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Type 1 cannabinoid receptor modulates water deprivation-induced homeostatic responses

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1Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil; 2Department of Physiological Sciences, Biomedical Sciences Institute, Federal University of Alfenas, Alfenas, Minas Gerais, Brazil; and 3Department of Physiological Sciences, State University of Londrina, Londrina, Paraná, Brazil

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Ruginsk SG, Vechiato FMV, Uchoa ET, Elias LLK, Antunes-Rodrigues J. Type 1 cannabinoid receptor modulates water deprivation-induced homeostatic responses. Am J Physiol Regul Integr Comp Physiol 309: R1358–R1368, 2015. First published October 14, 2015; doi:10.1152/ajpregu.00536.2014.—The present study investigated the type 1 cannabinoid receptor (CB1R) as a potential candidate to mediate the homeostatic responses triggered by 24 h of water deprivation, which constitutes primarily a hydroelectrolytic challenge and also significantly impacts energy homeostasis. The present results demonstrated for the first time that CB1R mRNA expression is increased in the hypothalamus of water-deprived (WD) rats. Furthermore, the administration of ACEA, a CB1R selective agonist, potentiated WD-induced dipsogenic effect, whereas AM251, a CB1R antagonist, attenuated not only water but also salt intake in response to WD. In parallel with the modulation of thirst and salt appetite, we confirmed that CB1Rs are essential for the development of appropriated neuroendocrine responses. Although the administration of ACEA or AM251 did not produce any effects on WD-induced arginine vasopressin (AVP) secretion, oxytocin (OXT) plasma concentrations were significantly decreased in WD rats treated with ACEA. At the genomic level, ACEA significantly decreased AVP and OXT mRNA expression in the hypothalamus of WD rats, whereas AM251 potentiated both basal and WD-induced stimulatory effects on the transcription of AVP and OXT genes. In addition, we showed that water deprivation alone upregulated proopiomelanocortin, Agouti-related peptide, melanin-concentrating hormone, and orexin A mRNA levels in the hypothalamus, and that CB1Rs regulate main central peptidergic pathways controlling food intake, being that most of these effects were also significantly influenced by the hydration status. In conclusion, the present study demonstrated that CB1Rs participate in the homeostatic responses regulating fluid balance and energy homeostasis during water deprivation.

type 1 cannabinoid receptor; ACEA; AM251; water deprivation

WATER INTAKE IN RODENTS IS mostly (but not exclusively) determined by the temporal distribution of feeding patterns (50). The association of food and water intake is intuitive, being supported by anatomical substrates, such as the activation of shared neural pathways. The nucleus of the solitary tract, for example, is implicated not only in the short-term control of feeding (11) but also integrates ascending pathways that constantly monitor extracellular fluid (ECF) volume and osmolality (1). This evidence gives support to the hypothesis that peripheral signals originated from both food and fluid ingestion are sensed at brain stem level, so that acute changes in hydration status and energy balance may affect each other’s response. Conversely, some reports argue against the association of food and drinking behaviors. Mietlicki et al. (23) showed that ghrelin, one of the main orexigenic peptides, actually inhibits ANG II and hyperosmolarity-induced water drinking. Furthermore, it has been demonstrated that, in satiated rats, acute electrical stimulation of the subfornical organ (SFO), primarily involved in osmoreception, elicits not only drinking (at low frequencies) but also feeding (at higher stimulation intensities) (40).

In healthy, nonobese individuals, the amount of ingested food is primarily determined by adiposity and satiety signals, which provide the control of body-fat stores and meal size, respectively. Peripheral signals reach the hypothalamic arcuate nucleus (ARC), where they activate first-order neurons (49). These primary ARC neurons are known to express critically important peptides, such as neuropeptide Y (NPY), Agouti-related peptide (AgRP), proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which selectively regulate orexigenic and anorexigenic signaling systems (9, 15, 18, 25, 30). Neural inputs are then retransmitted from the ARC to second-order neurons, located in the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA), whose activation determines coordinated homeostatic responses to match energy acquisition to its storage or expenditure. PVN neurons regulating energy balance are phenotypically characterized mainly as corticotrophin-releasing hormone (CRH)- and CART-positive cells (2, 15), whereas LHA neurons primarily express melanin-concentrating hormone (MCH) and orexin A (38). More recently, oxytocinergic neurons of the PVN have been also shown to mediate some of the CRH inhibitory actions on food intake (44). Together, the activation of proper integrated responses involving intrahypothalamic peptidergic systems and extra-hypothalamic arousal pathways determines the modulation of feeding, drinking, and seeking behaviors.

