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Molecular Mechanisms in Hippocampus Involved on Object Recognition Memory Consolidation and Reconsolidation

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Abstract—Acquired information is stabilized into long-term memory through a process known as consolidation. Though, after consolidation, when stored information is retrieved they can be again susceptible, allowing modification, updating and strengthening and to be re-stabilized they need a new process referred to as memory reconsolidation. However, the molecular mechanisms of recognition memory consolidation and reconsolidation are not fully understood. Also, considering that the study of the link between synaptic proteins is key to understanding of memory processes, we investigated, in male *Wistar* rats, molecular mechanisms in the hippocampus involved on object recognition memory (ORM) consolidation and reconsolidation. We verified that the blockade of AMPA receptors (AMPAr) and L-VDCCs calcium channels impaired ORM consolidation and reconsolidation when administered into CA1 immediately after sample phase or reactivation phase and that these impairments were blocked by the administration of AMPAr agonist and of neurotrophin BDNF. Also, the blockade of CaMKII impaired ORM consolidation when administered 3 h after sample phase but had no effect on ORM reconsolidation and its effect was blocked by the administration of BDNF, but not of AMPAr agonist. So, this study provides new evidence of the molecular mechanisms involved on the consolidation and reconsolidation of ORM, demonstrating that AMPAr and L-VDCCs are necessary for the consolidation and reconsolidation of ORM while CaMKII is necessary only for the consolidation and also that there is a link between BDNF and AMPAr, L-VDCCs and CaMKII as well as a link between AMPAr and L-VDCCs on ORM consolidation and reconsolidation.

Key words: AMPA receptors, L-VDCCs calcium channels, CaMKII, BDNF, ORM consolidation, ORM reconsolidation.

INTRODUCTION

The ability to learn and store information is crucial to survival and life management, allowing individuals to retrieve memories and act through the acquired knowledge (McGaugh, 2000; Kandel et al., 2014). The process of storage information is known as consolidation and it does not occur immediately after learning, but it occurs with time through a stabilization process dependent on protein synthesis that can take a few hours and is susceptible to modifications during this period (Bailey et al., 1996; Dudai, 2012; McGaugh, 2015). When stored information are retrieved they can be again susceptible, allowing modification, updating, strengthening or even loss due to further experiences and to be restabilized they need a new protein-dependent process referred to as memory reconsolidation (Lee, 2008; Monfils et al., 2009; Schiller et al., 2010; Inda et al., 2011; Cowansage et al., 2014; Furini et al., 2015). The reconsolidation may share some mechanisms with memory consolidation, but it can also require different molecular pathways and brain structures (Nader et al., 2000; Sara, 2000; Alberini, 2005; Lee et al., 2012; Einarsson et al., 2015; Furini et al., 2015; Izquierdo et al., 2016).

Recognition memory is the fundamental ability to recognize previously encountered objects, events and individuals. In rodents, the object recognition task has become a widely used paradigm to evaluate recognition memory, allowing the investigation of mechanisms related to the neurobiology of mammalian memory (Squire et al., 2007; Winters et al., 2008). It relies on rodents' natural propensity to explore for a longer time

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Abbreviations: AMPAr, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BDNF, brain-derived neurotrophic factor; CaMKII, calcium/calmodulin-dependent kinase II; LTP, long-term potentiation; L-VDCCs, L-type voltage dependent calcium channel; ORM, object recognition memory; PKC, protein kinase C; PLC, phospholipase C.

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novel objects than familiar ones when presented together (Ennaceur and Delacour, 1988; Ennaceur, 2010; Balderas et al., 2014; Furini et al., 2014). One of the brain structures that have been demonstrated to be involved on the consolidation and reconsolidation of object recognition memory (ORM) is the hippocampus, especially the CA1 region (Reed and Squire, 1997; de Lima et al., 2005; Myskiw et al., 2008; Broadbent et al., 2010; Clarke et al., 2010; Furini et al., 2010, 2014, 2015; Cohen et al., 2013; Mello-Carpes and Izquierdo, 2013).

Some molecular mechanisms that regulate memory processing are dependent on intracellular Ca^{2+} level, that can be regulated by L-type voltage dependent calcium channel (L-VDCCs), which contribute to several important molecular cascades associated with postsynaptic plasticity and memory (Power and Sah, 2005; Ou and Gean, 2007; Fourcaudot et al., 2009; Davis and Bauer, 2012; Perugini et al., 2012; Da Silva et al., 2013; de Carvalho Myskiw et al., 2014). The calcium/calmodulin-dependent kinase II (CaMKII) is one of the targets of Ca^{2+} (Wolfman et al., 1994; Mayford et al., 1996; Coultrap and Bayer, 2012; Lisman et al., 2012). CaMKII are necessary to the induction of long-term potentiation (LTP; (Redondo et al., 2010; Lisman et al., 2012; Fukushima et al., 2014) and are also related to memory processing (Wolfman et al., 1994; Vianna et al., 2000; Tinsley et al., 2009; de Carvalho Myskiw et al., 2014). One of the mechanisms through which CaMKII can act is modulating glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAr). AMPAr plays a critical role in different forms of synaptic plasticity including LTP and a variety of memory types (Bernabeu et al., 1997; Winters and Bussey, 2005; Rose and Rankin, 2006; Barker and Warburton, 2008; Sanderson et al., 2011; Rodriguez-Ortiz et al., 2012; Garcia-Delatorre et al., 2014; Santoyo-Zedillo et al., 2014). Its function can be regulated by CaMKII by phosphorylation of the AMPAr GluR1 subunit, which increases AMPA receptor conductance and its delivery to glutamatergic synapses (Barria et al., 1997; Derkach et al., 1999; Fink and Meyer, 2002; Lisman et al., 2002; Derkach, 2003).

