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Corticosteroid-endocannabinoid loop supports decrease of fear-conditioned response in rats

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Abstract
The endocannabinoid (eCB) and glucocorticoid systems contribute to the modulation of emotional states. Noteworthy, glucocorticoid hormones are released by adrenal glands during stressful events and endocannabinoids are released in the brain during fear-conditioned responses. Since it was already suggested that glucocorticoids may trigger the release of endocannabinoids in the brain, our objective was to investigate whether the interaction between these neuromodulatory systems contributes to the decrease of conditioned freezing behavior over successive 9-min exposures to the conditioning context. Present results suggest a bidirectional interdependence between glucocorticoid and endocannabinoid systems. CB_1 receptors blockade prevents glucocorticoid-induced facilitation of conditioned freezing decrease and inhibition of glucocorticoid synthesis renders boosting of endocannabinoid signaling innocuous, while preserving the efficacy of direct CB_1 receptors activation by an exogenous cannabinoid agonist. This suggests that CB_1 receptors are somehow "downstream" to glucocorticoid release, which in its turn, is reduced by CB_1 activation, contributing to the persistent reduction of conditioned freezing responses.

1. Introduction

Understanding the synaptic mechanisms and neural substrates of emotional modulation may lead to innovative approaches to a number of psychiatric diseases, especially those related to stress, such as anxiety disorders, depression and trauma-related diseases (Mahan and Ressler, 2012). In this context, memory extinction is particularly useful, since it helps achieving suppression of emotional responses through repetitive and/or sustained exposures to conditioned stimuli (Milad and Quirk, 2012). Extinction training represents an experimental correlate to investigate biological processes underlying exposure-based therapies for trauma-related diseases in laboratory animals (Kaplan et al., 2011). Of note, extinguished responses tend to spontaneously recover with the passage of

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time, illustrating the fact that the memory trace is not erased during extinction, but rather inhibited, with suppression of a fearful behavioral output upon exposure to the feared stimulus (Bouton et al., 2006). More recent protocols involving a retrieval procedure immediately before extinction claim to successfully "delete" the memory trace (Monfils et al., 2009). While this may be true for the cognitive aspects of the fear memory, there is evidence that sensitization components do not reduce after extinction with (Costanzi et al., 2011) or without previous retrieval (Pamplona et al., 2011). This is particularly true for remote memories (for a recent review on fear relief, see Riebe et al., 2012).

It is already known that the endocannabinoid system is essential to the modulation of emotional states and decrease of fear-conditioned response (Kamprath and Wotjak, 2004; Marsicano et al., 2002; and others). Besides that, boosting eCB signaling facilitates decrease of fear-conditioned response in different behavioral tasks (Bitencourt et al., 2008; Chhatwal et al., 2005; Lin et al., 2009; Pamplona et al., 2008). The glucocorticoid system is also largely involved in decrease of fear-conditioned response (Barrett and Gonzalez-Lima, 2004; Clay et al., 2011; Yang et al., 2006; Yang et al., 2007; and others). Glucocorticoids are steroid hormones (corticosterone in animals and cortisol in humans), released from the adrenal cortex, that can easily cross the blood-brain barrier and bind to glucocorticoid (GC) receptors located in many brain regions, included that relevant areas within the fear circuitry in the brain (Bentz et al., 2009; Fuxe et al., 1985; Korte, 2001).

As the glucocorticoids – "stress hormones" – are also a fundamental part of the mechanisms leading to fear expression and modulation, we wanted to know whether endocannabinoid and glucocorticoids systems interact in the process of contextual conditioned fear extinction, recorded as the reduction of conditioned freezing response over consecutive exposure to the conditioning context (Fanselow, 1980). Previous evidence showed that endogenous release of corticosterone is necessary for decrease of fear-conditioned response (Barrett and Gonzalez-Lima, 2004) and a putative fast inhibitory feedback of endocannabinoid on glucocorticoid secretion was suggested in the paraventricular nucleus of the hypothalamus (Malcher-Lopes et al., 2006). On top of that, a slower component of endocannabinoid-mediated glucocorticoid feedback in the mPFC alleviates the consequences of stress (Hill et al., 2011). Therefore, we hypothesized that endocannabinoids participate in a feedback loop of suppression of glucocorticoid release during decrease of fear-conditioned response; this hypotheses can be explored by trying to understand whether this mechanism would include initial contribution of glucocorticoid release, assuming that a certain level of glucocorticoid release would trigger endocannabinod "on demand" synthesis.

