

Original articles

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INTERACTION BETWEEN OREXIN A AND CANNABINOID SYSTEM IN THE LATERAL HYPOTHALAMUS OF RATS AND EFFECTS OF SUBCHRONIC INTRAPERITONEAL ADMINISTRATION OF CANNABINOID RECEPTOR INVERSE AGONIST ON FOOD INTAKE AND THE NUTRITIVE UTILIZATION OF PROTEIN

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Crosstalk may occur between cannabinoids and other systems controlling appetite, since cannabinoid receptors are present in hypothalamic circuits involved in feeding regulation, and likely to interact with orexin. In this study, an immunohistochemical approach was used to examine the effect of the intracerebroventricular administration of cannabinoid receptor inverse agonist AM 251 on orexin neuropeptide in the hypothalamic system. AM-activated neurons were identified using c-Fos as a marker of neuronal activity. The results obtained show that AM 251 decreases orexin A immunoreactivity, and that it increases c-Fos-immunoreactive neurons within the hypothalamus when compared with the vehicle-injected control group. We also studied the effects of subchronic intraperitoneal administration of AM 251 on food intake, body weight, and protein utilization. The administration of AM 251 at 1, 2, or 5 mg/kg led to a significant reduction in food intake, along with a significant decrease in the digestive utilization of protein in the groups injected with 1 and 2 mg/kg. There was a dose-related slowdown in weight gain, especially at the doses of 2 and 5 mg/kg, during the initial days of the trial. The absence of this effect in the pair-fed group reveals that any impairment to digestibility was the result of administering AM 251. These data support our conclusion that hypothalamic orexigenic neuropeptides are involved in the reduction of appetite and mediated by the cannabinoid receptor inverse agonist. Furthermore, the subchronic administration of AM 251, in addition to its effect on food intake, has significant effects on the digestive utilization of protein.

Key words: *cannabinoid, food intake, body weight, protein digestibility, c-Fos expression, orexin A, immunoreactivity, cannabinoid CB1 receptor agonist*

INTRODUCTION

Endocannabinoids (*via* cannabinoid CB1 receptor activation) are physiological regulators of feeding behaviour (1), and CB1 receptors are widely expressed in the central and peripheral nervous systems (2). In the brain, CB1 receptors have been identified in pathways responsible for reward processes and for energy balance (3). In the periphery, cannabinoid CB1 receptors are expressed in several areas of the body including fat, muscle, liver, and the digestive tract (4).

Further evidence that endogenous cannabinoids are involved in central nervous system appetite regulation is derived from observations that the direct administration of anandamide into the ventromedial hypothalamus stimulates food intake (5). Moreover, it has been observed that concentrations of 2-arachidonoyl glycerol in the limbic forebrain and hypothalamus correlate positively with the stimulation of food intake in rats (6). Low doses of the exogenous cannabinoid agonist THC are known to induce hyperphagia in rats through the stimulation of cannabinoid CB1 receptors (7). This action is blocked or

reversed by the selective CB1 receptor antagonists rimonabant (SR141716A) (8, 9) and AM 251 (10, 11). The appetite suppressant effects of rimonabant have been observed after both acute and chronic administration (12), and also for various diets differing in palatability (13).

Several studies have suggested that the chronic administration of cannabinoid receptor antagonists induces a decrease in the total number of CB1 receptors within brain structures, including the limbic system and the cerebral cortex (14). In addition, lower levels of mRNA (which codifies for cannabinoid CB1 receptor), have been observed in the forebrain region (15).

Cannabinoid CB1 receptors regulate feeding by interacting with both peripheral sensory terminals and hypothalamic circuits (16, 17). At the level of the hypothalamus, they may interact with neuropeptide circuits involved in feeding regulation, including neuropeptide Y, melanocortin, and orexins, which are a type of neuropeptides earlier described as hypocretins (18). Orexin A administered to the lateral hypothalamus increases appetite in a dose-dependent manner (19). CB1 and OX1 receptors are expressed in similar brain regions, such as the lateral

hypothalamus (20, 21). Therefore, crosstalk between orexin A and the cannabinoid may occur within the brain since both are co-expressed in the same regions as the hypothalamic neurons involved in feeding behavior and energy homeostasis (22-24).

The presence of CB1 receptors in the nerve terminals that innervate the gastrointestinal tract (25) suggests that they might not only affect daily food intake, but also nutrient utilization at the digestive and metabolic level. The present study reports novel data regarding the effect of cannabinoids on protein utilization.

Following previous research showing that CB1 receptor agonists enhance feeding behaviour (26) and that CB1 receptor antagonists reduce food intake, the aim of the present study was to examine the effects that the subchronic administration of the cannabinoid receptor inverse agonist AM 251 at different doses would have on food intake, body weight, and protein utilization by rats. In order to identify the hypothalamic circuits implicated in these effects, c-Fos expression was used to determine neuronal activity at these levels. In addition, potential interactions between AM 251 and orexin A were examined.

MATERIALS AND METHODS

Animals

Male Wistar rats with an initial body weight of 150 ± 20 g were housed in individual cages designed for the separate collection of faeces and urine. The cages were located in a well ventilated thermostatically controlled room ($21 \pm 1^\circ\text{C}$, 12 h light/dark period) and the animals were given a standard rat chow and water *ad libitum*. All experiments were undertaken according to Directional Guides Related to Animal Housing and Care. The European guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Ethics Committee for Animal Experimentation of the University of Granada (Spain).

