_{(7)} = 2.736$, $p = 0.0291$; PACAP 6–38 $t_{(7)} = 2.934$, $p = 0.0219$; Fig. 4c: Veh $t_{(6)} = 2.498$, $p = 0.0467$; PACAP 6–38 $t_{(6)} = 4.107$, $p = 0.0063$). Two-way ANOVA showed significant effect of juvenile (Fig. 4b: $F_{(1,28)} = 52.87$, $p < 0.0001$; Fig. 4c: $F_{(1,24)} = 33.68$; $p < 0.0001$), but no significant effect of treatment (Fig. 4b: $F_{(1,28)} = 0.00$, $p > 0.9999$; Fig. 4c: $F_{(1,24)} = 0.00$, $p > 0.9999$) or interaction between the variables (Fig. 4b: $F_{(1,28)} = 2.061$, p

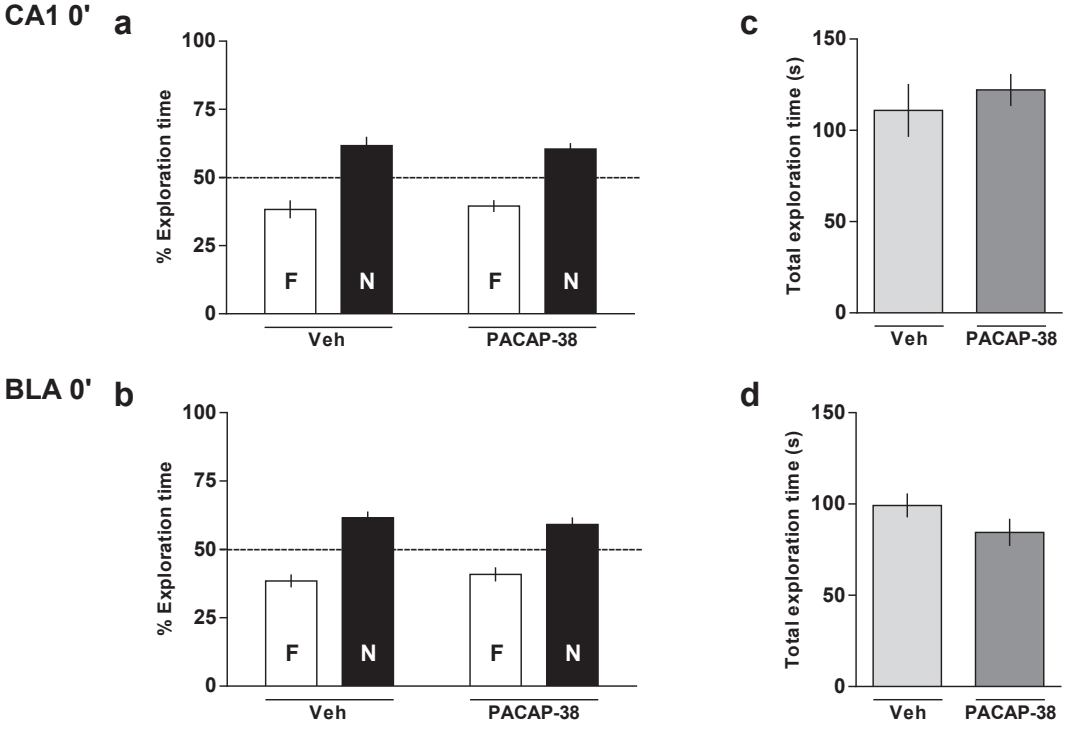

Fig. 3. Effect of PACAP-38 intra-CA1 and intra-BLA on the consolidation of SRM. Immediately after the sample phase, animals received infusions of Vehicle (Veh) or PACAP-38 (endogenous neuropeptide and potent stimulator of adenylyl cyclase; 40 pg/side) intra-CA1 (1.0 μl/side) or intra-BLA (0.5 μl/side). Twenty-four hours later, animals were submitted to a 5 min retention test in the presence of the familiar and a novel juvenile. Dashed line indicates the theoretical means of 50%. Data are expressed as means ± SEM. Percentages of exploration time (a and b) were analyzed by two-way ANOVA followed by Bonferroni's Multiple Comparison Test. Total exploration time (c and d) were analyzed by unpaired *t*-test. CA1: Veh n = 9, PACAP-38n = 7; BLA: Veh n = 9, PACAP-38n = 8.