Likewise, brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, plays important role in synaptic plasticity as well as learning and memory (Pang et al., 2004; Bekinschtein et al., 2007; Furini et al., 2010; Lee and Hynds, 2013; Rodríguez-Serrano et al., 2014; Rosas-Vidal et al., 2014, 2018; Signor et al., 2017). BDNF seems to interact with L-VDCCS and CaMKII, since membrane depolarization-triggered Ca^{2+} influx through L-VDCCs induce an increase in BDNF mRNA expression in cultured neurons and this effect is blocked by inhibitors of CaMKII (Zafra et al., 1992). Also, BDNF may enhance phosphorylation of AMPAr GluR1 subunit through changes in the NMDA receptor function, activation of protein kinase C (PKC) and CaMKII, and the expression of AMPAr by a exocytotic pathway triggered by intracellular Ca^{2+} (Narisawa-Saito et al., 2002; Wu et al., 2004; Caldeira et al., 2007).

However, the molecular mechanisms in hippocampus involved on recognition memory consolidation and reconsolidation are not fully understood (Walker et al., 2003; Alberini, 2005, 2011; Forcato et al., 2007, 2010). Thus, through the object recognition task and a pharmacological approach with AMPAr antagonist, CNQX; L-VDCCs inhibitor, Nife; CaMKII inhibitor, AIP; AMPAr agonist, AMPA and neurotrophin BDNF, administered into the CA1 region of hippocampus of rats, we aimed to study the involvement of L-VDCCs, CaMKII, AMPAr and the relationship of the neurotrophin BDNF with these proteins, as well as the relationship of AMPAr with LVDCCs and CaMKII on the consolidation and reconsolidation of recognition memory. Our hypothesis is that AMPAr, L-VDCCs and CaMKII are necessary to the consolidation and reconsolidation of ORM and that there is a link between BDNF and AMPAr, L-VDCCs and CaMKII and, a link between AMPAr and L-VDCCs and CaMKII on the consolidation and reconsolidation of ORM.

EXPERIMENTAL PROCEDURES

Subjects

To this study 397 male *Wistar* rats (CrlCembe:WI; 3-month-old, 300–330 g) from Centro de Modelos Biológicos e Experimentais (CeMBE) of the Pontifical Catholic University of Rio Grande do Sul, Porto Alegre – Brazil, were housed four to a polypropylene cage (49 × 35 × 17 cm, Alesco®), kept with free access to food and water under a 12-h light/dark cycle (lights on at 7:00 a.m.) and the experiments were performed during the light phase of the cycle. The temperature of the animals' room was maintained at 22–24 °C. Experimental procedures were approved by the Animal Committee on Ethics for the Care and Use of Laboratory Animals (number 15/00477) of the Pontifical Catholic University of Rio Grande do Sul, Brazil.

Surgery

All animals were anesthetized with i.p. infusion of 75 mg/kg of ketamine plus 10 mg/kg of xylazine and through a stereotaxic surgery were bilaterally implanted with stainless steel 22-gauge guide cannulae aimed 1.0 mm above of the CA1 region of the dorsal hippocampus (anterior −4.2 mm; lateral ±3.0 mm; ventral −1.8 mm) (Paxinos and Watson, 1986; Myskiw et al., 2008; Furini et al., 2015; Izquierdo et al., 2016). The guide cannulae were fixed to the skull with dental acrylic. After the procedure, animals were allowed 7 days to recover and were handled once daily for 3 consecutive days before behavioral experiments.

Drug administration

The drugs (purchased from Sigma–Aldrich; St. Louis, MO, USA) and the doses used were: Nifedipine (Nife, 3.4 μg per side), autocamtide-2-related inhibitory peptide (AIP, 1.7 μg per side), AMPA receptor antagonist (CNQX, 1 μg per side), AMPA receptor agonist (AMPA, 2 μg per side) and neurotrophin BDNF (0.25 μg per side) (Jerusalinsky et al., 1992; Quillfeldt et al., 1994; Furini et al., 2010; Da Silva et al., 2013; de Carvalho

Myskiw et al., 2014). To the bilateral microinfusion a tight fitting 30-gauge injection needle, connected by a polyethylene tube to a 10-μl Hamilton microsyringe, was introduced into the guide cannula. The injection needles extended 1.0 mm beyond the cannulae tip. The volume infused was 1.0 μl per side into the CA1 area of the dorsal hippocampus, injected at a rate of 1.0 μl/min. The injection needle was kept in place for 1.0 additional minute to maximize diffusion and to prevent backflow of drug into the cannula. Control groups received equal volumes of sterile saline (0.9%), the vehicle in which drugs were dissolved.

Object recognition task

The experimental apparatus used was an open field arena (60 × 40 × 50 cm) placed in a dimly illuminated room. The objects to be discriminated were made of glass and varied in shape and texture and were chosen based on previous observations that demonstrated a lack of preferential exploration for one object over the other. The objects were secured to the floor of the arena with Velcro tape. The open field arena and the stimulus objects were carefully cleaned with 70% ethanol after each animal to ensure the absence of olfactory cues. The role (familiar or novel) as well as the relative position of the two stimulus objects were counterbalanced and randomly permuted for each experiment.

Exploration was defined as sniffing or touching the stimulus object with the nose and/or forepaws. Sitting on or moving around the objects was not considered exploratory behavior. During the sample phase, reactivation phase and test phase the time animals spent exploring the objects was measured by a trained experimenter who was blind to the treatment condition. Before the sample phase, animals were habituated to the experimental apparatus, allowing them to freely explore it for consecutive 4 days, 20 min per day. No stimuli object was placed inside the arena during habituation.

Consolidation protocol: To study ORM consolidation, animals were individually placed in the open field arena with two identical objects (A and A) and left to freely explore them for 5 min (sample phase). Twenty-four hours later, animals were placed again in the same apparatus for a 5-min retention test phase, with a familiar and a novel object (A and B). After the sample phase, animals were randomly divided in control (Veh) or drug groups. Drugs administration into the CA1 region of the hippocampus were carried out immediately or three hours after the sample phase. As rats naturally prefer novelty, animals with intact memory spend significantly more time exploring the novel object than the familiar one during test phase (Ennaceur, 2010).

