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The type-1 cannabinoid receptor modulates the hydroelectrolytic balance independently of the energy homeostasis during salt load

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ABSTRACT

Hydroelectrolytic imbalances, such as saline load (SL), trigger behavioral and neuroendocrine responses, such as thirst, hypophagia, vasopressin (AVP) and oxytocin (OT) release and hypothalamus–pituitary–adrenal (HPA) axis activation. To investigate the participation of the type-1 cannabinoid receptor (CB1R) in these homeostatic mechanisms, male adult Wistar rats were subjected to SL (0.3 M NaCl) for four days. SL induced not only increases in the water intake and plasma levels of AVP, OT and corticosterone, as previously described, but also increases in CB1R expression in the *lamina terminalis*, which integrates sensory afferents, as well as in the hypothalamus, the main integrative and effector area controlling hydroelectrolytic homeostasis. A more detailed analysis revealed that CB1R-positive terminals are in close apposition with not only axons but also dendrites and secretory granules of magnocellular neurons, particularly vasopressinergic cells. In satiated and euhydrated animals, the intracerebroventricular administration of the CB1R selective agonist ACEA (0.1 μg/5 μL) promoted hyperphagia, but this treatment did not reverse the hyperosmolality-induced hypophagia in the SL group. Furthermore, ACEA pretreatment potentiated water intake in the SL animals during rehydration as well as enhanced the corticosterone release and prevented the increase in AVP and OT secretion induced by SL. The same parameters were not changed by ACEA in the animals whose daily food intake was matched to that of the SL group (Pair-Fed). These data indicate that CB1Rs modulate the hydroelectrolytic balance independently of the food intake during sustained hyperosmolality and hypovolemia.

Introduction

Increases in plasma osmolality and decreases in extracellular fluid (ECF) volume are continuously monitored by sensory afferents that centrally trigger behavioral and neuroendocrine homeostatic responses. The main osmotic sensors are located in the circumventricular organs (CVOs) which comprise part of the *lamina terminalis* (LT) [subfornical organ (SFO) and *organum vasculosum* of the *lamina terminalis* (OVLT)] as well as the area postrema (AP) in the brainstem. The projections from the CVOs reach the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus, which synthesize and release the neurohormones oxytocin (OT) and vasopressin (AVP) (Oliet and Bourque, 1993; McKinley et al., 1999) during osmotic stimulation and hypovolemia. In parallel, behavioral responses are triggered by LT activation that acts in coordination with limbic and cortical structures to evoke or inhibit the water/sodium appetite and acquisition (Antunes-Rodrigues and McCann, 1970). It has also been extensively demonstrated that hyperosmolality by itself induces thirst and inhibits sodium consumption and that the detection of a concomitant ECF deficit results in the additional stimulation of sodium appetite (Stricker et al., 1992). Furthermore, dehydrated animals show hyperosmolality-induced hypophagia to correct hyperosmolality (Watts and Boyle, 2010).

ECF osmolality and volume changes also activate the hypothalamus–pituitary–adrenal axis (HPA), promoting glucocorticoid release (Lauand et al., 2007) that, in turn, inhibits OT and AVP release (Di et al., 2003, 2005, 2009; Ruginsk et al., 2007, 2010, 2012) through retrograde messengers, such as nitric oxide, which stimulate the release of γ-aminobutyric acid (GABA) and lipid-derived mediators (endocannabinoids, ECBs), which inhibit the release of glutamate within the PVN and SON (Di et al., 2003, 2005, 2009).

ECBs have been recognized as important signaling molecules that regulate energy homeostasis, not only by stimulating food intake and increasing body weight gain, but also by reducing energy consumption (Pagotto et al., 2006). Furthermore, several experimental findings indicate that ECBs may also act as important mediators in the hydromineral balance. Indeed, CB1Rs have been identified not only in the hypothalamic PVN and SON (Herkenham et al., 1991) but also in the CVOs (Suárez et al., 2010), suggesting that these receptors may be implicated in the integration of osmosensory and neuroendocrine pathways. In

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fact, the administration of rimonabant, a CB1R antagonist, enhances the hyperosmolality-induced OT and AVP secretion and reverses the inhibitory effect of dexamethasone on OT release under the same experimental conditions (Ruginsk et al., 2012). However, the mechanisms through which ECBs affect the behavioral and endocrine responses induced by osmotic stimulation remain poorly understood. Therefore, the present study aims to evaluate the effects of prolonged osmotic stimulation (four days of salt load, SL) on PVN, SON and LT structures CB1Rs expression as well as the central modulation exerted by ACEA (arachidonoyl-2′-chloroethylamide), a highly sensitive CB1R agonist, on the behavioral and endocrine responses induced by SL.

Material and methods

Animals

Male Wistar rats (260–300 g) from the Central Animal Facility of the Campus of Ribeirão Preto, University of São Paulo, were housed in cages with a maximum of five rats per cage under controlled conditions of temperature (23 ± 2 °C) and lighting (lights on from 0600 to 1800 h) and with free access to standard chow, tap water and 0.3 M NaCl. All of the experimental procedures were in accordance with the Ethical Committee for Animal Use of the School of Medicine of Ribeirão Preto, University of São Paulo (Protocol #93/2012).