Within this context, the endocannabinoid (ECB) system appeared in the last few years as an important central modulator of ingestive behavior (20), and several studies have demonstrated that the type 1 cannabinoid receptor (CB1R) is the main isof orm implicated in the central orexigenic effects of ECBs (8). Ultrastructural studies have consistently demon-
stratified CB1R-immunoreactive terminals in several hypothalamic nuclei regulating energy balance, including the PVN, ARC, and LHA (48). In the PVN, CB1R agonists have been implicated in the glucocorticoid-induced inhibition of both magnocellular and parvocellular groups, mediated by parallel and antagonistic effects on glutamate and GABA presynaptic release (7). Similarly, whole cell patch-clamp of ARC POMC neurons revealed that CB1R stimulation decreases the amplitude of evoked, glutamatergic excitatory postsynaptic currents in these cells (13). An increased K+-induced GABA release from ARC NPY/AgRP neurons has also been reported in response to the incubation of hypothalamic explants with a CB1R agonist (22).

Interestingly, CB1Rs and the machinery for ECB production and degradation have been identified in the SFO (42), which constitute a primary sensory target for sodium and circulating ANG II levels (21). CB1R-positive terminals were also observed within the supraoptic nucleus (SON), in close apposition with magnocellular secretory neurons (37), suggesting that ECBS, through CB1Rs, could also play an important role in fluid balance. Therefore, the present study aimed to investigate CB1R as a potential candidate to mediate the homeostatic responses triggered by 24 h of water deprivation (WD), which constitutes not only a hydroelectrolytic challenge but also significantly impacts energy homeostasis.

**METHODS**

**Animals.** The present study used adult male Wistar rats, weighing between 250 and 300 g, obtained from the Central Animal Facility of the Campus of Ribeirão Preto (University of São Paulo). Initially, the animals were maintained in collective plastic cages (maximum of five animals per cage) at the Animal Facility of the Department of Physiology of the School of Medicine of Ribeirão Preto, under standard conditions of temperature (22 ± 2°C) and light-dark cycle (lights on at 6 AM, lights off at 6 PM). All of the experimental procedures, detailed below, were performed between 0700 and 1200 and have been previously approved by the Ethical Committee for Animal Use in Experimentation of the School of Medicine of Ribeirão Preto (University of São Paulo) under the protocol number 37/2010.

During habituation, animals were housed individually in metabolic cages (for the evaluation of behavioral responses) or polycarbonate cages (for all other experiments) for at least 3 days with free access to food, 0.3 M NaCl and water. After habituation, the animals were randomly divided into two groups: the first one remained in the cages (for all other experiments) for at least 3 days with free access to cages (for the evaluation of behavioral responses) or polycarbonate cages (lights on at 6 AM, lights off at 6 PM). All of the experimental procedures, detailed below, were performed between 0700 and 1200 and have been previously approved by the Ethical Committee for Animal Use in Experimentation of the School of Medicine of Ribeirão Preto (University of São Paulo) under the protocol number 37/2010.

After specific extraction processes, plasma hormone concentrations were determined by radioimmunoassays, using the following primary antibodies: rabbit anti-OXT (cat. no. T-4084; Bachem); rabbit anti-AVP (cat. no. T-4563, Bachem). The assay sensitivities were 0.3 pg/ml for AVP and OXT. For nitrate determinations, plasma aliquots of 20 μl were deproteinized with 40 μl of absolute ethanol and kept at −20°C for 30 min. Next, they were centrifuged, and the supernatant was applied to a chemiluminescent analyzer (Sievers 280 NO Analyzer). Nitrate contents were normalized by total protein concentration, which was determined from plasma samples by the Bradford colorimetric method (Bio-Rad). Considering that nitrate is a metabolite of nitric oxide (NO) production, the assessment of plasma nitrate concentrations was used as an indirect estimate of NO peripheral production.

**Semiquantitative analysis of mRNA expression.** A set of EH and WD rats was euthanized by decapitation for the determination of the mRNA expression of CB1R in the hypothalamus. Another set of experimental animals was submitted to vehicle, ACEA, or AM251 administration, as previously described, and decapitated 90 min later. All of the brains were collected under RNase-free conditions and kept at −80°C until total RNA extraction. The target brain structures were obtained in a cryostat from two consecutive thick coronal sections (1,500 μm each, from coordinates 0.6 to 2.1 mm and 2.1 to 3.6 mm posterior to bregma). For this purpose, a stainless-steel punch needle with 1.5 mm of internal diameter was used. The PVN and SON were isolated from the first thick section, whereas the ARC and LHA were punched from the following section, according to the anatomic clues provided by the rat brain atlas (29). The tissue samples were collected into sterile polypropylene tubes containing 30 μl of RNA later solution (Ambion). The tissue samples were initially homogenized in phosphate buffer, followed by the addition of TRIzol (Invitrogen), chloroform, and glycogen. Total RNA was then quantified by UV spectrophotometry (260 nm, Biophotometer, Eppendorf), and the integrity of each sample was further confirmed by electrophoresis in agarose gel (1.2%, 60 min at 60 V). After this procedure, cDNA was synthesized from 500 ng of RNA (high-capacity cDNA reverse transcription; Applied Biosystems), diluted 1:5 in sterile water and assayed in triplicate (7500 real-time PCR System; Applied Biosystems) with the appropriate mixture of reagents (Master Mix; Applied Biosystems) and probes (β-actin: Rn00667869_m1 (VIC), OXT: Rn00564446_g1 (FAM), AVP: Rn00566449_m1 (FAM), CRH: Rn01462137_m1 (FAM), CART: Rn00567382_m1 (FAM), NPY: Rn01410145_m1 (FAM), AgRP: Rn01431703_g1 (FAM), POMC: Rn00595020_m1 (FAM), CB1: Rn00562880_m1 (FAM) and orexin A: Rn00565995_m1 (FAM), Applied Biosystems). Data were obtained.
in real time (Real Time PCR System, Applied Biosystems), and the relative expression of target genes in each sample was determined either by the variation of threshold cycles (ΔΔCt) between target and housekeeping mRNAs or by the relative standard curve method, according to the assay efficiency. The results were expressed as relative to EH or vehicle + EH groups (arbitrary units, AU).