Drugs

The CB₁ receptor inverse agonist, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM 251) (Tocris Cokson, Bristol, UK) was dissolved in vehicle (Tween-80: dimethyl sulfoxide (DMSO): 0.9% NaCl in 1:2:97 ratio).

Experimental groups

In order to study the effect of the subchronic (i.p.) administration of AM 251 on food intake, body weight and nutritive utilization, 40 male Wistar rats ($n=10$ animals per group) were distributed among the following experimental groups:

- 1 - Vehicle-injected control group
- 2 - Group injected with AM 251 at dose of 1 mg/kg
- 3 - Group injected with AM 251 at dose of 2 mg/kg
- 4 - Group injected with AM 251 at dose of 5 mg/kg

A fifth pair-fed experimental group was included after the previous four groups were completed to separate drug effects to those related to differences in food intake. This fifth experimental group was vehicle-injected and pair-fed to the amount of food consumed by the group injected with AM 251 at the intermediate dose of 2 mg/kg.

For the immunohistochemistry study, four groups of male Wistar rats ($n=5$ animals per group) were used. In two groups, we studied the effect of the intracerebroventricular administration of AM 251 on orexin expression, as follows:

- 1 - Vehicle-injected control group, reflecting orexin A expression in the hypothalamic neurons.
- 2 - Experimental group injected with AM 251 at a dose of 1 $\mu\text{g}/5 \mu\text{l}$.

In the other two groups, we studied the effect of the intracerebroventricular administration of AM 251 on c-Fos expression, as follows:

- 1 - Vehicle-injected control group
- 2 - Experimental group injected with AM 251 at a dose of 1 $\mu\text{g}/5 \mu\text{l}$.

Food intake and nutritive utilization studies

The effects of the subchronic (i.p.) administration, for eight days, of CB₁ receptor inverse agonist on food intake were analysed in presatiated rats as follows: the animals were allowed a period of ten days to adapt to the experimental conditions and diet. Then, after 24 hours fasting a presatiation procedure was applied, in which the rats were allowed to eat the experimental diet *ad libitum* for 1 hour before drug administration to permit a standardised observation of the drug-induced feeding effect (27). After this initial presatiation period, the drugs were administered daily (i.p.) during eight days. Three different fresh solutions of drug + vehicle were prepared daily. The solutions were prepared for 0.4, 0.8 and 2 mg/mL concentrations by diluting AM 251 in vehicle which was a mixture of Tween-80: dimethyl sulfoxide (DMSO): 0.9% NaCl 100 (1:2:97 ratio). Animals were daily weighted and solution volume for each animal was then calculated according to their weight to get a final concentration of 1, 2 and 5 mg/kg body weight following the protocol previously reported by Chambers *et al.* (28). Administration was performed in darkness. Thirty minutes after drug or vehicle administration, the rats were allowed free access to food. A previous study (29) examined the effect of acute administration, and the present study considers the effect of subchronic administration, using the same doses. Throughout the experimental period, the animals' body weight and food consumption were recorded daily 24 hours after the injection of vehicle or drugs, at 10 a.m. Faeces and urine were collected daily for each rat, frozen at -20°C , weighed and ground for analysis of protein content.

Composition analysis of diet, faeces and urine

According to the manufacturer, the macronutrient composition of the standard diet used in the present study was adequate to meet the nutrient requirements of the experimental animals for crude energy, total nitrogen and ash content. The diet composition was as follows: energy, 13.0 MJ/kg; nitrogen, 26.1 g/kg; fat, 41 g/kg; carbohydrates, 690 g/kg; fibre, 45 g/kg; ash, 60.8 g/kg. Moisture content was determined by drying to constant weight in an oven at $105 \pm 1^\circ\text{C}$. Total nitrogen (N) was measured in diet, faeces and urine according to Kjeldahl's method in order to determine the intake and faecal and urinary excretion of nitrogen to assess the digestive and metabolic utilization of nitrogen (30). Crude protein was calculated as nitrogen $\times 6.25$.

Biological indices

The following indices and parameters were determined for each group according to the formulas given below: apparent digestibility coefficient (ADC) (1), retention (balance) (2), and percent nitrogen retention/nitrogen absorption (%R/A) (3):

- (1) $\text{ADC} = \{(I - F) / I\} \times 100$
- (2) $\text{Balance} = I - (F + U)$
- (3) $\% \text{R/A} = \{I - (F + U) / (I - F)\} \times 100$

where I = intake, F = faecal excretion and U = urinary excretion.

Immunohistochemical study

The animals were anaesthetized with chloral hydrate (400 mg/kg) and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). A stainless-steel cannula guide was implanted into the brain above the third ventricle, following the method described by Paxinos & Watson (31) (AP -0.72 mm, L 0.0 mm, DV -6.2). As described previously (29), a 10d post-surgical recovery period was allowed in order to stabilize food intake before the experimental period.

The effects of AM 251 on hypothalamic neuropeptides were analysed in partially satiated rats; to this end, the rats were deprived of food but not of water for 24 hours before the beginning of the experiments. They were then given free access to food for 1 hour (29). Infusions of 1 µg cannabinoid inverse agonist AM 251 solutions were made at a rate of 1 µl/min and a volume of 5 µl was injected into the third ventricle. The injector was maintained in place for 1 min to allow diffusion of the drug into the brain and to reduce backflow through the cannula track.