Reconsolidation protocol: To study ORM reconsolidation, animals were individually placed in the open field arena with two identical objects (A and A) and left to freely explore them for 5 min (sample phase). Twenty-four hours later, animals were placed again in the same apparatus for a 5-min reactivation phase, with a familiar object and a novel object (A and B). After another 24 h, animals were placed again in the same apparatus for a 5-min retention test phase, with a familiar object and another novel object (A and C). The experimental protocol was based on Balderas et al. (2013); Santoyo-Zedillo et al. (2014). After the reactivation phase, animals were randomly divided in control (Veh) or drug groups. Drugs administration into the CA1 region of the hippocampus were carried out immediately or three hours after the reactivation phase. Object recognition memory undergoes reconsolidation when information of a new object is presented with a previously experienced object. Amnesic agents given after reactivation of memory by presenting one of the initial objects together with a novel object can led to impairment of memory for the reactivated initial object. This can be observed in different protocols in which a novel object is presented during the reactivation phase (Myskiw et al., 2008; Balderas et al., 2013; Furini et al., 2015).

Histology

To verify cannula placement, 2 days after the end of the behavioral procedure, 1 μl of 4% methylene-blue was infused as described above and rats were anesthetized and killed by decapitation 15 min later. The spread of the dye was taken as an estimate of the drugs' diffusion. Placements were considered correct when the spread was 1 mm^3 or less from the intended infusion sites, this occurred in 98% of the animals. Fig. 1 depicts a schematic drawing of the infusion sites.

Statistical analyses

Data are expressed as mean ± S.E.M. In order to quantify ORM, discrimination indexes (DI) were calculated as follows: time spent exploring the novel object less the time spent exploring the familiar object, divided by the total exploration time spent exploring both objects $(tnov - tfam)/(tnov + tfam)$ (Ennaceur and Delacour, 1988; Barker and Warburton, 2008; Romero-Granados et al., 2010). One-sample *t*-test was used to determine whether the DI differed from zero (i.e. chance

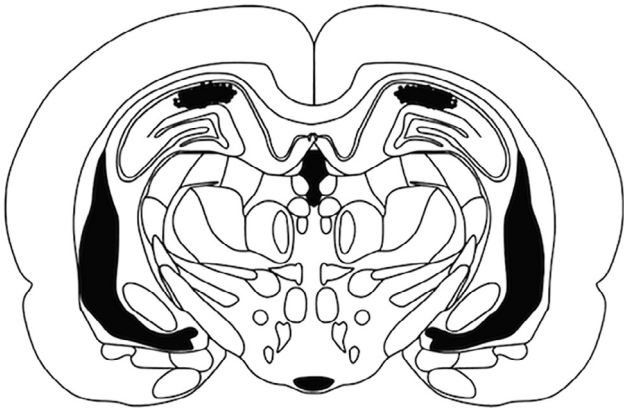

Fig. 1. Bilateral cannulae placements in the CA1 region of the dorsal hippocampus. Schematic drawing representing the infusion sites (black spots) in the CA1 region of the dorsal hippocampus for each animal included in the final analyses. Adapted from Paxinos and Watson (1986).

level) and thus whether learning had occurred. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used to determine differences between groups on DI on the test phase of object recognition task and to determine differences between groups on total exploration time. $P < 0.05$ was considered statistically significant. The number of rats per group is indicated in the figure legends. Data were analyzed using the GraphPad Prism® software.

RESULTS

Effect of CNQX, Nife and AIP given into the CA1 region of hippocampus on the consolidation and reconsolidation of ORM

To verify the participation of AMPAr, L-VDCCs and CaMKII on the consolidation and reconsolidation of ORM, after the sample phase or reactivation phase, respectively, animals received intra-CA1 infusions of Veh; AMPAr antagonist, CNQX; L-VDCCs inhibitor, Nife or CaMKII inhibitor, AIP.

Consolidation of ORM. As shown in Fig. 2A, the groups that received Veh ($t_{(9)} = 7.83$, $p < 0.001$) or AIP ($t_{(8)} = 4.11$, $p < 0.001$) immediately after the sample phase presented a DI significantly different from zero on the test phase (i.e., chance level), while CNQX ($t_{(8)} = 1.69$, $p = 0.12$) and Nife ($t_{(8)} = 0.65$, $p = 0.5335$) did not. One-way ANOVA on the test phase showed significant differences in DI between groups ($F_{(3,33)} = 13.76$, $p < 0.0001$). Bonferroni's multiple comparison test revealed differences in CNQX ($p < 0.001$) and Nife ($p < 0.001$) groups *vs*. the Veh group.

Since the blockade of AMPA receptors and L-VDCCs impaired ORM consolidation immediately after the sample phase, but the blockade of CaMKII did not, we decided to test their effects 3 h after the sample phase, considering