**Immunofluorescence for alpha-melanocyte stimulating hormone and CB1R in the hypothalamus.** EH and WD rats were deeply anesthetized with a solution of ketamine, xylazine, and 0.9% NaCl (0.1 ml/100 g body wt) and perfused with 150 ml of PBS, followed by 350 ml of 4% formaldehyde. Perfused brains were removed, postfixed in 4% formaldehyde for 4 h, and sectioned (30-350 μm) on a cryostat. The frozen sections were washed in PBS and incubated with 10% normal horse serum for at least 1 h for blocking of nonspecific binding. The sections were then incubated overnight at room temperature with a mixture of two primary antibodies: anti-CB1R, 1:1,000 (rabbit, cat. no. ab23703, Abcam) and anti-alpha-melanocyte stimulating hormone (α-MSH), 1:20,000 (sheep, cat. no. AB5087, Chemicon International). Thereafter, the sections were incubated for 4 h with the secondary antibodies (Alexa Fluor 488 donkey-anti-sheep, cat. no. 705-545-147; Alexa Fluor 594 donkey anti-rabbit, cat. no. 711-585-152, Jackson ImmunoResearch) at a dilution of 1:250 each. The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1:25,000, cat. no. D1306, Invitrogen) for 2 min, mounted on coated slides, dried, and covered with medium (Fluormount). The negative controls (not shown) were obtained by the omission of both primary and secondary antibodies (5). Representative photomicrographs of the PVN and ARC of EH and WD rats were obtained on a confocal microscope (Leica TCS SP5).

**Table 1. Plasma sodium concentrations, hematocrit, food intake, and body weight variation in euhydrated and water-deprived animals**

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma Sodium, mEq/l</th>
<th>Hematocrit, %</th>
<th>Food Intake, g·100 g body wt⁻¹·24 h⁻¹</th>
<th>Body Weight Variation, g/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>EH</td>
<td>135.50 ± 0.91</td>
<td>41.69 ± 1.12</td>
<td>9.53 ± 0.20</td>
<td>+5.10 ± 0.91</td>
</tr>
<tr>
<td>WD</td>
<td>142.16 ± 1.02***</td>
<td>51.71 ± 1.48***</td>
<td>4.90 ± 0.16***</td>
<td>-18.76 ± 0.86***</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; analyses were performed by t-test. EH, euhydrated; WD, water-deprived. ***P < 0.001 vs. EH group.

**Fig. 1.** Type 1 cannabinoid receptor (CB1R) mRNA relative expression in the paraventricular nucleus of the hypothalamus (PVN) (A), supraoptic nucleus of the hypothalamus (SON; B), arcuate nucleus (ARC; C), and lateral hypothalamic area (LHA; D) of euhydrated (EH) and water-deprived (WD) animals. Analyses were performed by t-test. *P < 0.05 and **P < 0.01 vs. EH group.

**Results.**

Effects of WD on food intake, body weight gain, plasma sodium concentrations, hematocrit, and CB1R mRNA expression in the hypothalamus. Table 1 demonstrates that WD induced a 50% reduction in food intake and a remarkable decrease in body weight. Still according to these data, WD rats are hypovolemic, as assessed by their increased hematocrit, and have increased plasma sodium concentrations. Furthermore, WD animals showed increased CB1R mRNA expression in the PVN (1.01 ± 0.17 vs. 1.62 ± 0.67 AU, P < 0.01, Fig. 1A), SON (1.04 ± 0.30 vs. 1.66 ± 0.70 AU, P < 0.05, Fig. 1B), ARC (1.02 ± 0.11 vs. 1.43 ± 0.07 AU, P < 0.05, Fig. 1C), and LHA (1.01 ± 0.07 vs. 1.56 ± 0.15 AU, P < 0.01, Fig. 1D).