One hour after drug administration, animals were anaesthetised and transcardially perfused through the aorta with 50 ml saline followed by 300 ml of a fixative solution containing 4% paraformaldehyde in 0.1 M-phosphate buffer (PB), pH 7.4. The brains were removed, postfixed for 8 hours in the same fixative solution, and then in 30% sucrose in PB for 48 hours at 4°C. Transverse 40-µm sections were cut on a sliding microtome and stored in protecting solution (30% ethylene glycol /30% glycerol in 0.1 M NaK₂PO₄) at -20°C until processing.

The expression of orexin-A and c-Fos within the hypothalamus (six sections per rat) was determined using rabbit polyclonal antibodies raised against orexin-A protein (AbCam, Cambridge, UK) or c-Fos (Calbiochem, Darmstadt, Germany).

The brain sections were incubated for 24 hours at room temperature with the primary c-Fos or orexin A antiserum (1/10000; 1/1000). The sections were then incubated for 1 hour at room temperature with a biotinylated goat anti-rabbit antiserum (1/200) (Vector Laboratories, Paris, France); the sections were then rinsed in PBS and incubated for 1 hour at room temperature with the standard avidin-biotin-peroxidase (1/50) (Vector Laboratories). Peroxidase was revealed according to the method of Shu *et al.* (32), using diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St Louis, MO) as a chromogen. The reaction was stopped and the sections were washed, mounted on polylysine coated slides using a 0.1M PB and then dehydrated and cover-slipped before examination in

microscope. The photomicrographs were taken on an Olympus BX-41 microscope (Olympus Inc., Hamburg, Germany) coupled to a Sony 3CCD video camera. Quantification of the number of c-Fos or orexin A immunopositive cells was performed using Image J software.

Statistical analysis

Statistical differences among groups for daily food intake and weight changes were analysed by time-repeated ANOVA with time, treatment and time × treatment interaction as the main effects. Pairwise comparisons were made among the different experimental groups at each time points selected (days 1–8) (n=10). Differences in the digestive utilization of protein between the experimental group given 2 mg/kg of AM 251 and the pair-fed vehicle-administered group (*Fig. 3*) were evaluated by Student's t-test (n=10). Statistical differences in the nutritive utilization of protein were evaluated by one-way ANOVA. Multiple mean comparisons were performed using Duncan's test (*Table 1*) (n=10). Statistical analysis was applied to the data using Statgraphic Statistical Graphics 2.1 System Software (Statistical Graphics Corporation, Rockville, MD). The level of significance was set at P <0.05. The results of immunostaining were expressed as mean values (n=5) ± standard errors and statistical significance were determined using Student's t-test. Differences with a P-value <0.05 were considered significant.

RESULTS

Effect of the subchronic administration of AM 251 on food intake and weight changes

Following the administration of AM 251, a reduction in food intake was observed in animals injected with doses of 1, 2, and 5 mg/kg, compared with the vehicle-injected control group (*Fig. 1*). These differences persisted throughout the experimental period. Significant differences in food intake among the different doses tested were observed during the first four days of drug administration, but disappeared 24 hours after the fourth injection. The AM 251-derived reduction in food intake was linked to significantly lower (P <0.05) cumulative weight changes during the experimental period (*Fig. 2*), which were particularly evident for the 2 and 5 mg/kg doses, compared with the vehicle-injected control group (time × treatment interaction, P <0.0001). Furthermore, weight changes in the experimental group receiving AM 251 at a dose of 2 mg/kg were smaller than in pair-fed control animals.

Table 1. Effect of subchronic intraperitoneal administration of AM 251 at different doses on the nutritive utilization of protein. Data are mean ± S.E.M. of n=10 animals. ADC, apparent digestibility coefficient, (%R/A) percent nitrogen retention/nitrogen absorption.

	Vehicle	1 mg/kg	2 mg/kg	5 mg/kg
Intake				
(g 100/g body weight)	9.68±0.18 ^c	8.12±0.12 ^b	8.37±0.19 ^b	7.98±0.11 ^a
N intake (mg/d)	509.2±10.0 ^b	442.1±14.8 ^a	451.6±14.1 ^a	420.1±14.1 ^a
Faecal N (mg/d)	126.3±5.33 ^b	140.8±9.86 ^c	124.9±5.08 ^b	100.8±6.05 ^a
Urinary N (mg/d)	136.3±9.0 ^b	121.0±9.3 ^a	111.9±9.3 ^a	105.9±12.0 ^a
Absorbed N (mg/d)	367.1±9.9 ^b	309.7±15.0 ^a	341.0±14.7 ^a	329.2±16.0 ^a
ADC (%)	74.4±0.89 ^b	66.7±1.23 ^a	70.9±1.18 ^a	76.3±1.18 ^b
Balance (mg/d)	230.8±11.0 ^a	188.7±17.6 ^a	229.1±17.7 ^a	223.3±20.3 ^a
(%) R/A	61.2±2.45 ^a	60.1±3.6 ^a	66.4±3.21 ^a	67.1±4.04 ^a

^{a,b,c}. Mean values within a row with unlike superscript letters were significantly different (P <0.05).

