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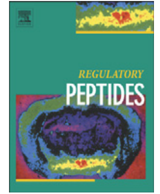

Tolerance to hypophagia induced by prolonged treatment with a CB_1 antagonist is related to the reversion of anorexigenic neuropeptide gene expression in the hypothalamus

Rodrigo Rorato *, Cristiano Miyahara, José Antunes-Rodrigues, Lucila Leico Kagohara Elias

Department of Physiology, School of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil

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ABSTRACT

It is well established that treatment with rimonabant, a CB_1 antagonist, decreases food intake and body weight gain. In part, these responses are mediated by increased activity of hypothalamic neurons related with energy homeostasis. However, food consumption is reversed to basal level during prolonged CB_1 antagonist treatment, suggesting tolerance to its anorexigenic effect. This study investigated the effects of acute or prolonged CB_1 receptor blockade on the expression of hypothalamic neuropeptides involved with energy homeostasis. Male Wistar rats received vehicle, a single dose or daily doses of rimonabant (10 mg/kg by gavage) over 7 days. Food intake, body weight, CRF and CART immunoreactivity, as well as, mRNA expression of hypothalamic neuropeptides were evaluated. In comparison with vehicle treatment, single dose of rimonabant decreased food intake and body weight. Acute rimonabant treatment also increased Fos-CRF and Fos-CART double labeled neurons in the PVN and Fos immunoreactivity in the ARC. We also observed that acute rimonabant treatment increased CRF, CART and TRH mRNA expression in the PVN, while it decreased POMC and NPY mRNA expression in the ARC with no changes in the CART mRNA expression in this nucleus. There was an increase in CB_1 mRNA expression in the PVN of rats that received both acute and prolonged-rimonabant treatment. Interestingly, rats subjected to prolonged rimonabant treatment had no changes in food intake, body weight gain, hypothalamic mRNA expression, Fos expression and CRF and CART neuron activation. These data indicate that tolerance to hypophagic effects of CB_1 antagonist, rimonabant, is associated with reversion of hypothalamic neuropeptide gene expression related to regulation of energy homeostasis.

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1. Introduction

The endogenous cannabinoid system is involved in several physiological and pathological functions, such as control of food intake and body weight [1–3]. As such, the lipid-derived cannabinoid mediators have an important orexigenic role [4–7], as demonstrated by the increased hypothalamic endocannabinoid content in obese Zucker rats [8]. The effects of endocannabinoids on food intake are mediated by cannabinoid type-1 (CB_1) receptor signalling, as shown by the reversion of cannabinoid-induced food intake with the administration of the selective CB_1 receptor antagonist rimonabant [6,9]. Consistent with CB_1-mediated orexigenic effects of endocannabinoids, transgenic mice that lack its receptor exhibit decreased food consumption [8].

In part, rimonabant-induced decrease of food intake is due to changes in endocannabinoid signalling via CB_1 receptors in the hypothalamus, a crucial area associated with the homeostatic control of food intake [10–13]. In the hypothalamus, CB_1 receptors are found in neurons expressing neuropeptides with anorexigenic role, such as the cocaine-amphetamine regulated transcript (CART) and corticotrophin-releasing factor (CRF) in the paraventricular nucleus (PVN). In the PVN, CB_1 receptors are also located in axons that have synaptic association with thyrotropin-releasing hormone (TRH) [13–20]. Increased PVN neuron activity is observed following CB_1 blockade and in CB_1 knockout mice [13,21]. Additionally, Verty et al. [21] demonstrated that rimonabant treatment increases the activity of proopiomelanocortin (POMC) and CART neurons in the arcuate nucleus (ARC) and decreases neuropeptide Y (NPY) neuron activity.