Effects of CB1R on behavioral and cardiovascular responses in EH and WD rats. Figure 2 shows that WD-induced pronounced dipsogenic (F5,319 = 425.72, P < 0.001, Fig. 2A) and

**Statistical analysis.** The results were expressed as means ± SE and analyzed by factorial ANOVA. Repeated measures were performed to analyze the variables along time (behavioral studies). Once the ANOVA-null hypothesis of equal means has been rejected using the F-test, the Newman-Keuls multiple-comparisons post hoc test was performed (SigmaStat Software, version 3.1). The t-test was employed to analyze the effects of WD on body weight gain, plasma sodium concentrations, hematocrit, 24-h food intake and CB1R mRNA expression. The significance level was set at 5% (two-sided).
natriorexigenic responses ($F_{5,324} = 165.11, P < 0.001$, Fig. 2B). WD-induced 0.3 M NaCl intake was not affected by ACEA administration, whereas water intake was significantly potentiated by ACEA in WD (3.57 ± 0.73 vs. 5.94 ± 1.06 ml/100 g body wt, $P < 0.01$, t = 40 min) but not in EH animals. The dipsogenic effect of ACEA was accompanied by a parallel increase in urinary volume in WD rats (0.85 ± 0.16 vs. 1.68 ± 0.47 ml/100 g body wt, $P < 0.01$, Fig. 2C), whereas total sodium excretion was not altered by WD or ACEA/AM251 administration (Fig. 2D). Conversely, AM251 administration decreased WD-induced water intake (6.97 ± 0.59 vs. 4.16 ± 1.09 ml/100 g body wt, $P < 0.05$, t = 90 min) and 0.3 M NaCl intakes (3.39 ± 0.44 vs. 1.60 ± 0.46 ml/100 g body wt, $P < 0.05$, t = 90 min). The amount of food ingested by experimental animals injected with ACEA or AM251 did not differ between EH and WD rats (data not shown). According to Table 2, neither WD nor ACEA/AM251 administration significantly changed plasma nitrate concentrations or MAP.

Table 2. Mean arterial pressure and plasma nitrate concentrations in EH and WD animals administered vehicle, ACEA, and AM251

<table>
<thead>
<tr>
<th>Group</th>
<th>ΔPAM, mmHg</th>
<th>Plasma nitrate, μmol/μg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EH</td>
<td>1.66 ± 2.04</td>
<td>168.76 ± 11.07</td>
</tr>
<tr>
<td>WD</td>
<td>4.65 ± 3.01</td>
<td>145.92 ± 7.72</td>
</tr>
<tr>
<td>ACEA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WD</td>
<td>8.00 ± 4.00</td>
<td>166.55 ± 14.93</td>
</tr>
<tr>
<td>EH</td>
<td>7.50 ± 1.50</td>
<td>185.73 ± 11.93</td>
</tr>
<tr>
<td>AM251</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WD</td>
<td>-1.33 ± 1.30</td>
<td>172.28 ± 14.71</td>
</tr>
<tr>
<td>EH</td>
<td>0.50 ± 1.89</td>
<td>134.76 ± 19.11</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; analyses were performed by two-way ANOVA, followed by Newman-Keuls post hoc test. EH and WD animals were administered vehicle (V); arachidon-2'-chlorelloxylamide (ACEA; 10 mg/kg), a CB1R agonist; or N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; 3 mg/kg), a CB1R antagonist.

Figure 2. Effects of the administration of vehicle, ACEA (10 mg/kg), or AM251 (3 mg/kg) on water (A) and 0.3 M NaCl (B) intakes, as well as on total urinary volume (C) and sodium (D) in EH and WD animals. Analyses were performed by two-way ANOVA (repeated measures), followed by Newman-Keuls post hoc test. **$P < 0.01$ and ***$P < 0.001$ vs. EH group. +$P < 0.05$ and ++$P < 0.01$ vs. the respective vehicle-treated group.

Effects of CB1R on plasma AVP and OXT concentrations and mRNA expression in the hypothalamus of EH and WD rats. Figure 3 shows that WD rats injected with vehicle exhibited a significant increase in AVP (1.38 ± 0.12 vs. 5.16 ± 0.16 pg/ml, $P < 0.001$, $F_{5,79} = 54.38$, Fig. 3A) and OXT plasma concentrations (1.55 ± 0.18 vs. 5.16 ± 0.16 pg/ml, $P < 0.001$, $F_{5,72} = 49.49$, Fig. 3B). ACEA/AM251 administration did not change plasma AVP concentrations. However, ACEA administration attenuated OXT secretion in WD animals (5.16 ± 0.16 vs. 3.45 ± 0.43 pg/ml, $P < 0.01$), whereas AM251 treatment significantly enhanced OXT plasma concentrations under basal (1.55 ± 0.18 vs. 2.97 ± 0.42 pg/ml, $P < 0.05$) but not stimulated conditions, evidencing a statistically significant effect of treatment on OXT secretion ($F_{5,72} = 4.22, P < 0.05$).