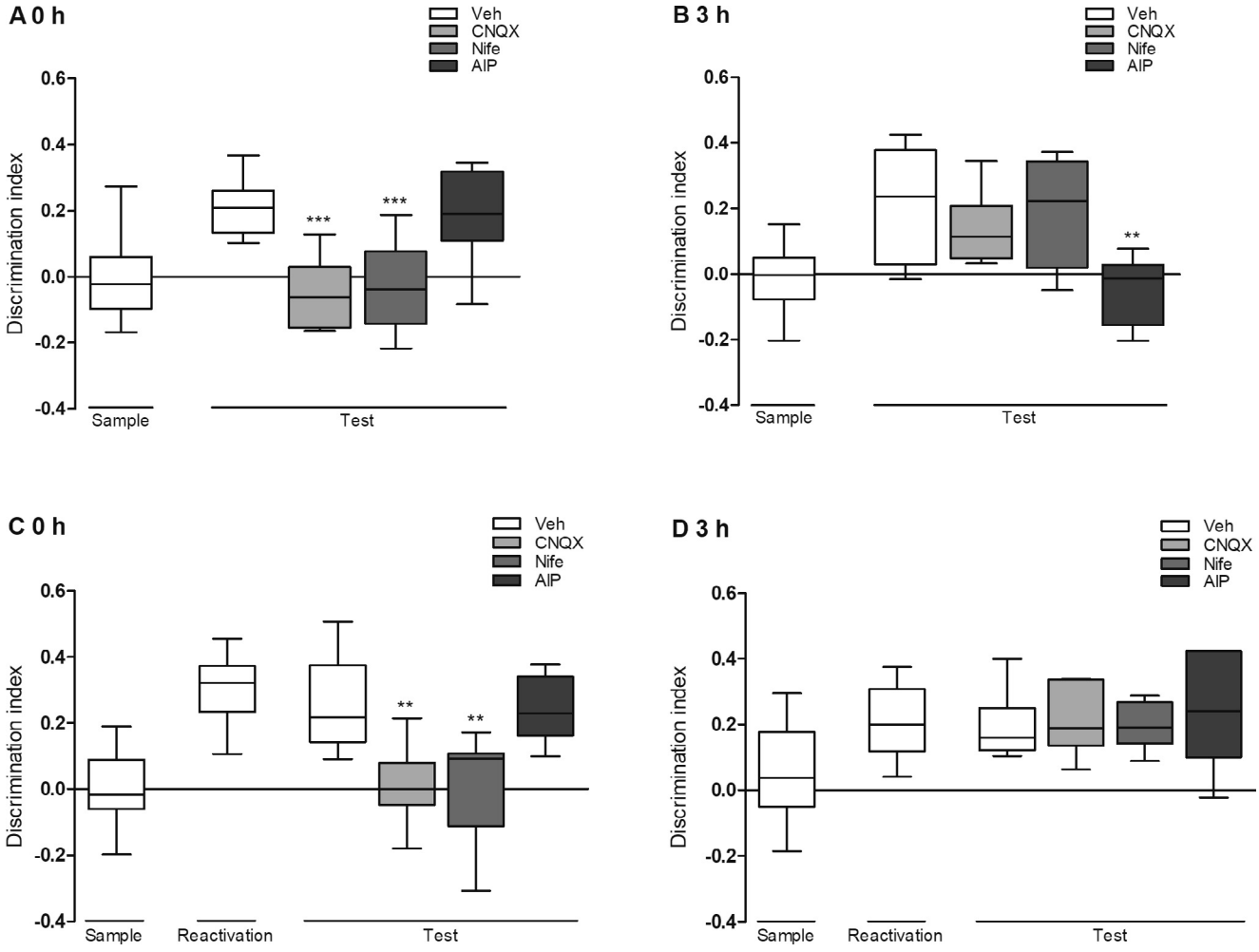

Fig. 2. Effect of CNQX, Nife and AIP given into the CA1 region of hippocampus on the consolidation and reconsolidation of ORM. **(A)** and **(B)** The AMPAr antagonist, CNQX and the L-VDDCs inhibitor, Nife impaired the consolidation of ORM when given into CA1 immediately but not 3 h after the sample phase, while the CaMKII inhibitor, AIP, impaired the consolidation of ORM when given into CA1 3 h but not immediately after the sample phase (**(A)**: $n = 9$–10 rats/group; **(B)**: $n = 7$–9 rats/group). *(C and D)* CNQX and Nife also impaired ORM reconsolidation when administered into CA1 immediately but not 3 h after the reactivation phase, while AIP had no effect on ORM reconsolidation (**(C)**: $n = 7$–9 rats/group, **(D)**: $n = 7$–8 rats/group). Discrimination indexes at the test phase were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. Data expressed as median, 25th to 75th percentiles, and minimum and maximum values. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs*. Veh group.

that it has been demonstrated that the consolidation of some memories can be modulated at distinct post-acquisition time-windows (McGaugh, 2000; Igaz et al., 2002; Myskiw et al., 2008; Romero-Granados et al., 2010; Marcondes et al., 2019).

Therefore, when administered 3 h after the sample phase (Fig. 2B), the groups that received Veh ($t_{(8)} = 3.59$, $p < 0.01$), CNQX ($t_{(7)} = 3.57$, $p < 0.01$) and Nife ($t_{(6)} = 3.09$, $p < 0.05$) showed a DI significantly different from zero on the test phase, whereas AIP ($t_{(7)} = 1.17$, $p = 0.27$) did not. One-way ANOVA on the test phase showed significant differences in DI between groups ($F_{(3,28)} = 5.34$, $p < 0.01$) and Bonferroni's multiple comparison test revealed differences in AIP ($p < 0.01$) *vs*. the Veh group. These results demonstrate that CNQX and Nife impaired ORM consolidation when administered into CA1 immediately after the sample phase, and AIP impaired ORM consolidation when administered 3 h after the sample phase.

Reconsolidation of ORM. As shown in Fig. 2C, the groups that received Veh ($t_{(7)} = 5.19$, $p < 0.01$) or AIP ($t_{(7)} = 6.99$, $p < 0.001$) immediately after the reactivation phase, presented a DI significantly different from zero on the test phase, while CNQX ($t_{(6)} = 0.24$, $p = 0.81$) and Nife ($t_{(8)} = 0.13$, $p < 0.89$) did not. One-way ANOVA on the test phase showed significant differences in DI between groups ($F_{(3,28)} = 8.54$, $p < 0.001$). Bonferroni's multiple comparison test revealed differences in CNQX ($p < 0.01$) and Nife ($p < 0.01$) groups *vs*. the Veh group.

Like memory consolidation, memory reconsolidation could also be modulated during a post-reactivation time-window (Romero-Granados et al., 2010; Schmidt et al., 2017). So, we verified the effect of blockade of AMPAr, L-VDCCs and CaMKII 3 h after reactivation phase of ORM.

As can be observed on Fig. 2D, when administered 3 h after the reactivation phase, the groups that received Veh ($t_{(7)} = 5.61$, $p < 0.001$), CNQX ($t_{(7)} = 5.71$, $p < 0.001$), Nife ($t_{(6)} = 7.26$, $p < 0.001$) and AIP ($t_{(6)} = 3.62$, $p < 0.05$) showed a DI significantly different from zero on the test phase. One-way ANOVA on the test phase showed no significant differences in DI between groups ($F_{(3,26)} = 0.24$, $p = 0.86$). These results demonstrate that CNQX and Nife impaired ORM reconsolidation when administered into CA1 immediately after the reactivation phase and AIP had no effect on the ORM reconsolidation.

Effect of AMPAr agonist given into the CA1 region of hippocampus in the impairment induced by CNQX, Nife and AIP on the consolidation and reconsolidation of ORM

Consolidation of ORM. To investigate the effect of AMPAr agonist, AMPA, as well as its effect on the impairment induced by the blockade of AMPAr, LVDCCs and CaMKII on the consolidation of ORM, animals received intra-CA1 infusion of Veh, AMPA, CNQX, Nife or co-infusion of CNQX + AMPA or Nife + AMPA immediately after the sample phase, and the infusion of Veh, AMPA, AIP or AIP + AMPA 3 h after the sample phase.