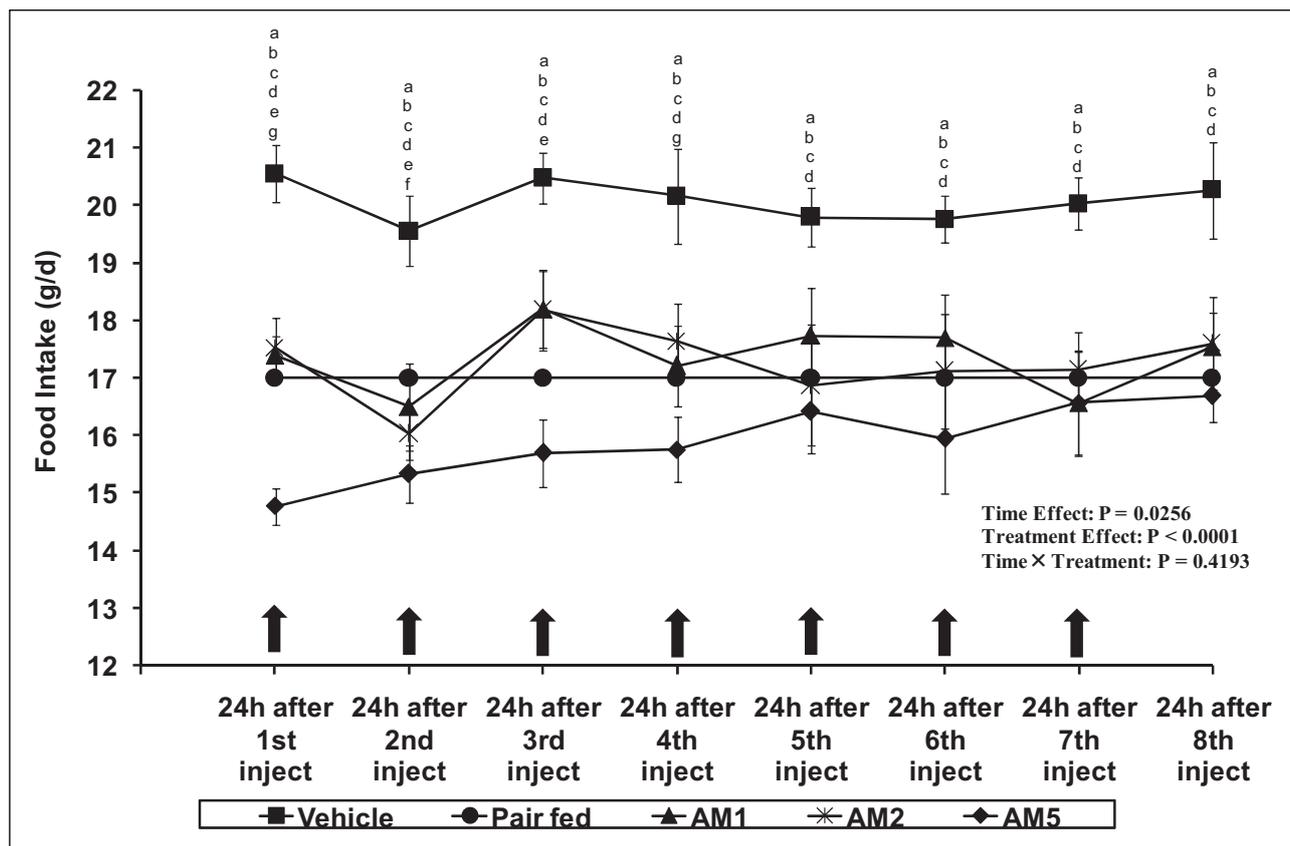


Fig. 1. Effect of subchronic administration of AM 251 at different doses on food intake (g/day). Arrows indicate where different treatments were given. Values are means and the standard errors are depicted by vertical bars (n=10). (a) vehicle vs. pair-fed, (b) vehicle vs. AM 251 (1 mg/kg), (c) vehicle vs. AM 251 (2 mg/kg), (d) vehicle vs. AM 251 (5 mg/kg), (e) pair-fed vs. AM 251 (5 mg/kg), (f) AM 251 (1 mg/kg) vs. AM 251 (5 mg/kg), (g) AM 251 (2 mg/kg) vs. AM 251 (5 mg/kg), $P < 0.05$.

Table 2. Effect of subchronic intraperitoneal administration of AM 251 (2 mg/kg) on the nutritive utilization of protein. Data are mean \pm S.E.M. of n=10 animals. ADC, apparent digestibility coefficient, (%R/A) percent nitrogen retention/nitrogen absorption.

	Pair fed	AM 2 mg/ kg
Intake (g 100/g body weight)	8.26 \pm 0.31 ^b	8.37 \pm 0.19 ^b
N Intake (mg/d)	450.47 ^a	451.6 \pm 14.1 ^a
Faecal N (mg/d)	89.81 \pm 4.16 ^a	124.9 \pm 5.08 ^b
Urinary N (mg/d)	120.4 \pm 8.5 ^a	111.9 \pm 9.3 ^a
Absorbed N (mg/d)	360.66 \pm 4.16 ^a	341.0 \pm 14.7 ^a
ADC (%)	80.71 \pm 0.78 ^b	70.9 \pm 1.18 ^a
Balance (mg/d)	240.26 \pm 8.02 ^a	229.1 \pm 17.7 ^a
(%) R/A	66.68 \pm 2.33 ^a	66.4 \pm 3.21 ^a

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Effect of the subchronic administration of AM 251 on protein utilization

Nitrogen intake was significantly lower in all AM 251-injected animals compared with the vehicle-injected control group (Table 1). Faecal N excretion was significantly increased by the 1 mg/kg dose, while it was significantly reduced by the 5 mg/kg dose. The digestive utilization of protein (expressed as ADC) was significantly lower in the groups injected with the 1 and 2 mg/kg doses than in the control group, whereas no significant differences were found between these groups and the animals injected with the 5 mg/kg dose. On the other hand, a progressive increase in the digestive utilization of protein was observed with growing doses of AM 251.