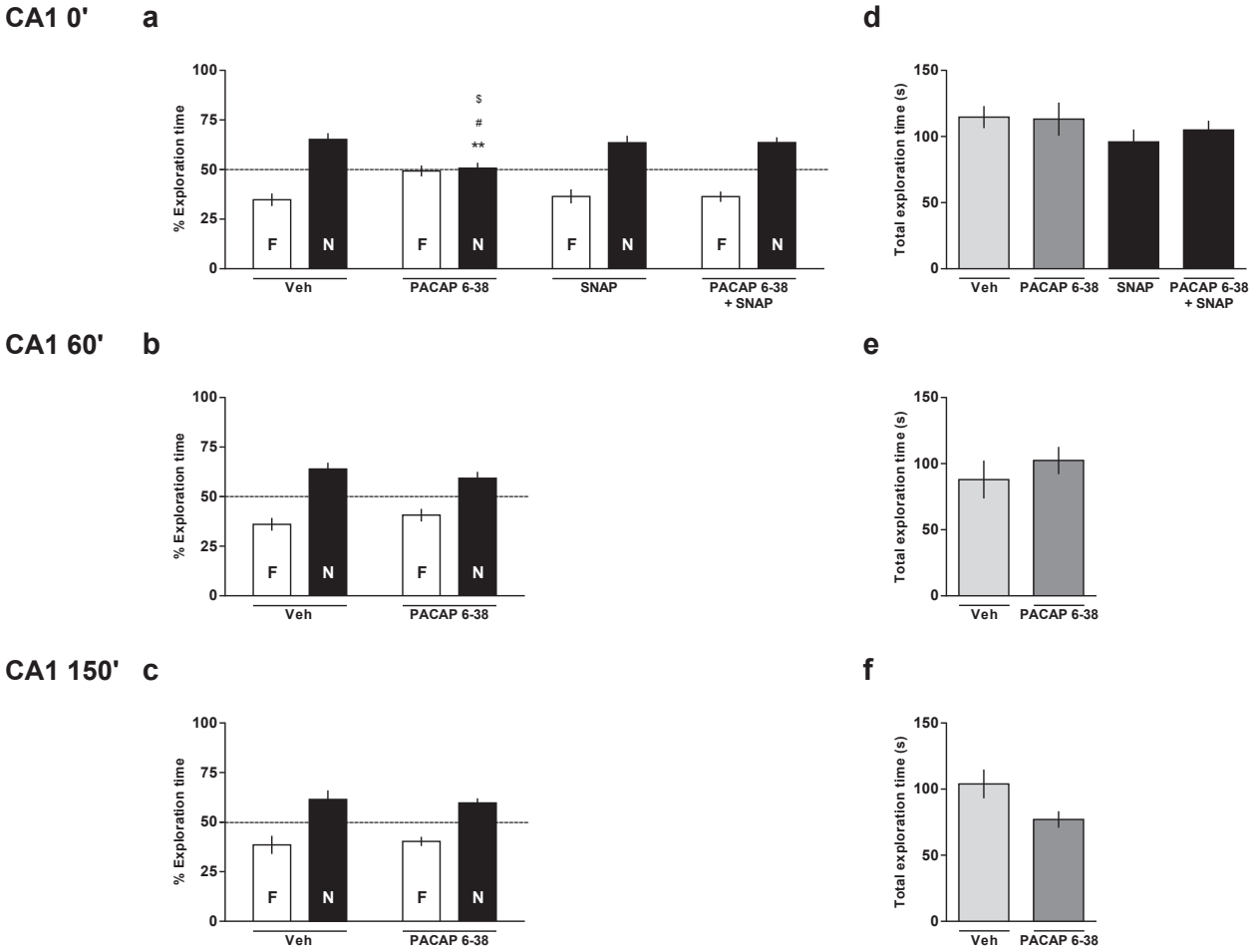

Fig. 4. Effect of PACAP 6–38 and SNAP intra-CA1 on the consolidation of SRM. Immediately, 60 or 150 min after the sample phase, animals received intra-CA1 (1.0 μl/side) infusions of Vehicle (Veh), PACAP 6–38 (PAC1/VPAC2 receptors antagonist; 40 pg/side), SNAP (NO donor; 5 μg/side) or PACAP 6–38 + SNAP. Twenty-four hours later, animals were submitted to a 5 min retention test in the presence of the familiar and a novel juvenile. Dashed line indicates the theoretical means of 50% and data are expressed as means ± SEM. Percentages of exploration time (a, b and c) were analyzed by two-way ANOVA followed by Bonferroni's Multiple Comparison Test. Total exploration time (d, e and f) were analyzed by unpaired *t*-test or one-way ANOVA followed by Bonferroni's Multiple Comparison Test. ** $p < 0.01$ Veh-N *vs.* PACAP 6–38-N; # $p < 0.05$ PACAP 6–38-N *vs.* SNAP-N; $ $p < 0.05$ PACAP 6–38-N *vs.* PACAP 6–38 + SNAP-N. CA1 0′: Veh n = 7, PACAP 6–38 n = 7, SNAP n = 5, PACAP 6–38 + SNAP n = 7; CA1 60′: Veh n = 8, PACAP 6–38 n = 8; CA1 150′: Veh n = 7, PACAP 6–38 n = 7.