The analysis of mRNA expression in the hypothalamus revealed that WD significantly triggered AVP and OXT gene transcription. In the PVN, WD increased both AVP (1.00 ± 0.06 vs. 1.96 ± 0.16 AU, $P < 0.01$, $F_{5,53} = 20.55$, Fig. 3C) and OXT mRNA expression (1.02 ± 0.16 vs. 2.35 ± 0.36 AU, $P < 0.01$, $F_{5,51} = 5.21$, Fig. 3D). ACEA administration completely blocked WD-induced mRNA expression, whereas AM251 treatment significantly increased the content of AVP and OXT transcripts in both EH (1.00 ± 0.06 vs. 1.60 ± 0.13 AU, $P < 0.05$, for AVP; 1.02 ± 0.16 vs. 3.28 ± 0.28 AU, $P < 0.001$, for OXT) and WD groups (1.96 ± 0.16 vs. 2.76 ± 0.38 AU, $P < 0.01$, for AVP; 2.35 ± 0.36 vs. 4.11 ± 0.67 AU, $P < 0.001$, for OXT), showing a clear effect of treatment on this parameter ($F_{5,53} = 18.99$ and $P < 0.001$, $F_{5,51} = 24.52$ and $P < 0.001$, respectively, for AVP and OXT).

The pattern of WD-induced responses on mRNA expression was very similar in the SON. WD significantly increased the content of both mRNAs [$F_{5,53} = 34.10$ and $P < 0.001$, $F_{5,54} = 20.93$ and $P < 0.001$, respectively, for AVP (Fig. 3E) and OXT (Fig. 3F)]. Again, ACEA administration completely disrupted WD-induced increase in both mRNAs. However, a positive effect of AM251 potentiating mRNA expression under both basal and WD conditions was observed for OXT (1.00 ± 0.08.
Fig. 3. Effects of the administration of vehicle, ACEA, (10 mg/kg), or AM251 (3 mg/kg) on plasma AVP (A), OXT (B), as well as on the relative expression of AVP and OXT mRNAs in the PVN (C and D) and SON (E and F) of EH and WD animals. Analysis by two-way ANOVA, followed by Newman-Keuls post hoc test. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. EH group; +P < 0.05, ++P < 0.01 and +++P < 0.001 vs. the respective vehicle-treated group. AVP, vasopressin; OXT, oxytocin; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus of the hypothalamus.

Effects of CB1R on mRNA expression for orexigenic and anorexigenic peptides in the hypothalamus of EH and WD rats. Figure 4 represents the effects of ACEA or AM251 administration on mRNA expression for CART in the SON (Fig. 4A) and PVN (Fig. 4B) and CRH in the PVN (C) of EH and WD animals. Neither WD nor ACEA/AM251 treatment produced a significant effect on CART mRNA expression in the SON (Fig. 4A). In the PVN, WD alone was again ineffective in changing CART transcript levels. However, both the agonism (1.55 ± 0.12 vs. 2.13 ± 0.12 AU, P < 0.05) and the antagonism to CB1Rs (1.55 ± 0.12 vs. 2.48 ± 0.29 AU, P < 0.001) produced a stimulatory effect on CART mRNA expression, showing a positive effect of treatment on this parameter (F5,52 = 10.48, P < 0.001). CRH mRNA expression, in turn, was not affected by water deprivation alone or in association with ACEA treatment. In AM251-treated rats, however, the PVN contents of CRH transcript were increased in both EH (1.13 ± 0.14 vs. 3.09 ± 0.71 AU, P < 0.01) and WD groups (1.53 ± 0.24 vs. 3.20 ± 0.73, P < 0.05), evidencing a positive effect of treatment on CRH mRNA expression (F5,52 = 11.24, P < 0.001).

In the ARC, WD itself increased AgRP (1.00 ± 0.14 vs. 2.04 ± 0.14, P < 0.001, F5,52 = 75.92, Fig. 5A) and POMC mRNA expression (1.04 ± 0.08 vs. 1.43 ± 0.14, P < 0.05, F5,50 = 13.13, Fig. 5D) but did not change CART (Fig. 5B) or NPY (Fig. 5C) mRNA expression. ACEA administration had no effect on NPY mRNA expression, but significantly reduced CART (1.01 ± 0.07 vs. 0.10 ± 0.01, P < 0.001) and AgRP mRNA expression (1.00 ± 0.14 vs. 0.15 ± 0.01, P < 0.001) in EH but not in WD rats. On the other hand, ACEA decreased both basal (1.03 ± 0.12 vs. 0.11 ± 0.01, P < 0.001) and WD-induced POMC mRNA expression (1.43 ± 0.14 vs. 0.93 ± 0.08, P < 0.01). AM251 treatment, in turn, increased CART mRNA expression only in the ARC of WD (0.94 ± 0.04 vs. 1.59 ± 0.12 AU, P < 0.001) but not EH rats. Similarly to the effect observed for CRH transcript in the PVN, AM251 administration significantly increased POMC mRNA expression irrespective to the experimental group (1.04 ± 0.08 vs. 2.14 ± 0.24 AU, P < 0.001, for EH rats; 1.43 ± 0.14 vs. 2.52 ± 0.24 AU, P < 0.001, for WD rats). AM251 treatment also increased AgRP mRNA levels in both EH (1.03 ± 0.09 vs. 1.66 ± 0.21 AU, P < 0.05) and WD groups (2.04 ± 0.14 vs. 3.09 ± 0.29 AU, P < 0.001). However, AM251-induced effect on AgRP mRNA expression was greater in WD rats. Finally, AM251 administration did not change NPY mRNA levels in the ARC of EH rats, but significantly increased the expression of NPY transcript in WD group (1.13 ± 0.06 vs. 1.49 ± 0.07 AU, P < 0.001).
**Effects of WD on α-MSH and CB₁R immunoreactivity in the hypothalamus.** The qualitative analysis of immunofluorescence results revealed a clear increase in CB₁R immunoreactivity in the hypothalamus of WD rats, particularly in the PVN (Fig. 7, E and F). A poor colocalization of CB₁Rs and α-MSH was observed, either under basal (EH) or stimulated conditions (WD), suggesting that CB₁Rs may be predominantly expressed by presynaptic terminals innervating ARC α-MSH-positive neurons.