Regarding the metabolic utilization of protein, expressed as the percentage of retained to absorbed N, no significant differences were observed after comparing the groups administered varying doses of AM 251 (Table 1) and the control group.

Pair-fed experiment

Although the amount of N ingested was similar in the vehicle-injected and in the animals injected 2 mg/kg (Table 2), a significantly lower digestive utilization of protein was observed for the latter experimental group compared with the pair-fed control group (Fig. 3).

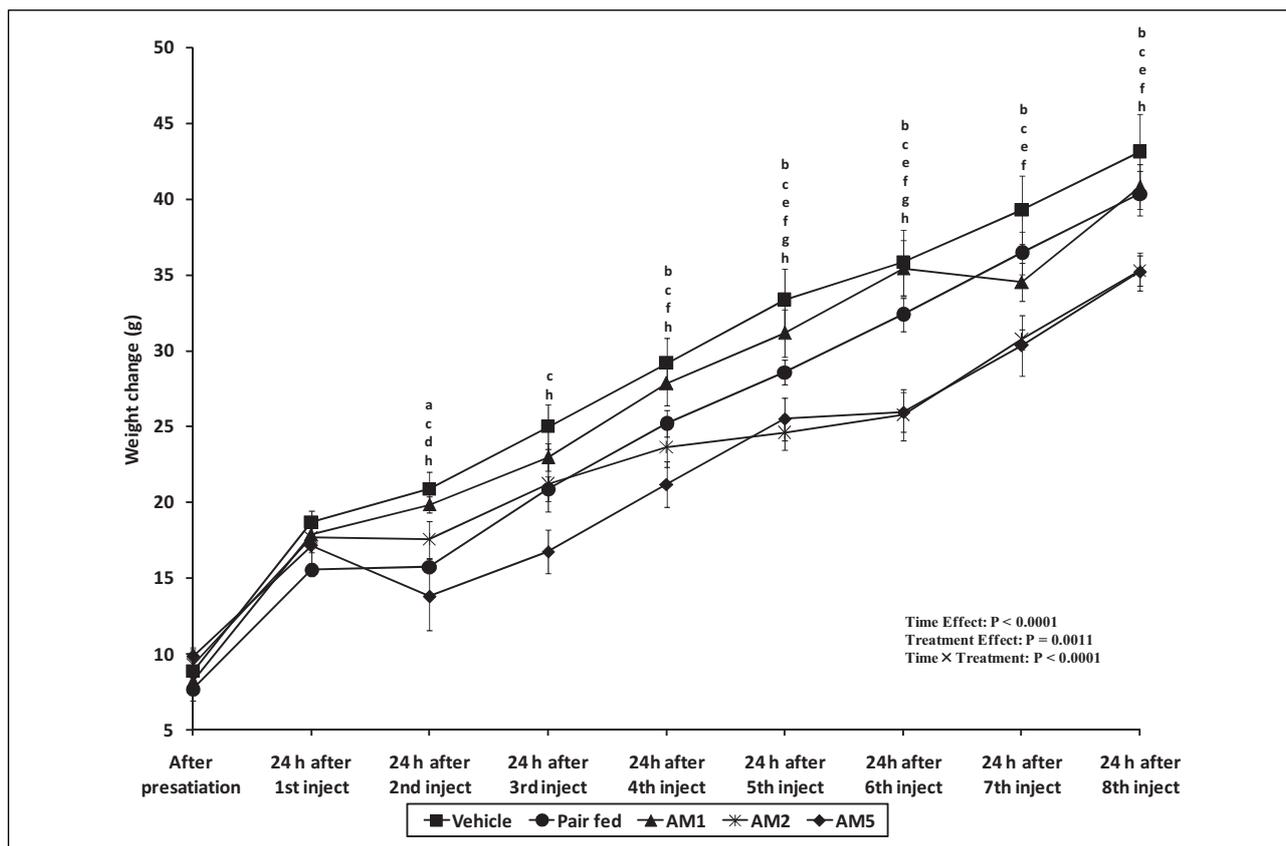


Fig. 2. Effect of subchronic administration of AM 251 at different doses on body weight change (g). Values are means and the standard errors are depicted by vertical bars ($n=10$). Changes are calculated taking the 24 h-fasted body weight as reference for all time points. (a) vehicle vs. pair-fed, (b) vehicle vs. AM 251 (2 mg/kg), (c) vehicle vs. AM 251 (5 mg/kg), (d) pair-fed vs. AM 251 (1 mg/kg), (e) pair-fed vs. AM 251 (2 mg/kg), (f) pair-fed vs. AM 251 (5 mg/kg), (g) AM 251 (1 mg/kg) vs. AM 251 (2 mg/kg), (h) AM 251 (1 mg/kg) vs. AM 251 (5 mg/kg), $P < 0.05$.