= 0.1622; Fig. 4c: $F_{(1,24)} = 0.2242$, p = 0.6402). Bonferroni's post-test showed no significant differences between Veh-N *vs.* PACAP 6–38-N groups on the retention test (Fig. 4b: $p > 0.05$, n = 8; Fig. 4c: $p > 0.05$, n = 7). Unpaired *t*-test revealed no differences between groups in the total exploration time during the retention test (Fig. 4e: $t_{(14)} = 0.8176$, p = 0.4273, n = 8 animals per group; Fig. 4f: $t_{(12)} = 2.145$, p = 0.0531, n = 7 animals per group).

Together, these results suggest that the animals that received intra-CA1 infusions of PACAP 6–38 immediately but not 60 min or 150 min after the sample phase presented an impairment on the consolidation of SRM and this deficit was blocked by the coinfusion of PACAP 6–38 plus SNAP.

3.3. Effect of PACAP 6–38 and SNAP in the basolateral amygdala on the consolidation of SRM

Immediately (Fig. 5a and 5d), 60 min (Fig. 5b and 5e) or 150 min (Fig. 5c and 5f) after the sample phase, animals received intra-BLA infusions of Veh, PACAP 6–38 (40 pg/side), SNAP (5 μg/side) or PACAP 6–38 + SNAP.