**DISCUSSION**

The present results demonstrate for the first time that CB₁R mRNA expression is increased in the PVN and SON WD rats. These findings were further supported at the protein level by the clear WD-induced increase in CB₁R immunoreactivity in the PVN. Together with evidence from the literature showing that osmosensory areas also express CB₁Rs (42), these data strongly support the hypothesis that ECBs participate in body fluid homeostasis. In this regard, we also show here that CB₁Rs are critically implicated in behavioral responses triggered by WD. The administration of ACEA, a CB₁R selective agonist, potentiated the dipsogenic effects of WD, whereas AM251, a CB₁R antagonist, attenuated this response. These effects on water ingestion were accompanied by a parallel increase in free water excretion in WD rats injected with ACEA.

In fact, very few reports in the literature have investigated the effects of cannabinoids on fluid intake. Pioneer studies using natural cannabinoids initially proposed a predominantly inhibitory role for these compounds on thirst (41). More recently, corroborating our findings, Verty et al. (45) showed that SR141716 (rimonabant), a CB₁R inverse agonist, partially attenuated water intake induced by the administration of an OXT receptor antagonist. In the present study, CB₁R blockade by AM251 also significantly attenuated salt appetite in WD rats. On the other hand, ACEA administration could not overcome WD-induced natriorexic effects, which corroborates previous findings showing that the administration of delta-9-tetrahydrocannabinol, a phytocannabinoid, could not change the spontaneous ingestion of isotonic NaCl solution in rats (24).

In parallel with the modulation of drinking and salt appetite, we confirmed previous findings from our group showing that CB₁Rs are essential for the development of appropriate neuroendocrine responses. These effects on neuroendocrine output may be accomplished by the close relationship between CB₁R-positive terminals and magnocellular cell bodies, as previously demonstrated by our group (37). In the present study, we demonstrated that neither the administration of ACEA nor AM251 produced any effects on WD-induced AVP secretion. OXT plasma concentrations, in turn, were significantly decreased in WD + ACEA group, as previously described in response to the intracerebroventricular administration of anandamide to volume-expanded rats (36). On the other hand, AM251 failed to produce a further increase in WD-induced OXT secretion, diverging from previous reports in which rimonabant was administered previously to extracellular volume expansion (33). In this regard, we have already demonstrated that, under systemic acute increases of ECF volume and osmolality, vasopressinergic neurons may be less susceptible than oxytocinergic neurons to dexamethasone-induced inhibi-

Figure 6 shows the pattern of MCH (Fig. 6A) and orexin A (Fig. 6B) mRNA expression in the LHA of EH and WD rats treated with vehicle, ACEA, or AM251. WD alone significantly increased the expression of both mRNAs (0.88 ± 0.08 vs. 1.47 ± 0.24 AU, *P* < 0.05; 0.95 ± 0.11 vs. 1.65 ± 0.16 AU, *P* < 0.001, respectively, for MCH and orexin A). ACEA administration significantly increased the expression of both transcripts under basal conditions (MCH: 0.88 ± 0.08 vs. 2.55 ± 0.34 AU, *P* < 0.001; *P* < 0.05; orexin A: 0.95 ± 0.11 vs. 2.13 ± 0.24 AU, *P* < 0.001) and only MCH mRNA expression in WD rats (1.47 ± 0.24 vs. 2.62 ± 0.17 AU, *P* < 0.001). AM251 treatment, in turn, significantly attenuated WD-induced orexin A mRNA expression in the LHA (1.65 ± 0.16 vs. 1.14 ± 0.12 AU, *P* < 0.05).
tory effects (32), which are known to be mediated by CB1Rs (35). Furthermore, we demonstrated that AVP, but not OXT secretion, is directly affected by the metabolism of ECBs by fatty acid amide hydrolase (36), suggesting that local mechanisms may account for a differential regulation of AVP and OXT during hypertonic hypervolemia.

