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Rapid Communication

Involvement of the infralimbic cortex and CA1 hippocampal area in reconsolidation of a contextual fear memory through CB1 receptors: Effects of CP55,940

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ABSTRACT

The endocannabinoid system (ECS) has a pivotal role in different cognitive functions such as learning and memory. Recent evidence confirm the involvement of the hippocampal CB1 receptors in the modulation of both memory extinction and reconsolidation processes in different brain areas, but few studies focused on the infralimbic cortex, another important cognitive area. Here, we infused the cannabinoid agonist CP55,940 either into the infralimbic cortex (IL) or the CA1 area of the dorsal hippocampus (HPC) of adult male Wistar rats immediately after a short (3 min) reactivation session, known to labilize a previously consolidated memory trace in order to allow its reconsolidation with some modification. In both structures, the treatment was able to disrupt reconsolidation in a relatively long lasting way, reducing the freezing response. To our notice, this is the first demonstration of ECS involvement in reconsolidation in the Infralimbic Cortex. Despite poorly discriminative between CB1 and CB2 receptors, CP55,940 is a potent agent, and these results suggest that a similar CB1-dependent circuitry is at work both in HPC and in the IL during memory reconsolidation.

1. Introduction

Converging evidence from several studies does not cease to provide consistent support to a pivotal role for the endocannabinoid system (ECS) in different cognitive processes, with emphasis in learning and memory (e.g., Basavarajappa, Nagre, Xie, & Subbanna, 2014; Quillfeldt & de Oliveira Alvares, 2015, chap. 3; Ratano, Everitt, & Milton, 2014). CB1 receptors are widely expressed throughout the brain, with significant levels expressed in areas such as the dorsal hippocampus, the basolateral amygdala and the prefrontal cortex, all involved in learning and memory processes (Herkenham et al., 1990; Marsicano & Kuner, 2008). Endogenous cannabinoids such as anandamide (AEA) or 2-AG, synthesized on demand, act as retrograde modulators of GABA and Glutamate transmission, inhibiting their release inhibit neurotransmitter release by a retrograde action (Katona & Freund, 2012; Kortleven, Fasano, Thibault, Lacaille, & Trudeau, 2011; Szabó et al., 2014).

A considerable amount of evidence indicates that previously consolidated memories can become labile/unstable after retrieval under certain "boundary" conditions: a reactivation session consisting of a short-lasting re-exposition to the training context, in the absence of the unconditioned stimulus, allows for the memory trace to become susceptible again to pharmacological and behavioral disruption, undergoing a subsequent re-stabilization process known as reconsolidation (Duvarci & Nader, 2004). However, when this reactivation session is prolonged beyond a certain critical period, a different process takes place, with the creation of a new trace where the conditioned response has a decreased expression – a process called extinction (Bouton, Westbrook, Corcoran, & Maren, 2006; De Oliveira Alvares, Genro, Diehl, Molina, & Quillfeldt, 2008; Myers & Davis, 2007; Pedreira & Maldonado, 2003).

Previous findings from our lab show that the administration of the agonist/endogenous CB1 ligand AEA into the hippocampus impaired memory reconsolidation, while the selective CB1

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antagonist AM251 enhanced it (De Oliveira Alvares, Genro, Diehl, Molina, et al., 2008). Both drugs were also effective upon extinction – when infused after a longer, 25 min re-exposure session – however, with remarkable "opposite" effects: AEA facilitated and AM251 impaired extinction (De Oliveira Alvares, Genro, Diehl, Molina, et al., 2008).

Effects are not usually that clear when the endogenous ligand anandamide is the drug of choice, since it is difficult to estimate endogenous levels and predict the consequences of the unavoidable fact that they will *pool* with the exogenously administered quantity (De Oliveira Alvares, Genro, Diehl, & Quillfeldt, 2008). Despite AM251 having a clearcut effect – amnestic upon memory consolidation and facilitatory upon retrieval – AEA was facilitatory upon consolidation and had no effect upon retrieval (De Oliveira Alvares, Genro, Diehl, & Quillfeldt, 2008). This may be due, at least in part, to the fact that anandamide also acts as a TRPV1 endogenous ligand (Ross, 2003). Indeed, a detailed study from our lab revealed some involvement of the endovanilloid system in memory modulation, but only when strong aversive stimulus (shock) is present: TRPV1 antagonist capsazepine was able to impair memory consolidation, while the agonist capsaicin did not affect any of two different aversive tasks (Genro, de Oliveira Alvares, & Quillfeldt, 2012).

Recent reports have suggested that endocannabinoid CB1 receptors in the infralimbic cortex (IL), located in the ventromedial region of the prefrontal cortex, may have an important role in the extinction of fear memories: the infusion of the CB1 agonist WIN55212-2 (Lin, Mao, Su, & Gean, 2009), or the CB1 antagonist cannabidiol (Do Monte, Souza, Bitencourt, Kroon, & Takahashi, 2013) into the IL was shown to facilitate fear memory extinction in rats: despite being a poorly selective indirect CB1 antagonist, cannabidiol is know to potentiate the effects of agonists, and in this work, its effect was blocked by rimonabant, suggesting a CB1-mediated action.