In the Fig. 5a and 5b, one-sample *t*-test revealed that the animals that received intra-BLA infusions of Veh, SNAP or PACAP 6–38 + SNAP immediately or 60 min after the sample phase were able to recognize the familiar juvenile on the retention test (Fig. 5a: Veh $t_{(7)} = 8.061$, $p < 0.0001$; SNAP $t_{(5)} = 3.107$, p = 0.0264; PACAP 6–38 + SNAP $t_{(7)} = 2.798$, p = 0.0266; Fig. 5b: Veh $t_{(7)} = 15.81$, $p < 0.0001$; SNAP $t_{(7)} = 5.748$, p = 0.0007; PACAP 6–38 + SNAP $t_{(7)} = 3.108$, p = 0.0171), while the animals that received PACAP 6–38 were not (Fig. 5a: $t_{(5)} = 1.385$, p = 0.2248; Fig. 5b: $t_{(5)} = 0.5391$, p = 0.6130). Two-way ANOVA showed no effect of treatment (Fig. 5a: $F_{(3,48)} = 0.00$, $p > 0.9999$; Fig. 5b: $F_{(3,52)} = 0.00$, $p > 0.9999$), but significant effect of juvenile (Fig. 5a: $F_{(1,48)} = 43.26$, $p < 0.0001$; Fig. 5b: $F_{(1,52)} = 88.25$, $p < 0.0001$) and interaction between factors (Fig. 5a: $F_{(3,48)} = 12.31$, $p < 0.0001$; Fig. 5b: $F_{(3,52)} = 13.64$, $p < 0.0001$). Bonferroni's multiple comparisons test showed significant differences between the following groups on the retention test: Veh-N *vs.* PACAP 6–38-N (Fig. 5a: $p < 0.01$, n = 6–8; Fig. 5b: $p < 0.001$, n = 6–8), PACAP 6–38-N *vs.* SNAP-N (Fig. 5a: $p < 0.01$, n = 6; Fig. 5b: $p < 0.01$, n = 6–8) and PACAP 6–38-N *vs.* PACAP 6–38 + SNAP-N (Fig. 5a: $p < 0.01$, n = 6–8; Fig. 5b: $p < 0.01$, n = 6–8). One-way ANOVA revealed no differences between groups in the total exploration time during the retention test (Fig. 5d: $F_{(3,24)} = 0.4331$, p = 0.7313, n = 6–8 animals per group; Fig. 5e: $F_{(3,26)} = 0.5170$, p = 0.6743, n = 6–8 animals per group).

In the Fig. 5c, one-sample *t*-test revealed that the animals that received intra-BLA infusions of Veh or PACAP 6–38 150 min after the sample phase were able to recognize the familiar juvenile on the