After three to four days of acclimatization, the rats were subjected to surgical cannulation of the lateral cerebral ventricle. For this procedure, the rats were anesthetized through an intraperitoneal injection of ketamine (60 mg/kg body weight, Ceva Santé Animale) and xylazine (7.5 mg/kg b.w., Ceva Santé Animale), and a stainless steel guide cannula (12.0 × 0.7-mm outer diameter; 0.4-mm internal diameter) was placed into the right lateral ventricle using the following stereotaxic coordinates (relative to the bregma): 0.5 mm posterior, 1.4 mm lateral and 3.6 mm vertical (Paxinos and Watson, 2004). The cannula was then fixed to the cranial surface using dental cement and two small jeweler screws. After surgery, the rats were administered a prophylactic dose of pentabiotic (0.1 mL/100 g of b.w., IM, Fort Dodge) of and were placed in individual cages, where they were allowed to recover for at least five days. On the day of the experiment, the rats were administered arachidonoyl-2′-chloroethylamide (ACEA, selective CR1R agonist, 0.1 μg/5 μL, Tocris, #1319) or the respective vehicle (0.4% ethanol in 0.15 M NaCl) via the icv route, according to the experimental design described below. The dose of ACEA was chosen according to a pilot experiment result regarding food intake in satiate animals, in which the doses of 0.1, 1.0 and 10 μg/5 μL were tested (data not shown). The position of the cannula was confirmed after decapitation by administration (5 μL) of 2% Evans Blue at the end of each experiment.

Experimental design

All of the animals were assigned to three experimental groups: 1) control: provided regular chow and tap water ad lib; 2) Salt Loaded (SL): provided regular chow and 0.3 M NaCl for 4 days; and 3) Pair-Fed (PF): provided tap water and regular chow (daily paired to the mean ingestion of the SL group) for four days. All of the experiments (except the evaluation of food intake) were performed in the morning (0700–1200) of the fifth day after the beginning of the SL protocol.

Evaluation of fluid and food intake

For the evaluation of food consumption and fluid intake, the rats were maintained in individual metabolic cages during the four-day acclimatization and experimentation period. On the day of the experiment, tap water and 0.3 M NaCl solution were offered in volumetric and graduated burettes 10 min after the icv administration of ACEA or vehicle. The fluid ingestion was evaluated 20, 40, 60 and 90 min after icv injection. For the evaluation of food intake, food was removed at 1600 and returned at 1800, 10 min after the icv administration of ACEA or vehicle. The food ingestion was then evaluated after 1, 2, 4, 14 and 24 h.

Determination of hematocrit, plasma osmolality, sodium and hormone concentrations

Immediately before or 30 min after fluid (water and 0.3 M NaCl) presentation (FP), animals were killed by decapitation. Trunk blood was collected into heparinized (50 μL/mL) plastic tubes, which were then centrifuged (3000 rpm, 4 °C, 20 min) to separate the plasma fraction for the determination of osmolality, hormone and sodium concentrations, or into heparinized capillaries, which were then centrifuged (2500 rpm, 25 °C, 5 min) and read against a volumetric scale for the determination of the hematocrit. The determinations of the plasma sodium concentrations were performed using a flame photometer (Micronal, B262). The plasma osmolality was determined by the water freezing point method using a micro-osmometer (GENOTEC, Inc.).

All of the plasma hormone concentrations were determined through radioimmunoassays (RIA) after specific extraction processes. Briefly, for determination of the vasopressin (AVP) and oxytocin (OT) concentrations, the plasma samples (1 mL) were extracted using acetone and petroleum ether (Haanwinckel et al., 1995). For the corticosterone RIA, 25 μL of plasma was extracted using ethanol (Castro et al., 1995). The assay sensitivities and intra- and inter-assay coefficient of variation were 0.1 pg/mL, 1.4% and 10.3% for AVP; 0.1 pg/mL, 3.1% and 11.2% for OT; and 0.4 μg/dL, 3.3% and 10.0% for corticosterone.

Immunofluorescence for CB1R and AVP

The experimental animals subjected to the same procedures described above were deeply anesthetized with ketamine (60 mg/kg b.w.) and xylazine (7.5 mg/kg b.w.) immediately before or 120 min after FP. Thereafter, they were perfused with 250 mL of 0.1 M phosphate buffer saline (PBS) and then with 500 mL of 4% formaldehyde in 0.1 M PBS. The brains were removed and post-fixed for 12 h in 4% formaldehyde solution before being stored at 4 °C in 0.1 M PBS containing 30% sucrose. Coronal sections (30 μm) were obtained in a cryostat in three serial sets of tissues and stored at −20 °C in anti-freezing solution until processing. All of the incubation solutions were prepared in 0.01 M PBS containing 0.1% triton and 0.04% NaN_3 (sodium azide). Initially, the free-floating sections were incubated in 10% normal horse serum for at least 1 h and then transferred to a solution containing the primary antibodies [anti-CB1R (rabbit, 1:2000, Abcam, #Ab23703) and anti-AVP (guinea pig, 1:20,000, Peninsula Laboratories, Inc., #T-5048)]. After overnight incubation at room temperature, the sections were incubated for 4 h with the respective secondary antibodies labeled with fluorescent dyes (1:250, FITC-conjugated donkey anti-rabbit, #711-095-152, and Cy5-conjugated donkey anti-guinea pig, #715-175-150, Jackson ImmunoResearch Laboratories, Inc.). Thereafter, the sections were mounted on slides, allowed to dry and covered with the mounting medium for visualization. The PVN, SON, and SFO were identified and delimited according to the atlas published by Paxinos and Watson (2004). Representative photomicrographs of these areas were acquired with a confocal microscope (Leica TCS SP5) to qualitatively represent the expression of CB1Rs in the LT and hypothalamus. Color adjustments (sharpness, contrast and brightness) were carried out with ImageJ image-editing software to more accurately represent microscope displays.

Western blotting for CB1R

Immediately before or 120 min after FP, the animals were euthanized by decapitation, and their brains were collected and stored at −80 °C until protein extraction. Using a stainless-steel punch needle with an internal diameter of 1.5 mm, microdissections (1200 μm)