2. Experimental procedures

2.1. Animals

Male adult Wistar rats (3 months old) bred and reared at the animal facility of Department of the Pharmacology of the Universidade Federal de Santa Catarina (Florianopólis, Brazil) were used for behavioral experiments. The animals were kept in collective plastic cages (4–5 rats/cage) with food and water available ad libitum and maintained in a room at a controlled temperature ($23 \pm 2\ ^{\circ}C$) under a 12:12-h light/dark cycles (light on at 7:00 A.M.). Each behavioral test was conducted during the light phases of the cycle (9:00 A.M. to 5:00 P.M.) using independent experimental groups consisting of 8–12 animals per group. All experimental procedures were performed according to the Principles of Laboratory Animal Care of the NIH and approved by the local Animal Care Committee of the institution involved in the study.

2.2. Drugs

The compounds used in behavioral experiments were dexamethasone, a synthetic GR agonist (Tocris, USA); AM404, an inhibitor of endocannabinoid uptake (Tocris, USA); WIN55212-2, a cannabinoid receptor agonist (Tocris, USA); mifepristone, a glucocorticoid receptor (GR) antagonist (Tocris, USA); SR141716A, a CB_1 cannabinoid receptor antagonist (Sanofi-Aventis, France); metyrapone, a corticosteroid synthesis inhibitor (Sigma-Aldrich, USA); and spironolactone, a mineralocorticoid receptor (MR) antagonist (Tocris, USA). For i.c.v. injections, all drugs were stored in DMSO stock solutions (50 mM) and freshly diluted in 0.1 M PBS, pH 7.4, yielding a final concentration of 10% DMSO. For i.p. and s.c. injections, a similar procedure was used, but 0.1% Tween 80 was added to the final solution to enhance solubility. The respective vehicle was used as control for i.c.v., i.p. and s.c. injections. Drug doses were selected based on previous reports (Bitencourt et al., 2008; Oitzl et al., 1998; Rahmouni et al., 2001; Van Acker et al., 2001; Yang et al., 2006, 2007).

2.3. Stereotaxic surgery

The rats were deeply anesthetized with a 1:1 mixture of ketamine (75 mg/kg) and xylazine (15 mg/kg) and placed in a stereotaxic apparatus (Kopf, model 957), with bregma and lambda being kept on the same horizontal plane. A hole was drilled into the skull and a stainless steel guide cannula (23 G, 10 mm long) was lowered aiming at the right lateral ventricle. The following stereotaxic coordinates were used: LL = −1.6 mm; DV = −3.6, AP = −0.8 mm from bregma according to the rat brain atlas (Paxinos and Watson, 2002). Two screws were implanted into the skull and fixed with dental acrylic. A 30-G stainless steel stylet was placed into the guide cannula to prevent entry of foreign materials. The experimental procedure started 5–7 days after surgery.

2.4. Infusion procedure

Dexamethasone (0.1–1 μg/μl), AM404 (0.5 μg/μl, i.c.v.), WIN (0.1 μg/μl, i.c.v.), mifepristone (100 ng/μl, i.c.v.) and spironolactone (100 ng/μl, i.c.v.) were infused through a 30-G injector (11 mm long) connected to a 10 μl micro-syringe (Hamilton, USA) by polyethylene tube (PE10). The injector was carefully inserted into the guide cannula and the drug solution was injected with the aid of an automatic infusion pump (Insight, Brazil), at a rate of 2 μl/min and total

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