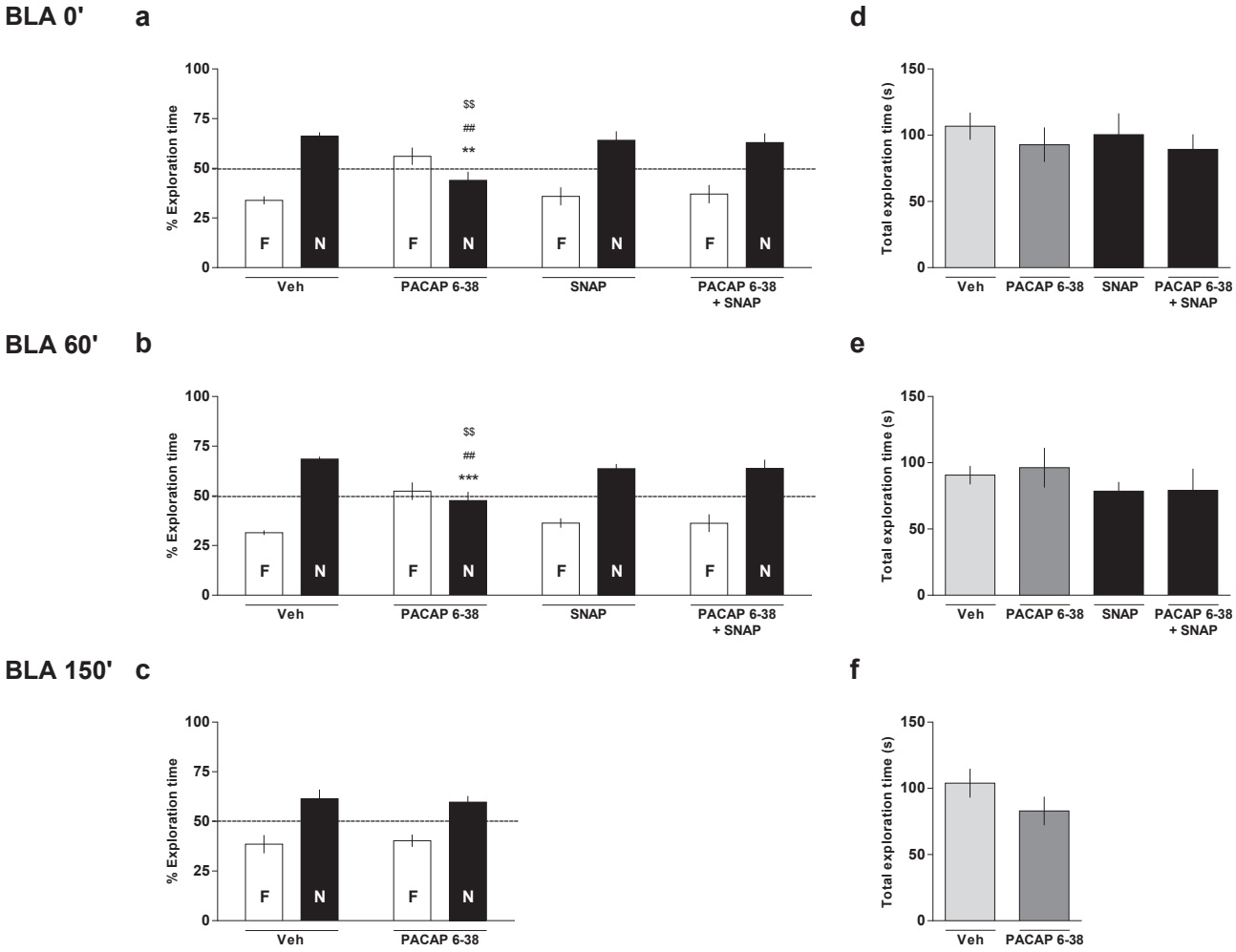

Fig. 5. Effect of PACAP 6–38 and SNAP intra-BLA on the consolidation of SRM. Immediately, 60 or 150 min after the sample phase, animals received intra-BLA (1.0 µl/side) infusions of Vehicle (Veh), PACAP 6–38 (PAC1/VPAC2 receptors antagonist; 40 pg/side), SNAP (NO donor; 5 µg/side) or PACAP 6–38 + SNAP. Twenty-four hours later, animals were submitted to a 5 min retention test in the presence of the familiar and a novel juvenile. Dashed line indicates the theoretical means of 50% and data are expressed as means ± SEM. Percentages of exploration time (a, b and c) were analyzed by two-way ANOVA followed by Bonferroni's Multiple Comparison Test. Total exploration time (d, e and f) were analyzed by unpaired *t*-test or one-way ANOVA followed by Bonferroni's Multiple Comparison Test. ** $p < 0.01$ *** $p < 0.001$ Veh-N *vs.* PACAP 6–38-N; ## $p < 0.01$ PACAP 6–38-N *vs.* SNAP-N; $$ $p < 0.01$ PACAP 6–38-N *vs.* PACAP 6–38 + SNAP-N. BLA 0′: Veh n = 8, PACAP 6–38 n = 6, SNAP n = 6, PACAP 6–38 + SNAP n = 8; BLA 60′: Veh n = 8, PACAP 6–38 n = 6, SNAP n = 8, PACAP 6–38 + SNAP n = 8; BLA 150′: Veh n = 7, PACAP 6–38 n = 7.

retention test (Veh $t_{(6)} = 2.498$, $p = 0.0467$; PACAP 6–38 $t_{(6)} = 3.168$, $p = 0.0194$). Two-way ANOVA showed significant effect of juvenile ($F_{(1,24)} = 29.46$, $p < 0.0001$), but no significant effect of treatment ($F_{(1,24)} = 0.00$, $p > 0.9999$) or interaction between factors ($F_{(1,24)} = 0.1942$, $p = 0.6634$). Bonferroni's post-test showed no significant differences between Veh-N *vs.* PACAP 6–38-N groups ($p > 0.05$, n = 7). Unpaired *t*-test revealed no differences between groups in the total exploration time during the retention test (Fig. 5f*:* $t_{(12)} = 1.371$, $p = 0.1956$, n = 7 animals per group).