As shown in Fig. 3A, animals that received Veh ($t_{(7)} = 3.94$, $p < 0.01$), AMPA ($t_{(7)} = 3.39$, $p < 0.05$), CNQX + AMPA ($t_{(8)} = 4.81$, $p < 0.01$) and Nife + AMPA ($t_{(6)} = 4.104$, $p < 0.01$) immediately after the sample phase showed a DI significantly different from zero on the test phase, while CNQX ($t_{(7)} = 1.26$, $p = 0.24$) and Nife ($t_{(8)} = 1.31$, $p = 0.22$) did not. One-way ANOVA on the test phase showed significant differences in DI between groups ($F_{(5,43)} = 8.11$, $p < 0.001$). Bonferroni's multiple comparison test showed differences in CNQX ($p < 0.05$) *vs*. Veh, AMPA and CNQX + AMPA groups and differences in Nife ($p < 0.01$) *vs*. Veh, AMPA and Nife + AMPA groups.

In Fig. 3B, animals that received Veh ($t_{(7)} = 3.08$, $p < 0.05$) or AMPA ($t_{(6)} = 5.04$, $p < 0.01$) 3 h after the sample phase showed a DI significantly different from zero on the test phase, while AIP ($t_{(7)} = 0.04$, $p = 0.96$) and AIP + AMPA ($t_{(8)} = 1.10$, $p = 0.30$) did not. One-way ANOVA on the test phase showed significant differences in DI between groups ($F_{(3,28)} = 7.93$, $p < 0.001$). Bonferroni's multiple comparison test showed differences in AIP ($p < 0.05$) and AIP + AMPA ($p < 0.05$) *vs*. Veh and AMPA groups. These results demonstrate that AMPA administration immediately or 3 h the after the sample phase did not affect the consolidation of ORM but blocked the impairment induced by CNQX and Nife. However, AMPA did not block the impairment induced by AIP 3 h after the sample phase.

Reconsolidation of ORM. To investigate the effect of AMPAr agonist, AMPA, as well as its effect on the impairment induced by the blockade of AMPAr and LVDCCs on the reconsolidation of ORM, animals received intra-CA1 infusion of Veh, AMPA or co-infusion of CNQX + AMPA or Nife + AMPA immediately after the reactivation phase. As can be seen in Fig. 3C, the groups that received Veh ($t_{(8)} = 5.07$, $p < 0.01$), AMPA ($t_{(7)} = 5.26$, $p < 0.01$), CNQX + AMPA ($t_{(7)} = 7.81$, $p < 0.001$) and Nife + AMPA ($t_{(7)} = 5.51$, $p < 0.001$) immediately after the reactivation phase showed a DI significantly different from zero on the test phase, while CNQX ($t_{(6)} = 0.80$, $p = 0.45$) and Nife ($t_{(8)} = 0.30$, $p = 0.77$) did not. One-way ANOVA on the test phase showed significant differences in DI between groups ($F_{(5,43)} = 7.75$, $p < 0.001$). Bonferroni's multiple comparison test showed differences in CNQX ($p < 0.05$) *vs*. Veh, AMPA and CNQX + AMPA groups and differences in Nife ($p < 0.05$) *vs*. Veh, AMPA and Nife + AMPA groups. Thus, AMPAr agonist on its own did not affect the reconsolidation of ORM but blocked the impairment induced by CNQX and Nife.

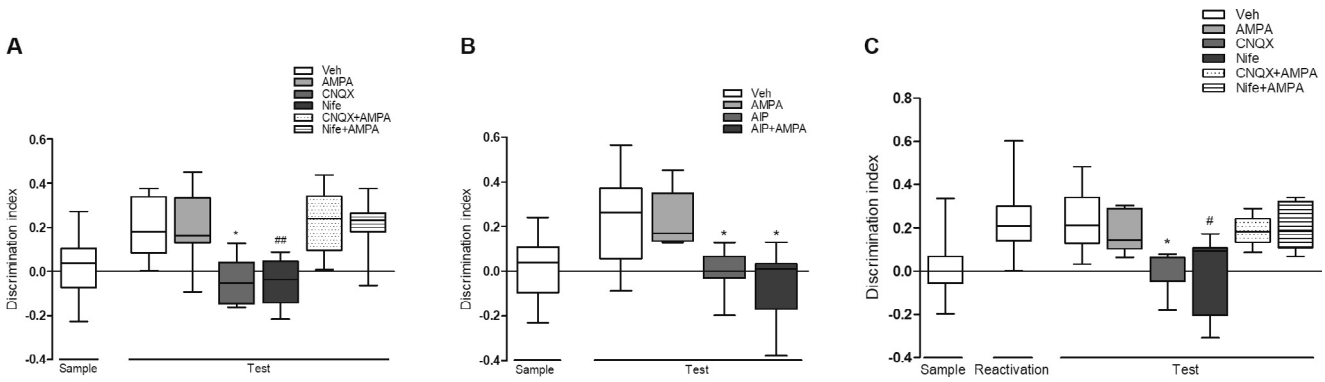

Fig. 3. Effect of AMPAr agonist given into the CA1 region of hippocampus on the impairment induced by CNQX, Nife and AIP on the consolidation and reconsolidation of ORM. The AMPAr agonist, AMPA, had no effect on the consolidation of ORM when infused into CA1, immediately **(A)** or 3 h **(B)** after the sample phase, but was able to block the impairment induced by CNQX and Nife (A: n = 7–9 rats/group; *p < 0.05 *vs*. Veh, AMPA and CNQX + AMPA groups; $^{\#\#}p$ < 0.01 *vs*. Veh, AMPA and Nife + AMPA groups). However, AMPA did not block the impairment on the consolidation of the ORM induced by AIP infused 3 h after the sample phase (B: n = 7–9 rats/group; *p < 0.05 *vs*. Veh and AMPA groups). Likewise, AMPA infusion into CA1 immediately after the reactivation phase had no effect, but blocked the impairment induced by CNQX and Nife on the reconsolidation of ORM (**C**: n = 7–9 rats/group; *p < 0.05 *vs*. Veh, AMPA and CNQX + AMPA groups; $^{\#}p$ < 0.05 *vs*. Veh, AMPA and Nife + AMPA groups). Discrimination indexes at the test phase were analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparison Test. Data expressed as median, 25th to 75th percentiles, and minimum and maximum values.