From a therapeutic point of view, extinction has been employed to supress maladaptive memories, but not without its limitations: the progressive decay of emotional response obtained usually do not last and fear response is (spontaneously) recovered over time (Liu et al., 2014; Revillo, Paglini, & Arias, 2014; Schiller et al., 2008). Since memory reconsolidation seems able to modify the original memory trace and different reports suggest that pharmacological and behavioral inhibition of the reconsolidation process prevent the re-expression of the previously consolidated emotional memories, reconsolidation seems quite promising in clinical terms (Schiller et al., 2010; Yang, Huang, & Hsu, 2011). The endocannabinoid system has also been proposed as a promising therapeutic target for drugs devised to decrease the impact of maladaptive memories such as those verified in PTSD – post-traumatic stress disorder (De Carvalho, Pamplona, Cruz, & Takahashi, 2014; Ratano et al., 2014), despite – to this point – clinical trials having been mostly inconclusive (Bucherelli, Baldi, Mariottini, Passani, & Blandina, 2006; Gazarini, Stern, Piornedo, Takahashi, & Bertoglio, 2014; Lee & Flavell, 2014).

The aim of this study was to verify the effect of the cannabinoid agonist CP55,940, when infused either into the IL cortex, or into the CA1 area of the dorsal HPC after a reactivation session of a contextual fear conditioning.

2. Materials and methods

One hundred twenty-one Wistar rats (270–320 g) from our breeding colony were used. Animals were housed in plastic cages, four to five per cage, under a 12 h light/dark cycle and at a constant temperature of 24 ± 1 °C, with water and food *ad libitum*. All experiments were conducted in accordance to our federal legislation (Law 11794/2008) and local guidelines for animal care, and the project, approved by the University Ethics Committee (CEUA/UFRGS Project # 17862).

Rats were deeply anesthetized by an i.p. injection of ketamine/xylazine (75 and 10 mg/kg, respectively) and bilaterally implanted with 27-gauge guide cannulae aimed 1 mm above the CA1 area of the dorsal hippocampus (AP: −4.0 mm, LL: ±3.0 mm, DV: 1.6 mm) or IL (AP: +3.2 mm, LL: ±0.6 mm, DV: 4.0 mm) from bregma (Paxinos & Watson, 1998). After a 1 week recovery from surgery, animals were submitted to the behavioral procedures. Following the behavioral experiments, subjects were sacrificed and their brains dissected and preserved in 10% formaldehyde to verify the correct position of the cannula. Only the 106 animals with the correct cannula placement (see Fig. 3) were considered in the statistical analysis.

The potent, non-selective cannabinoid receptor agonist CP55,940, was dissolved in phosphate buffered saline (PBS, isotonic) with 8% dimethylsulfoxide to a final concentration of 5 μg/μL (a safe hydrophobic vehicle regularly used in our and other labs – see, e.g., De Oliveira Alvares et al., 2005, 2006; De Oliveira Alvares, Genro, Diehl, & Quillfeldt, 2008; De Oliveira Alvares, Genro, Diehl, Molina, et al., 2008). At the time of infusion, a 30-gauge infusion needle was fitted into the guide cannulae, with its tip protruding 1.0 mm beyond the guide cannula end, and aimed either to the pyramidal cell layer either of the infralimbic cortex, or – form comparative reasons – the CA1 area of the dorsal hippocampus. In all experiments, a 0.5 μL volume was bilaterally infused in each structure at a slow rate (20 μL/h), and the needle removed after waiting for an additional 30 s.

The conditioning chamber consisted of an (indirectly) illuminated Plexiglas box (20 × 23 × 22 cm), with a metallic grid floor of parallel 0.1-cm caliber stainless steel bars spaced 1.0 cm apart. In the training session of the Contextual Fear Conditioning (CFC), rats were left to habituate for 3 min to the conditioning chamber before receiving two 2-s, 0.7-mA footshocks separated by a 30-s interval (the US or unconditioned stimulus) and kept in the conditioning environment for an additional minute before returning to their homecages.

In experiment I subjects were intrahippocampally infused with CP55,940 immediately after training, in three different concentrations (1, 5 and 10 μg/μL) in order to verify the effective concentration able to disrupt memory *Consolidation* (Fig. 1A). In experiment II we evaluated if the observed effect was mediated by CB1 receptors verifying if CP55,940 effect could be reversed by a concomitant, subthreshold concentration of the CB antagonist AM251 (Fig. 1B). Since the effective concentration for intrahippocampally infused AM251 was found to be 5.5 ng/side or 20 μM, dissolved in 0.5 μL volume of vehicle (De Oliveira Alvares, Genro, Diehl, & Quillfeldt, 2008; De Oliveira Alvares et al., 2005, 2006), with 2 mM being proven ineffective: that is why we employ 0.2 μM of AM251 to revert the CP55.940 effect, a concentration well bellow the minimum effective value. In experiments III and IV subjects were infused with the effective CP55,940 concentration right after a 180 s context re-exposure (reactivation session) 48 h after training, in order to observe *Reconsolidation* effects in two successive tests, one 48 h after reactivation, and the other, 7 days later: these Reconsolidation effects were verified for two different brain structures, the CA1 area of the dorsal Hippocampus (Fig. 2A), or the Infralimbic cortex (Fig. 2B). In all tests, animals have their freezing behavior recorded for 4 min in the same conditioning context without the US.

Since data from all experimental groups was proven to be both homoscedastic and normally distributed (Kolmogorov–Smirnov test with Lilliefors' correction, $P > 0.05$), results were analyzed either with One-way ANOVA followed by a Tukey HSD *post hoc* test (if needed) – experiments I and II has four independent groups