At the transcriptional level, ACEA significantly decreased AVP and OXT mRNA expression in both the PVN and SON of WD rats, whereas AM251 potentiated both basal and WD-induced stimulatory effects on the transcription of AVP gene in the PVN and OXT gene in both hypothalamic nuclei. This dissociation between secretory and transcriptional functions had already been reported for the hypothalamic neurohypophyseal system (33), reinforcing the hypothesis that hormone synthesis and secretion may be controlled by the ECB system under diverse and critical steps. Taken together with previous reports, the present findings suggest that the coexistence of hypovolemia and hyperosmolality in WD rats may selectively decrease the sensitivity of vasopressinergic neurons to the negative modulation by regulatory systems, such as the ECB system, probably through a fine-tuned control of local inputs to these cells, thus allowing a proper secretory response, which is essential to regulate not only water reabsorption at the renal level but also blood pressure during dehydration.

Accordingly, no changes in MAP were observed in WD rats, suggesting that the cardiovascular mechanisms controlling tissue perfusion are still operative. Nitrate plasma concentrations, which indirectly reflect the peripheral production of the relaxing factor NO by vascular endothelial cells, were also unaltered in WD group. Furthermore, no changes were induced by ACEA or AM251 on MAP or nitrate plasma concentrations, corroborating previous findings of Bátkai et al. (4), who demonstrated that the ECB system is only a little active under normotensive conditions. The same report also revealed that
the ECB system becomes tonically active under several hypertensive stimuli, so that the stimulation of CB₁Rs in hypertensive rats determines a decrease in MAP, originated from a decrease in cardiac contractility and peripheral vascular resistance.

The present results also confirmed the anorexic effect induced by plasma hyperosmolality in WD rats, as evidenced by the significant reduction in food intake observed in this experimental group. WD animals also exhibited a reduction in body weight, caused not only by the reduced food intake itself, but also by the prominent reduction in ECF volume, as assessed by their increased hematocrit. Indeed, it has been demonstrated that the activation of gastrointestinal osmoreceptors by hyperosmolality in experimentally induced hypertonic solution drinking inhibits additional food intake. The final output would be a reduction of osmolyte absorption at the gastrointestinal level, which would determine, albeit temporarily, a further increase in ECF osmolality (39). It has been proposed that,
although being upregulated by water deprivation, central orexigenic pathways would have their actions limited by inhibitory networks, overriding the effects of negative energy balance on food intake (46). According to this hypothesis, these systems would be organized in a way to rapidly reestablish feeding as soon as water becomes available.

We also showed here that water deprivation alone increased the expression of CB1R mRNA in the PVN, ARC, and LHA, confirming the participation of ECB-mediated signaling in central pathways controlling energy homeostasis. Considering anorexigenic peptides, WD did not induce any changes in CART or CRH mRNA expression, corroborating previous findings of our group (34). On the other hand, POMC mRNA was upregulated in the ARC of WD rats, in contrast to previous findings in the literature showing that POMC and CRH expression are reduced after dehydration, as a result of increased circulating corticosterone levels (14, 46). Within this context, it is worthwhile mentioning that these studies employed chronic models of hyperosmolality-induced hypophagia and that the hypothalamic-pituitary-adrenal (HPA) axis seems to differ-ently exceed its participation in the control of energy balance, as suggested by the experimental evidence that both magnocellular and parvocellular POMC neurons are CART-positive and respond to increased ECF volume and osmolality by enhancing c-Fos expression and CART mRNA levels (34). In the ARC, however, CART has been implicated in the anorexic responses induced by rimonabant, which strongly supports the participation of this peptide in food intake control (26). Furthermore, POMC mRNA expression was affected by CB1R stimulation of a different group of mRNAs. Conversely, the stimulation of CB1Rs by the selective agonist ACEA (right column) consistently potentiated thirst and decreased WD-induced OXT and AVP mRNA expression. Furthermore, WD rats injected with ACEA maintained high AgRP and CART mRNA levels, as well as increased NPY mRNA expression, suggesting that exogenous CB1R stimulation probably could not override most of endogenous WD-mediated responses. Therefore, these changes induced by ACEA or AM251 on hypothalamic mRNA expression were characterized as WD-dependent (when these effects appeared only in WD rats) or independent (when the responses were induced irrespective to the hydration status). AVP, arginine vasopressin; AgRP, Agouti-related peptide; CART, cocaine- and amphetamine-regulated transcript; CRH, corticotrophin-releasing hormone; ECF, extracellular fluid; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; OXT, oxytocin. POMC, proopiomelanocortin.