Effect of BDNF given into the CA1 region of hippocampus on the impairment induced by CNQX, Nife and AIP on the consolidation and reconsolidation of ORM

Consolidation of ORM. To investigate the effect of BDNF as well as its effect on the impairment induced by the blockade of AMPAr, LVDCCs and CaMKII on the consolidation of ORM, animals received intra-CA1 infusion of Veh, BDNF, CNQX, Nife or co-infusion of CNQX + BDNF or Nife + BDNF immediately after the sample phase, and the infusion of Veh, BDNF, AIP or AIP + BDNF 3 h after the sample phase.

As shown in Fig. 4A, animals that received Veh ($t_{(8)}$ = 5.28, p < 0.01), BDNF ($t_{(7)}$ = 3.40, p < 0.05), CNQX + BDNF ($t_{(7)}$ = 5.15, p < 0.01) or Nife + BDNF ($t_{(9)}$ = 5.69, p < 0.01) immediately after the sample phase showed a DI significantly different from zero on the test phase, while CNQX ($t_{(8)}$ = 1.69, p = 0.12) and Nife ($t_{(9)}$ = 0.93, p = 0.37) did not. One-way ANOVA on the test phase showed significant differences in DI between groups ($F_{(5,48)}$ = 10.99, p < 0.001). Bonferroni's multiple comparison test showed differences in CNQX (p < 0.01) *vs*. Veh, BDNF and CNQX + BDNF groups; Nife (p < 0.01) *vs*. Veh, BDNF and Nife + BDNF groups.

While on Fig. 4B, animals that received Veh ($t_{(7)}$ = 3.23, p < 0.05), BDNF ($t_{(6)}$ = 3.04, p < 0.05), AIP + BDNF ($t_{(7)}$ = 3.65, p < 0.01) 3 h after the sample phase showed a DI significantly different from zero on the test phase, while AIP ($t_{(6)}$ = 1.22, p = 0.26) did not. One-way ANOVA on the test phase showed significant

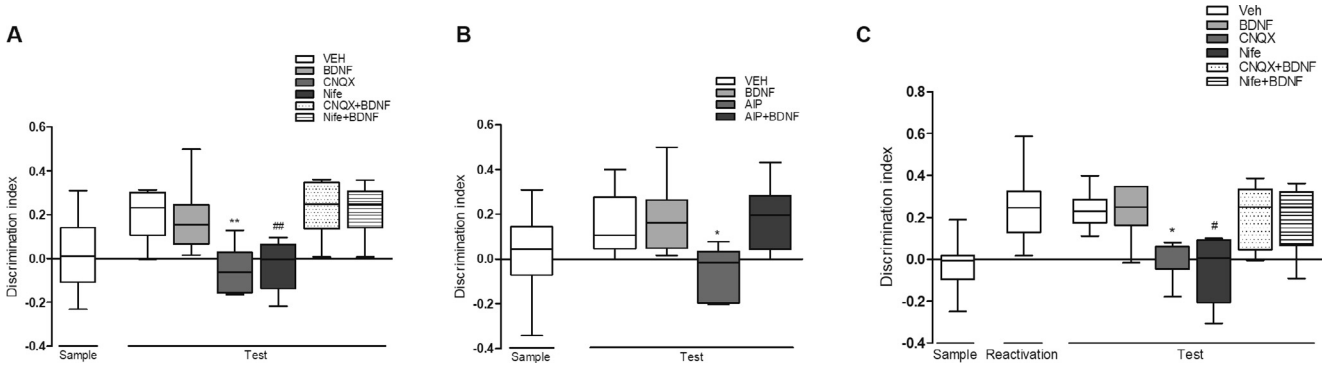

Fig. 4. Effect of BDNF given into the CA1 region of hippocampus on the impairment induced by Nife, AIP and CNQX on the consolidation and reconsolidation of ORM. The neurotrophin BDNF had no effect on the consolidation of ORM when infused into CA1 immediately **(A)** or 3 h **(B)** after the sample phase but was able to block the impairment on the consolidation of ORM induced by the administration of CNQX and Nife immediately after the sample phase (**A**: n = 8–10 rats/group; **p < 0.01 *vs*. Veh, BDNF and CNQX + BDNF groups; $^{\#\#}p$ < 0.01 *vs*. Veh, BDNF and Nife + BDNF groups) and AIP 3 h after the sample phase (**B**: n = 7–8 rats/group; *p < 0.05 *vs*. Veh, BDNF and AIP + BDNF groups). Also, BDNF infusion into CA1 immediately after the reactivation phase had no effect on the reconsolidation of ORM but blocked the impairment induced by CNQX and Nife (**C**: n = 7–8 rats/group; *p < 0.05 *vs*. Veh, BDNF and CNQX + BDNF groups; $^{\#}p$ < 0.05 *vs*. Veh, BDNF and Nife + BDNF groups) on the reconsolidation of ORM. Discrimination indexes at the test phase were analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparison Test. Data expressed as median, 25th to 75th percentiles, and minimum and maximum values.

differences in DI between groups ($F_{(3,26)} = 4.79$, $p < 0.01$). Bonferroni's multiple comparison test showed differences in AIP ($p < 0.05$) *vs*. Veh, BDNF and AIP + BDNF groups. These results demonstrate that BDNF administration immediately or 3 h after the sample phase did not affect the consolidation of ORM but blocked the impairment induced by CNQX and Nife administered immediately after the sample phase and the impairment of AIP administered 3 h after the sample phase.