Together, these results suggest that the animals that received intra-BLA infusions of PACAP 6–38 immediately or 60 min, but not 150 min after the sample phase presented an impairment on the consolidation of SRM and this deficit was blocked by the coinfusion of PACAP 6–38 plus SNAP.

4. Discussion

The ability to recognize a conspecific is essential for many aspects of social interaction and organization. Despite its importance, little is known about the neural mechanisms underlying the SRM (Ferguson et al., 2002; van der Kooij & Sandi, 2012).

In the present work, we show that the infusion of PAC1/VPAC2 receptors antagonist PACAP 6–38 in the CA1 region of the hippocampus and in the BLA impaired the consolidation of SRM. Moreover, we demonstrate that the deficit observed on the consolidation of SRM in the CA1 and in the BLA was abolished by the coinfusion of the NO donor SNAP. In particular, our results suggest that PACAP/PAC1/VPAC2 signaling is required in the CA1 and in the BLA during a restricted post-acquisition time-window for the consolidation of the SRM.

PACAP has a broad spectrum of biological functions such as neuromodulator, neuroprotective and/or neurotrophic factor (Brenneman et al., 1990; Cabezas-Llobet et al., 2018; Fahrenkrug, 1993; Kojro et al., 2006; Lioudyno et al., 1998; Pincus et al., 1990; Vaudry et al., 2009). Additionally, several studies have demonstrated that PACAP plays an important role on learning and memory (Borbély et al., 2013; Ciranna & Costa, 2019; Ladjimi et al., 2020; Meloni et al., 2019; Ogata et al., 2015; Sacchetti et al., 2001; Stevens et al., 2014; Telegdy & Kokavszky, 2000; Yang et al., 2010; Zhou et al., 2002).

PACAP exerts its effects through three GPCR receptors. Because of the high homology of the amino acid sequences of PACAP and VIP, VPAC1/VPAC2 receptors bind these peptides with similar affinities (Arimura, 1998; Hashimoto et al., 2006; Hattori et al., 2012; Joo et al.,

2004; Pantaloni et al., 1996; Roberto & Brunelli, 2000; Spengler et al., 1993; Vaudry et al., 2009; Yang et al., 2010). On the other hand, PAC1 receptor binds PACAP with an affinity at least 1000 times greater than for VIP, thereby PACAP exerts its effects mainly via its cognate receptor PAC1 (Harmar et al., 1998; Hirabayashi et al., 2018; Iemolo et al., 2016; Miyata et al., 1989, 1990; Pedersen et al., 2019). In the brain, PACAP receptors mRNA is especially expressed in key regions for mnemonic processing such as the CA1 region of the hippocampus and the BLA (Hashimoto et al., 1996; Hirabayashi et al., 2018; Joo et al., 2004; Sheward et al., 1995; Shioda et al., 1997; Usdin et al., 1994; Vaudry et al., 2009; Zhou et al., 2002).

Here we show that the infusion of the PACAP 6–38 antagonist intra-CA1 or intra-BLA impaired the consolidation of SRM. Although PACAP 6–38 is a potent and competitive antagonist of PAC1 receptor (Kojro et al., 2006; Leyton et al., 1998; Liao et al., 2019; Payet et al., 2003; Robberecht et al., 1992; Vaudry et al., 2009), it is important to note that PACAP 6–38 acts as a potent dual PAC1/VPAC2 antagonist, once that the IC50 value of binding of PACAP 6–38 for PAC1 and VPAC2 receptors are 30 and 40 nM, respectively (Gourlet et al., 1995; Laburthe et al., 2007).