![Diagram summarizing the integrated homeostatic control of hydroelectrolytic and energy balances in 24-h-water-deprived (24-h WD) animals. According to the results, WD animals are hypovolemic and exhibit increased plasma osmolality, as well as hypophagia. The response to WD (central gray column) is characterized, among other effects, by increased CB1R mRNA expression in the hypothalamus, increased natriorexigenic and dipegenic responses, increased mRNA expression and secretion of the neuropeptides OXT and AVP, increased hypothalamic orexigenic drive (AgRP, MCH, and orexin A), and a less prominent increase in POMC mRNA expression. A decreased CB1R activation (left column), induced in the present study by treatment with the CB1R antagonist AM251, not only decreased WD-induced natriorexigenic and dipegenic responses but also potentiated OXT and AVP mRNA expression. WD animals treated with AM251 also maintained a dual stimulatory effect on orexigenic and anorexigenic hypothalamic pathways, although AM251 treatment affected the expression of a different group of mRNAs. Conversely, the stimulation of CB1Rs by the selective agonist ACEA (right column) consistently potentiated thirst and decreased WD-induced OXT and AVP mRNA expression. Furthermore, WD rats injected with ACEA maintained high AgRP and CART mRNA levels, as well as increased NPY mRNA expression, suggesting that exogenous CB1R stimulation probably could not override most of endogenous WD-mediated responses. Therefore, these changes induced by ACEA or AM251 on hypothalamic mRNA expression were characterized as WD-dependent (when these effects appeared only in WD rats) or independent (when the responses were induced irrespective to the hydration status). AVP, arginine vasopressin; AgRP, Agouti-related peptide; CART, cocaine- and amphetamine-regulated transcript; CRH, corticotrophin-releasing hormone; ECF, extracellular fluid; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; OXT, oxytocin. POMC, proopiomelanocortin.](http://ajpregu.physiology.org/)}
manipulations regardless of the hydration status. CRH mRNA levels were upregulated by AM251 administration, confirming the participation of CB1Rs on the negative modulation of HPA axis (12) but remained unchanged in ACEA-treated groups, suggesting that the ECB tonus on HPA axis activity could not be overcome under basal conditions.

Counteracting CART, POMC, and CRH inhibitory effects on food intake, we investigated WD-induced changes in orexigenic pathways mediated by the ARC and LHA. We showed that AgRP (but not NPY) mRNA is upregulated in the ARC of WD rats, which partially corroborates previous findings (27, 46). In fact, AgRP has been also shown to reduce locomotor activity (43) and basal metabolic rate (19), which indirectly impacts food intake. Other authors also demonstrated that AgRP and NPY may act in completely opposite directions to control light-dark cycle-motivated food intake (31). Regarding CB1R-mediated control of orexigenic pathways, we demonstrated here that WD-induced stimulatory effect on AgRP mRNA expression was potentiated in the presence of AM251, whereas the administration of ACEA decreased AgRP mRNA expression in EH but not in WD rats, suggesting that ACEA-mediated effects may be suppressed during dehydration. Accordingly, it has been previously reported that chronic rimonabant treatment increased AgRP mRNA levels in animals fed with a high-fat diet (17). Furthermore, the present study also demonstrated that NPY mRNA levels were not altered in the ARC of WD rats injected with vehicle or ACEA. In AM251-treated WD rats, however, NPY transcript levels in the ARC were slightly increased. Indeed, studies indicate that CB1Rs and NPY-related pathways may interact to control energy homeostasis, since blockade of CB1Rs by rimonabant in NPY-knockout mice resulted in additive reductions in body weight and adiposity (51).

Neurons expressing the type 1 receptor for orexin A have been identified in the magnocellular PVN, SON, and ARC (3), consistently suggesting a role for orexin A not only in the control of energy but also fluid balance. Similarly, MCH, when injected into the third or lateral cerebral ventricles, is able to regulate fluid independently of food intake (6), as well as to produce a diuretic and natriuretic effect (28). In the present study, WD significantly increased the expression of both orexin A and MCH mRNAs in the LHA, diverging from previous findings in the literature that used chronic consumption of hypertonic saline (47). Although AM251 failed to induce any changes in orexin A and MCH mRNA expression, ACEA administration increased the expression of both transcripts in both EH and WD rats, suggesting that the lateral hypothalamus is not potentially affected by a CB1R-mediated tonus, but it is highly responsive to CB1R stimulation. Whether or not this increased MCH and orexin A mRNA expression in the LHA of WD rats injected with ACEA contributes to the increased diuretic and diuretic responses observed in these animals remains to be investigated.

Perspectives and Significance

The data obtained in the present study allow us to propose a novel action for the ECBs on behavioral responses triggered by water deprivation, since CB1Rs are essential for the development of appropriate natriorexigenic and dipsogenic responses in WD animals. Furthermore, CB1R activation during water deprivation avoids an exaggerated OXT and AVP production and secretion, acting predominantly at the genomic level to constrain OXT and AVP mRNA expression. In addition, these results suggest that CB1Rs regulate main central peptidergic pathways controlling food intake, being that most of these effects are also significantly influenced by the hydration status. The intriguing finding is that water deprivation alone upregulates POMC and AgRP mRNA levels in the ARC, as well as MCH and orexin A in the LHA, suggesting that this regulation may occur in both directions, through the simultaneous stimulation of anorexigenic and orexigenic signaling systems (Fig. 8). Upcoming studies may certainly determine whether these constitute the cause or a compensatory effect of WD-induced hypophagia.

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