The hypophagic effect of rimonabant is observed only after acute treatment. Animals become tolerant to this effect with chronic treatment, but show a sustained decrease in body weight gain [22,23]. Rimonabant modulates hypothalamic neuron activity, but the effects of chronic rimonabant treatment on neuronal activity and the mRNA levels of neuropeptides related to energy homeostasis are not known. In this work we investigated the effect of acute and prolonged rimonabant treatment on CRF and CART neuronal activation in the hypothalamus. We also investigated the mRNA expression of hypothalamic neuropeptides related to food intake control.

* Corresponding author at: Avenida Bandeirantes, 3900, 14049-900 Ribeirao Preto, Sao Paulo, Brazil. Tel.: +55 16 3602 3057; fax: +55 16 3602 0221.
E-mail address: rcrorato@yahoo.com.br (R. Rorato).

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2. Material and methods

2.1. Animals

Adult male Wistar rats weighing 220–300 g (Central Animal Facility of the University of Sao Paulo - Campus Ribeirao Preto) were individually housed under controlled light (12–12 h light–dark cycle; lights off at 18:00 h) and temperature (23 ± 1 °C) conditions with free access to water and standard rat pellet chow unless otherwise specified.

Daily food ingestion and body weight were recorded between 15:30 and 16:00 to acclimate the animals to the procedures of drug administration and food intake and body weight determination used during the experiments. To verify food consumption, rats were singly housed in metabolic cages with free access to chow in a metal container. On the 7th and last day of the experiment, food intake was measured based on the difference in weight between food initially placed in the container and that remaining after 24 h. Changes in body weight were determined by the difference between the body weight recorded at 16:00 on day 7 (before vehicle or rimonabant treatment) and on day 8.

2.2. Experimental procedures

2.2.1. Effect of acute or repeated rimonabant treatment on food intake and body weight

Animals were separated into 3 experimental groups (8–10 per group): daily vehicle (rats received 7 daily doses of vehicle; 0.1% Tween 80 in distilled water,), single rimonabant treatment (rats received 6 daily doses of vehicle and 1 dose of rimonabant on the day of the experiment; Acomplia®, Sanofi Aventis, 10 mg/kg, by gavage; Sanofi Winthrop Industrie, Tours, France) or repeated rimonabant treatment (rimonabant once a day for 7 days). On the 7th and last day of the experiment, food was removed at 15:30 h, when the animals had their initial body weight determined, and 30 min later the animals were treated with either rimonabant or vehicle. Food was re-offered at 18:00 and body weight and food consumption were recorded 24 h after drug administration. The dose of rimonabant used in the present study was based on reports in the literature describing its anorexigenic effect and tolerance after 3 to 4 days in lean rats [22,24].

2.2.2. Effect of acute or repeated rimonabant treatment on Fos, Fos-CRF and Fos-CART immunoreactivity in the hypothalamus

In this set of experiments, animals were divided into four groups: vehicle, acute treatment, prolonged treatment and pair-fed groups. The latter was added to exclude the possibility that the changes induced by rimonabant were due to decreased food intake, rather than a direct effect of rimonabant. The pair-fed group was treated with vehicle and received the same mean amount of food that was ingested on the previous day by the rats in the prolonged rimonabant treatment group. On the 7th and last day of the experiment, 4 h after treatment, the rats (5–6 per group) were anesthetized by intraperitoneal (i.p.) injections of 2.5% 2,2,2-tribromoethanol (TBE, 1 ml/100 g; Aldrich, St Louis, MO, USA) and transcardially perfused with 200 ml of saline containing heparin (50 UI/l) followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.2). The brain was removed, post-fixed in 4% paraformaldehyde for 1 h, placed in PBS containing 30% sucrose and stored at 4 °C.

2.2.3. Effect of acute or repeated rimonabant treatment on hypothalamic neuropeptide mRNA expression

In this experimental protocol, vehicle, acute and repeated rimonabant-treated and pair fed of non-anesthetized rats (6–7 per group) were decapitated on the 7th and last day of the experiment 4 h after treatment and the brains were collected under RNAse free conditions, immediately frozen on dry ice and stored at −80 °C until hypothalamic neuropeptide mRNA determination.