We have previously demonstrated that the blockade of PAC1/VPAC2 receptors by the infusion of PACAP 6–38 antagonist in the CA1 region of hippocampus and basolateral amygdala impairs the consolidation of contextual fear memory (Schmidt et al., 2015). Moreover, the increase in circulating PACAP and a polymorphism in the PAC1 receptor (ADCYAP1R1) genotype have been proposed as biomarkers for Post-Traumatic Stress Disorder (Hammack et al., 2009; Ressler et al., 2011; Uddin et al., 2013; Wang et al., 2013). The ADCYAP1R1 risk genotype have been associated with increased responses to fearful stimuli in the amygdala and hippocampus (Stevens et al., 2014).

In addition to the known involvement with fear memory, evidence suggests that PACAP is also implicated in the recognition memory and in the social behavior. Studies performed in PACAP-deficient mice have demonstrated that these animals present an impaired performance in the novel object recognition test (Ago et al., 2013; Shibasaki et al., 2015). More recently, Cabezas-Llobet and collaborators have demonstrated that daily intranasal administration of PACAP-38 counteract object recognition memory deficits in mouse models of Huntington's disease (Cabezas-Llobet et al., 2018). Furthermore, genetic analysis revealed that variations in the genes encoding PACAP and PAC1 receptor are associated with schizophrenia, a disease characterized by psychosis and profound disorders of cognition, emotion and social functioning (Hashimoto et al., 2007). Additionally, studies have shown that PACAP-deficient mice display impairments in social interaction (Ishihama et al., 2010; Takuma et al., 2014; Tanaka et al., 2006).

In this work SRM was impaired when the intra-CA1 infusion of the PACAP 6–38 antagonist was performed immediately but not 60 min or 150 min after the sample phase, while PACAP 6–38 intra-BLA impaired SRM when infused immediately or 60 min, but not 150 min after the sample phase. Based on these results, we can suggest that PACAP/PAC1/VPAC2 signaling is important during a restricted post-acquisition time window for consolidation of SRM. These findings are in agreement with the study of Meloni and collaborators, in which the authors demonstrated that intracerebroventricular infusions of PACAP produces a time-dependent effect on the conditioned fear memory (Meloni et al., 2016). Furthermore, the time-dependent effect shown in the present work indicates that the observed impairment was caused by the inhibition of the consolidation process and not by an insult on the CA1 or BLA functionality or by an unspecific impairment of behavioral performance.

Studies have demonstrate that the consolidation of some memories may activate molecular changes with different temporal progression in multiple brain areas, such as amygdala, hippocampus, entorhinal cortex and parietal cortex (Bambah-Mukku et al., 2014; Izquierdo et al., 1997, 2016). It has been well established that hippocampus and amygdala participate in the SRM (Bannerman et al., 2001; Feinberg et al., 2012; Garrido Zinn et al., 2016; Wang et al., 2014). The different roles recently assigned to each of these structures in the SRM may help to explain the difference in the temporal window between hippocampus and BLA observed here. While the amygdala is more involved in the regulation of social behaviors such as social interaction and approach, the hippocampus seems to act as an integrative center of brain networks to generate the SRM (Tanimizu et al., 2017).

Here we also show that the SRM impairment induced by PACAP 6–38 infusion intra-CA1 immediately after the sample phase, and intra-BLA immediately and 60 min after the sample phase, was abolished by the coinfusion of the NO donor, SNAP. Although these results do not identify signaling steps between PACAP, NO and synaptic targets, they indicate the existence of a strong relationship between PACAP signaling and NO on the SRM.