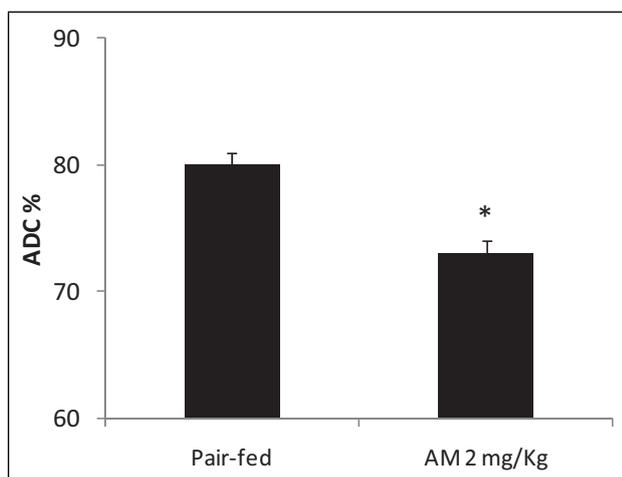


Fig. 3. Effect of subchronic administration of AM 251 (2 mg/kg) on digestive utilization of protein expressed as apparent digestibility coefficient (ADC). Values are means and the standard errors are depicted by vertical bars ($n=10$). *Significantly different ($P < 0.05$; Student's *t*-test).

Effect of the intracerebroventricular administration of AM 251 on *c-Fos* expression

Immunohistochemical analysis of the effects of administering AM 251 on neuronal activity at the lateral

hypothalamus showed that AM 251 significantly increased *c-Fos* expression (1.5-fold, $P < 0.05$), compared with the vehicle-injected control group (60.4 ± 4.0 and 40.4 ± 5.6 , respectively; Fig. 4a and 4b).

Effect of the intracerebroventricular administration of AM 251 on orexin A expression

Intracerebral injection of AM-251 induced a decrease in orexin expression in the lateral hypothalamus, compared with the vehicle-injected control group (16.3 ± 1.0 and 46.4 ± 3.5 , respectively $P < 0.01$; Fig. 5a and 5b).

DISCUSSION

We studied the effects of the subchronic (i.p.) administration of AM 251 at different doses, in order to better understand its potential therapeutic action regarding the alteration of food intake and of body weight. In addition to the effects observed on daily food intake and body weight, we provide data concerning the influence of the CB_1 receptor inverse agonist on protein utilization, together with data on the effect of the intracerebroventricular administration of AM 251 on *c-Fos* and orexin A expression in the lateral hypothalamus. The data provided complement those from prior studies conducted in our laboratory (29) concerning the effects of the acute administration of this inverse agonist on food intake and related levels of 5-HT in the ventromedial hypothalamus. In the present study, the

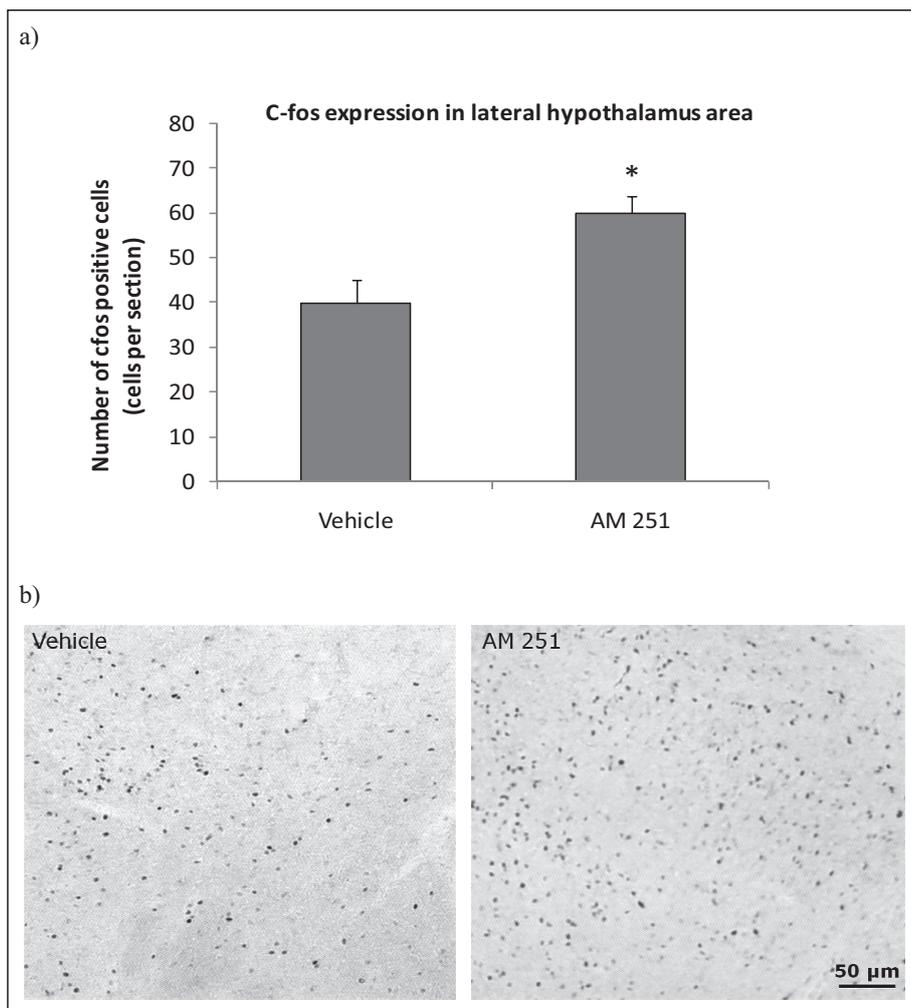


Fig. 4. Effect of intracerebroventricular injection of AM 251 (1 μ g/5 μ l) on lateral hypothalamus c-Fos expression. (a) Values are means and the standard errors are depicted by vertical bars ($n=5$; three sections counted per rat). *Significantly different ($P<0.05$, Student's t-test). (b) Photomicrographs showing 50 μ m sections of the lateral hypothalamus immunostained for c-Fos. Rats treated with vehicle (V) and AM251 (AM).