2.3. Immunohistochemistry for Fos, CRF and CART

Free-floating frozen coronal sections (30 μm) of brain were prepared using a cryostat. Immunohistochemistry procedures were carried out as previously described [25,26]. Briefly, sections were incubated overnight at room temperature with an anti-Fos antibody (1:10,000; Ab-5, Calbiochem-Novabiochem, La Jolla, CA, USA). After rinsing, sections were incubated with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA) for CRF and CART staining and then processed using the Vectastatin Elite avidin–biotin immunoperoxidase method (Vector Laboratories, Burlingame, CA, USA). Solutions of diaminobenzidine (DAB), nickel sulphate, and H_2O_2 were used to generate blue-black nuclear Fos immunolabelling. To verify neuronal phenotypes, sections were incubated with anti-CRF (1:10,000; Peninsula Laboratories Inc., San Carlos, CA, USA) or anti-CART antibody (1:10,000, Phoenix Pharmaceuticals Inc., Burlingame, CA, USA). Appropriate secondary antibodies were used to detect CRF and CART immunoreactivity. DAB without nickel sulphate was used to generate brown-labelled neurons.

Coordinates from the Paxinos and Watson [27] atlas were used to identify the hypothalamic regions. The medial (PaMP) and posterior parvocellular (PaPo) subdivisions of the paraventricular nucleus were considered to be −1.80 mm and −2.12 mm from bregma, respectively. The arcuate nucleus was considered to be between −2.12 mm and −2.8 mm from bregma. Quantitative analysis of Fos, Fos-CRF and Fos-CART labelling was performed using a Leica microscope equipped with a DC 200 digital camera that was attached to a contrast enhancement device. Fos-positive cells, indicated by black staining, were identified when the nuclear structure demonstrated clear immunoreactivity as compared to the background level of immunoreactivity. Cytoplasmic CRF and CART labelling (brown) were considered if there was a clearly labelled cell body surrounding a nucleus. Positively immunolabelled cells were counted in two or three sections to obtain a mean value for each animal. This was done in four or five animals from each experimental condition by two participants that were blind to the experimental protocols.

2.4. Microdissection, total RNA isolation and quantitative real-time PCR

PVN and ARC microdissections were obtained with a stainless steel punch needle (1.5 mm diameter) in a cryostat according to the coordinates from −0.92 to −2.12 (1200 μm) and −2.12 to −3.32 (1200 μm) from bregma, respectively [27]. Tissue samples were transferred to a microtube containing the RNAlater reagent (Ambion, Austin, TX, USA) and stored at 4 °C for a maximum of 24 h until RNA isolation. Total RNA was isolated from each micropunched hypothalamic tissue sample using the TRIzol reagent (Invitrogen®, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA concentration in each sample was determined using a UV spectrophotometer, and 250 ng of RNA from each sample was used for cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR (qPCR) was performed using an Applied Biosystems Step One Plus real-time PCR system. The TaqMan® Gene Expression Assay (Applied Biosystems) used in this study was Rn 01462137_m1 (CRF), Rn00564880_m1 (TRH), Rn 00567382_m1 (CART), Rn 00595020_m1 (POMC), Rn 01410145_m1 (NPY) and Rn00562880_m1 (Cnr1–CB_1). Each qPCR reaction was performed in triplicate. Water (instead of cDNA) was used as a negative control. Reference genes (β-actin and GAPDH) were run for each cDNA sample. The determination of transcript levels in each sample was performed using the ΔΔCT method. For each sample, the threshold cycle (Ct) was determined and normalized to the average of the reference genes ($\Delta Ct = Ct_{Unknown} - Ct_{reference\ genes}$). The fold-change of mRNA expression in the unknown sample relative to the control group (vehicle group) was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{Unknown} - \Delta Ct_{Control}$. Data are shown as mRNA expression relative to the control group.

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