The possible mechanisms underlying the results found here should be speculated with caution. One of them may be related to the work performed by Pugh and collaborators, in which they demonstrated that PACAP signaling modulates the synaptic function by increased vesicular acetylcholine release (quantal content) from presynaptic terminals and that this increase required activation of NO, since the PACAP-induced increase in quantal content was mimicked by NO donor SNAP and absent after inhibiting NO synthase enzyme (Pugh et al., 2010). Another possibility concerns about the fact that PACAP regulates synaptic plasticity mainly through Gαs/cAMP/PKA pathway, resulting in increase of NMDA receptor-mediated responses (Yaka et al., 2003). The activation of NMDA receptors produces several effects, including the induction of NO production which in turn regulates the presynaptic function in glutamatergic synapses (Bredt & Snyder, 1989; Garthwaite, 2018; Neitz et al., 2014; Qiu & Knöpfel, 2007; Regehr et al., 2009; Yamada & Nabeshima, 1997a, 1997b; Zorumski & Izumi, 1998).

The NO is implicated in an array of behaviors ranging from learning and memory to social interactions (Böhme et al., 1993; Jayakar et al., 2014; Jüch et al., 2009; Kirchner et al., 2004; Pugh et al., 2010). In the present study SNAP had no effect by itself on the SRM when administered intra-CA1 or intra-BLA, however, it was able to prevent the impairment of SRM caused by the PACAP 6–38 infusions on these regions. Previous studies have demonstrated that the effects of NO in memory are concentration-dependent (Furini et al., 2010; Gage & Nighorn, 2014; Zinn et al., 2009). Furthermore, this work suggests that the blocked of SRM impairment caused by inhibition of PACAP/PAC1/VPAC2 signaling is probably due to a mechanism of action involving the interaction between PACAP and NO.

To explain NO-mediated cellular effects, two main routes have been strongly established. One of them is the S-nitrosylation on cysteine residues of target proteins, resulting in significant conformational changes that affect the protein functional activity (Contestabile, 2008; Jaffrey et al., 2001). Evidence shows that the NO contributes via S-nitrosylation to the regulation of several molecular signaling pathways involved in mnemonic processing (Coultrap & Bayer, 2014; Gräff et al., 2014; Zoubovsky et al., 2011). Another critical route for the NO action is the activation of soluble guanylate cyclase (sGC) and the consequent increase in cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG) activity (Shelly et al., 2010; Sunico et al., 2010). The NO/sGC/PKG pathway is known to play an important role in the processes of plasticity and learning, including recognition memory (Arancio et al., 1995; Chetkovich et al., 1993; East & Garthwaite, 1991; Furini et al., 2010; Zhuo et al., 1994). In addition Akar and collaborators demonstrated that the inhibition of this pathway might disturb emotional, visual, and olfactory memory in mice (Akar et al., 2014).

The present study extends our knowledge about the role of PACAP in cognitive function, particularly in learning and memory. Here we suggested that the blockade of PACAP/PAC1/VPAC2 signaling in the hippocampus and in the basolateral amygdala impairs the consolidation of SRM in a time-dependent manner and that the SNAP is able to abolish this deficit in both brain structures. Our results may help to elucidate the underlying cellular mechanisms of psychiatric disorders in which the SRM processing is impaired. Additionally, our findings suggest PACAP

as a possible therapeutic target for treatment of disorders characterized by cognitive and social deficits, such as autism and schizophrenia.

CRediT authorship contribution statement

Scheila Daiane Schmidt: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Project administration. **Carolina Garrido Zinn:** Investigation. **Jonny Anderson Kielbovicz Behling:** Investigation. **Ana Flávia Furian:** Investigation. **Cristiane Regina Guerino Furini:** Conceptualization, Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Project administration. **Jociane Carvalho Myskiw:** Conceptualization, Resources, Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Ivan Izquierdo:** Conceptualization, Conceptualization, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Work supported by grants and fellowships from the National Research Council of Brazil (CNPq) and Coordination of Improvement of Higher Education Personnel of Brazil (CAPES - Finance Code 001).

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