administration of AM 251 at 1, 2, or 5 mg/kg body weight produced a significant reduction in food intake, accompanied by a significant decrease in the digestive utilization of protein in animals receiving 1 or 2 mg/kg of AM 251. These outcomes provoked a dose-related slowdown of weight gain, especially at the doses of 2 and 5 mg/kg during the initial days of the trial (observed with a dose of 2 mg/kg after the fourth injection and with a dose of 5 mg/kg after the second one), which persisted until the end of the experiment. The fact that the accumulated weight change was smaller in the group given AM 251 at a dose of 2 mg/kg than in the pair-fed control group led us to consider that this cannabinoid receptor inverse agonist could provoke effects other than those related to food intake, such as reducing absorption as measured by the coefficient of apparent digestibility (see below). Our results reflect a trend similar to that reported by Chambers *et al.* (11), and differences regarding methodological approach and experimental design are probably responsible for the comparably smaller effect observed. The reductions we measured in body weight could be related to changes in metabolism and/or increased energy expenditure, perhaps through the enhanced expression of adiponectin gene, a circulating hormone that produces weight loss through the oxidation of free fatty acids, which in turn leads to the lipolysis of adipose tissue and to increased oxygen consumption (33, 34). The weight loss could also be attributed to reduced locomotor activity caused by high doses of the drug. In some experiments, the dose of 1.0 mg/kg of SR-141716A led to hypoactivity (35) and to diminished nicotine-induced hypoactivity

(36). In other studies, a single injection of AM 251 (0.25 mg/kg, i.p.) antagonized the motor stimulation induced by WIN 55,212-2 (0.25 mg/kg, i.p.) in spontaneously hypertensive adolescent rats, suggesting that this effect was mediated by the cannabinoid CB1 receptor, which is densely expressed in brain structures such as the cerebellum and the basal ganglia. These structures are known to mediate the initiation and coordination of movement (37). A high density of cannabinoid CB1 receptors in the axon terminals of the striatal GABAergic neurons of the basal ganglia and of the glutamatergic granule cells of the cerebellum is probably involved in motor control (38). Cannabinoid receptors may modulate both inhibitory and excitatory neuronal transmission in the basal ganglia, thus providing dual regulation of movement (39, 40).

The inhibitory effect of AM 251 on food intake observed in the present study corroborates previous findings (10, 11, 28) in which varying doses and experimental periods of chronic administration were used. Sustained levels of AM 251 and its metabolites in plasma could induce a strong inhibitor effect on the hypothalamic nuclei involved in the regulation of food intake *via* the CB1 receptors (41) located in the structures responsible for the control of energy balance and the incentive value of food, which are present in the central and peripheral nervous systems (27, 42, 43). The hypophagic effects of AM 251 are caused, at least in part, by cannabinoid-induced alterations in both excitatory and inhibitory amino acid neurotransmission, and by consequent changes in the activity of cells comprising the hypothalamic feeding circuitry, such as melanin-concentrating