Reconsolidation of ORM. To investigate the effect of BDNF as well as its effect on the impairment induced by the blockade of AMPAr, LVDCCs and CaMKII on the reconsolidation of ORM, animals received intra-CA1 infusion of Veh, BDNF or co-infusion of CNQX + BDNF or Nife + BDNF immediately after the reactivation phase. As can be seen in Fig. 4C, the groups that received Veh ($t_{(7)} = 7.58$, $p < 0.001$), BDNF ($t_{(6)} = 4.80$, $p < 0.001$), CNQX + BDNF ($t_{(6)} = 3.90$, $p < 0.01$) or Nife + BDNF ($t_{6)} = 3.05$, $p < 0.05$) immediately after the reactivation phase showed a DI significantly different from zero on the test phase, while CNQX ($t_{(6)} = 0.80$, $p = 0.45$) and Nife ($t_{(7)} = 0.92$, $p = 0.38$) did not. One-way ANOVA on the test phase showed significant differences in DI between groups ($F_{(5,38)} = 7.65$, $p < 0.001$). Bonferroni's multiple comparison test showed differences in CNQX ($p < 0.05$) *vs*. Veh, BDNF and CNQX + BDNF groups and differences in Nife ($p < 0.05$) *vs*. Veh, BDNF and Nife + BDNF groups. Thus the results show that BDNF on its own did not affect the reconsolidation of ORM but blocked the impairment induced by CNQX and Nife.

Effect of the drugs alone or in combination on objects total exploration time

The mean of time (seconds) each experimental group spent exploring both objects is shown on Table 1 in Supplementary data for each of the above figures. As can be observed there were no differences in exploration time during the sample phase, reactivation phase and test phase. This suggests that the drugs had no apparent effects on locomotor activity or the normal tendency for exploration of objects.

DISCUSSION

Here we add a new understanding regarding the molecular mechanisms involved on the consolidation and reconsolidation of recognition memory and that these processes do not involve totally identical mechanisms, since into the hippocampus AMPAr and L-VDCCs are necessary to the consolidation and reconsolidation of ORM, while CaMKII is necessary only to the consolidation of ORM. Furthermore, the results suggest a link between the neurotrophin BDNF and AMPAr, L-VDCCs and CaMKII as well as a link between AMPAr and L-VDCCs but not CaMKII on the consolidation and reconsolidation of ORM.

First we demonstrated that the blockade of AMPAr and L-VDCCs into CA1 immediately but not 3 h after the sample phase or reactivation phase impaired the consolidation and reconsolidation of ORM, while the blockade of CaMKII impaired the consolidation of ORM when AIP was administered 3 h after the sample phase and had no effect on the reconsolidation of ORM. The investigation on the role of L-VDCCs on memory processing have demonstrated that its blockade in different brain structures impair fear memory consolidation and extinction (Bauer et al., 2002; Lashgari et al., 2006; Ou and Gean, 2007; Davis and Bauer, 2012; de Carvalho Myskiw et al., 2014), in CA1 impairs reconsolidation of spatial memory (Da Silva et al., 2013) and in perirhinal cortex impairs ORM consolidation (Seoane et al., 2009). Here we demonstrated that L-VDCCs of the CA1 region of the hippocampus also participate on the consolidation and reconsolidation of ORM.

Similarly, CaMKII have been demonstrated to be necessary on consolidation and extinction of fear memories (Wolfman et al., 1994; Vianna et al., 2000; Szapiro et al., 2003; Tinsley et al., 2009; Buard et al., 2010; Da Silva et al., 2013; Jarome et al., 2013; de Carvalho Myskiw et al., 2014) as well as on the consolidation of ORM in the perirhinal cortex (Tinsley et al., 2009). In agreement with these results, we verified that in CA1, CaMKII is necessary to ORM consolidation, however its participation did not occur immediately after learning, since AIP impaired ORM consolidation when infused 3 h after the sample phase. However, our results demonstrated that CaMKII in the CA1 region is not necessary to the reconsolidation of ORM, since the infusion of AIP up to 3 h after the reactivation phase did not affect memory retention. Other studies examining the role of CaMKII on reconsolidation process have found mixed results, with some also indicating normal memory retention after retrieval inhibition of CaMKII (Sakurai et al., 2007; Da Silva et al., 2013; Arguello et al., 2014; Jarome et al., 2016). One possible explanation for CaMKII inhibitors not to affect memory when administered after retrieval is that CaMKII could be acting regulating protein degradation upstream of its potential regulation of protein synthesis during the reconsolidation process. As observed by Jarome and collaborators, CaMKII activity is critical for retrieval-induced increases in proteasome activity, and pharmacological manipulation of CaMKII activity is able to rescue memory impairments resulted from post-retrieval blockade of protein synthesis in a fear memory (Jarome et al., 2016). This could also be the case of reconsolidation process of recognition memory, however other studies are necessary to verify this possibility.

Regarding AMPAr, its role in several brain structures on memory processing have been widely described with the blockade of these receptors impairing the consolidation and extinction of fear memories as well as consolidation and reconsolidation of spatial and recognition memory (Bernabeu et al., 1997; Pedreira et al., 2002; Winters and Bussey, 2005; Rose and Rankin, 2006; Rodriguez-Ortiz et al., 2012; Garcia-Delatorre et al., 2014; Santoyo-Zedillo et al., 2014). Despite studies demonstrating the participation of AMPAr from the perirhinal cortex on the consolidation and reconsolidation of ORM (Winters and Bussey, 2005;

Santoyo-Zedillo et al., 2014) the participation of AMPAr of CA1 region of hippocampus was unclear. Therefore, here we verified that AMPAr of CA1 region of hippocampus also participate on the consolidation and reconsolidation of ORM, since its blockade with CNQX immediately but not 3 h after the sample phase or the reactivation phase impaired memory retention.

We also demonstrated that the infusion of AMPAr agonist, AMPA, was able to block the impairment induced by CNQX and Nife on both consolidation and reconsolidation of ORM, but had no effect on the impairment caused by the blockade of CaMKII on consolidation of ORM. A link between L-VDCCs and AMPAr have been demonstrated in neurons culture (Kang et al., 2006) and this association increases the cell-surface localization of AMPAr (Seo et al., 2018), while the blockade of L-VDCCs impair activity-triggered surface delivery of AMPAr (Timpe et al., 2014). So, as demonstrated at cellular level, here we suggest that there is a link between L-VDDCs and AMPAr on the consolidation and reconsolidation of ORM. This link might be explained by the fact that increased $Na^{\pm}$ ions influx through AMPAr could lead to increased Ca^{2+} release from intracellular stores (Hoyt et al., 1998; Zhang and Lipton, 1999).

Moreover, CaMKII have been shown to regulate AMPAr incorporation into synapses during LTP (Barria et al., 1997; Derkach et al., 1999; Lisman et al., 2002, 2012). CaMKII phosphorylates Ser-831 in the GluR1 subunit of AMPAr, such phosphorylation allows the integration of new AMPAr at the postsynaptic density further potentiating synaptic transmission (Barria et al., 1997; Poncer et al., 2002; Kristensen et al., 2011). On the consolidation of inhibitory avoidance memory, it was verified an increase in the levels of GluR1 subunits of AMPAr in the CA1 region that was blocked by a CaMKII inhibitor, suggesting that consolidation of memory promotes the translocation of AMPAr to the post-synaptic terminal of CA1 neurons through a mechanism that requires the activation of CaMKII-dependent phosphorylation of GluR1 (Bevilaqua et al., 2005). Here we verified that the infusion of an AMPAr agonist was unable to abolish the impairment induced by CaMKII inhibitor, suggesting that on the consolidation of ORM there is no link between AMPAr and CaMKII. This lack of effect does not seem to be related to the dose of agonist used, since the same dose was able to abolish the impairment induced by both AMPAr antagonist, CNQX and L-VDDCs inhibitor, Nife, on the consolidation and reconsolidation of ORM. However, the results suggest that on the consolidation and reconsolidation of ORM there is no link between CaMKII and AMPAr.

Another interesting result obtained here is regarding the participation of neurotrophin BDNF, that when administered into CA1 did not affect the consolidation and reconsolidation of ORM but was able to abolish the impairment induced by the blockade of AMPAr, L-VDCCs and CaMKII on ORM consolidation and also to abolish the impairment induced by AMPAr and L-VDCCs blockade on ORM reconsolidation. BDNF has been widely related with synaptic plasticity, neurogenesis and plays a crucial role in learning and memory in different brain structures and distinct memory stages (Pang et al., 2004; Bekinschtein et al., 2007; Furini et al., 2010; Lee and Hynds, 2013; Rodríguez-Serrano et al., 2014; Rosas-Vidal et al., 2014, 2018; Signor et al., 2017). In this respect, it has been reported that hippocampal-specific deletion of the BDNF gene impairs ORM (Heldt et al., 2007), while intra-CA1 infusion of function-blocking anti-BDNF antibodies hinders ORM consolidation.

Moreover, BDNF may enhance phosphorylation of AMPAr GluR1 subunit and the expression of AMPAr (Narisawa-Saito et al., 2002; Wu et al., 2004; Caldeira et al., 2007) while the activation of AMPAr increases expression of BDNF (Zafra et al., 1990, 1992; Lauterborn et al., 2000), and the blockade of AMPAr reduces concentration of BDNF on both CA1 and perirhinal cortex (Kealy and Commins, 2009). Regarding BDNF and L-VDCCS relationship, it was demonstrated that BDNF-mediated LTP in hippocampal neurons requires activation of voltage-activated calcium channels (Kovalchuk et al., 2002) and that calcium entry through L-type channels play an important role in activity-dependent BDNF expression in hippocampal neurons (Zafra et al., 1990; Ghosh et al., 1994; Tao et al., 1998; Tabuchi et al., 2000; Kolarow et al., 2007). On memory process, fear-conditioning learning induced an increase in BDNF levels that was significantly reduced by inhibitors of L-VDCCs (Ou and Gean, 2007). Concerning the relationship of BDNF with CaMKII, it was demonstrated that BDNF transcription is promoted by CaMKII activation, and membrane depolarization-triggered Ca^{2+} influx through L-VDCCs induce an increase in BDNF mRNA expression in cultured neurons whose effect is blocked by inhibitors of CaMKII (Zafra et al., 1992). Also, BDNF in hippocampal neurons showed to enhance CaMKII autophosphorylation and consistent with upregulation of CaMKII activity, CaMKII-dependent phosphorylation of AMPAr was also increased in BDNF-treated slices (Zeng et al., 2010). In agreement with these results, here we suggest a link between BDNF and AMPAr, L-VDCCS and CaMKII on the consolidation of ORM and a link between BDNF and AMPAr and L-VDCCS on the reconsolidation of ORM.

The possible mechanisms linking BDNF with L-VDCCS and AMPAr could be related to the fact that binding of BDNF to TrkB receptors stimulates phospholipase C (PLC) pathway giving rise to inositol-1,4,5-trisphosphate (IP3), which mobilizes Ca^{2+} from intracellular stores, and diacylglycerol, which activates PKC (Kaplan and Miller, 2000; Huang and Reichardt, 2003; Reichardt, 2006). PKC can phosphorilate Ser-818 in the GluR1 subunit of AMPAr, controlling the delivery of AMPAr to the synapse (Boehm et al., 2006). Therefore, BDNF signaling through mobilization of Ca^{2+} from intracellular stores and PKC activation could explain the relationship of BDNF with L-VDCCs and AMPAr.

Regarding the mechanism that could explain the link between BDNF and CaMKII three hours after learning of ORM, it could also be related to the stimulation of PLC by BDNF signaling pathway, since it was demonstrated that BDNF both phosphorylates and activates CaMKII in

adult rat hippocampus via stimulation of PLC and subsequent intracellular Ca^{2+} mobilization (Blanquet and Lamour, 1997; Blanquet, 1999).

The study of the link between synaptic proteins is key to our understanding of memory processes. So, this study provides new evidence of the molecular mechanisms involved on the consolidation and reconsolidation of ORM in the hippocampus, demonstrating that AMPAr and L-VDCCs are necessary to the consolidation and reconsolidation of ORM while CaMKII is necessary only to the consolidation of ORM and also that there is a link between the neurotrophin BDNF and AMPAr, L-VDCCs and CaMKII as well as a link between AMPAr and L-VDCCs on ORM consolidation and reconsolidation. Taken together the results provide further insight about the molecular mechanisms underlying the consolidation and reconsolidation of recognition memory into the hippocampus.

DECLARATION OF INTEREST

None.

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APPENDIX A. SUPPLEMENTARY DATA

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