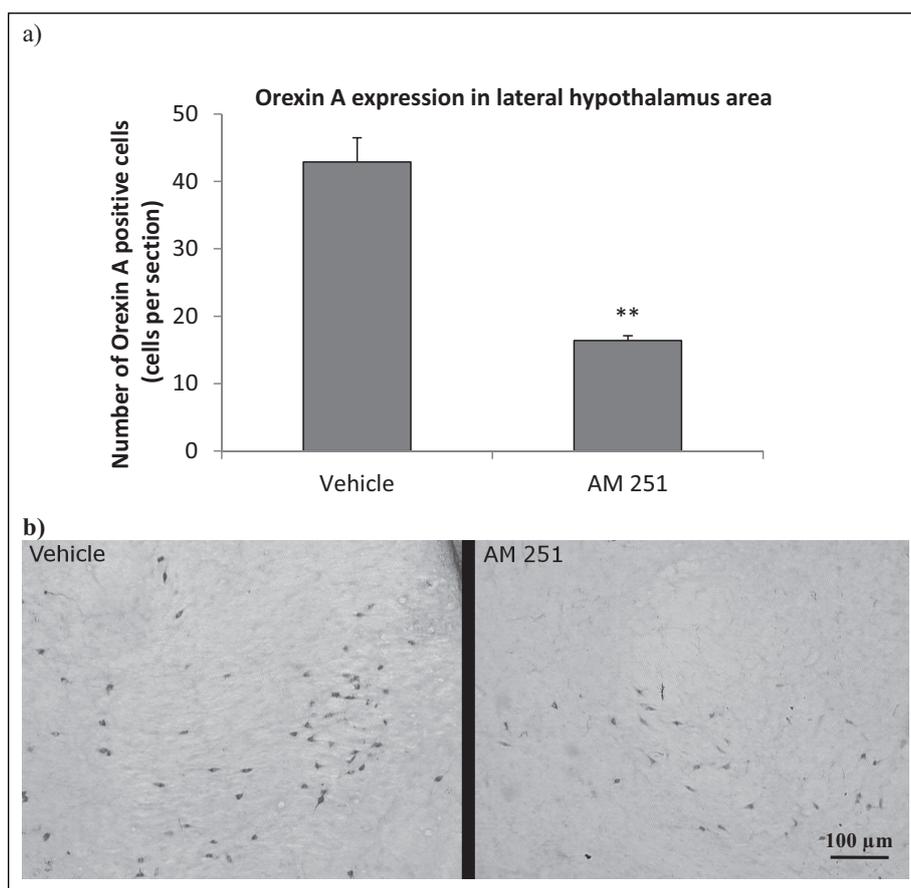


Fig. 5. Effect of intracerebroventricular injection of AM 251 (1 ug/5 ul) on lateral hypothalamus orexin A expression. (a) Values are means and the standard errors are depicted by vertical bars (n=5; six sections counted per rat). ** significantly different (P < 0.01, Student's t-test). (b) Photomicrographs showing 50 μm sections of the lateral hypothalamus immunostained for orexin A. Rats treated with vehicle (V) and AM251 (AM).

hormone (44, 45), NPY (46), POMC (47), and orexins (48). In addition, hypothalamic levels of endogenous cannabinoids are influenced by feeding-relevant humoral factors such as leptin (49) and ghrelin (50).

The present study reports data supporting the notion that the endocannabinoid system may influence food intake by regulating the expression and/or action of the hypothalamic neuropeptide orexin, which is involved in feeding behaviour but also takes part in the regulation of wakefulness affecting neuronal cellular morphology caused by GABA_A receptor dependent anaesthetics (51) and in protection of gastric mucosa (52). There is evidence that orexin A increases food intake by delaying the onset of behaviourally normal satiety. The selective orexin receptor antagonist suppresses food intake and advances the onset of the normal satiety sequence (48). According to Hilaiet *et al.* (22), cannabinoid CB1 receptors and orexin receptors are co-expressed by different hypothalamic regions involved in feeding behaviour and energy homeostasis, and their co-expression in structures implicated in feeding points out to the existence of an interaction between cannabinoids and orexin, as supported by our data.

Regarding the central nervous system, the intracerebroventricular injection of the cannabinoid receptor inverse agonist AM 251 produced a significant increase in c-Fos expression. This increase in neuronal activity was accompanied by a significant decrease in the number of neurons expressing orexin A in the hypothalamus. These data are in agreement with a previous study showing that rimobant produces a decrease in orexin expression and blocks the orexigenic effect of orexin A (53). Another study showed that the anorectic effect of AM 251-induced CB1 receptor blockade is due, at least in part, to the

inhibition of orexigenic peptide NPY production in the hypothalamus (54). Reduced orexin and NPY expression in animals given AM 251 indicates a mutual interaction at the level of the hypothalamic orexigenic neuropeptides. The hypophagic effect of AM 251 might be mediated to some extent by orexin and NPY. It has also been shown (29) that one of the neurochemical mechanisms underlying the anorectic effect of AM 251 could be related to its ability to differentially affect the functionality of serotonin-releasing neurons. Thus, AM 251 seems to produce hypophagia by stimulating the mechanisms producing the sensation of satiety.

The subchronic (i.p.) administration of AM 251 at doses of 1 and 2 mg/kg produced significant hypophagia and reduced digestive protein utilization. This reduced digestibility could be a result of either the reduced food intake or of the administration of AM 251. Therefore, we conducted an additional experiment in which a new group of animals was pair-fed to the experimental group, and given an intermediate dose of 2 mg/kg, thus avoiding the potential effect of the lower food intake. Although these two experimental groups had a similar daily food intake, the digestive utilization of nitrogen continued to be lower in the 2 mg/kg-injected animals than in the pair-fed vehicle-injected group. Therefore, the decrease in the digestive utilization of proteins was due to the effect of AM 251 rather than to reduced food intake.

It has been reported that the activation of CB1 receptors inhibit gastric motor function when given peripherally and provide gastric mucosal protection (55) whereas CB1 antagonists produce an increase in gastrointestinal motility (56, 57), an effect that primarily involves a peripheral site of action (58). However, we cannot exclude that at least part of our results are mediated at a central level, *via* the diffusion of AM 251

(containing CB1 receptors) to the central nervous system. The above findings support our results concerning the significant effect of AM 251 at the digestive level.

Regarding the experimental group given 5 mg/kg of AM 251, we observed a significant reduction in faecal excretion, and therefore a considerable increase in ADC. Greater digestive utilization of N, expressed as ADC, could be a compensatory effect in response to the lower N intake, aimed at meeting nutrient requirements.

The i.p. administration of AM 251 led to a significant reduction in renal excretion of N, which was not dose-dependent and resulted in similar N balances among the 1 mg/kg, 2 mg/kg, 5 mg/kg, and control animals. Nevertheless, no differences in urinary N excretion were found between the 2 mg/kg and the control animals in the pair-fed experiment. This finding links the urinary excretion of N to the significant hypophagia induced by AM 251, rather than to any drug-related effect. This was to be expected judging by the results of Deutsch *et al.* (59), who reported the presence of CB1 receptors at the renal level. In response to the lower daily intake of N, sufficient absorption of this nutrient took place to maintain the required levels of amino acids. This compensatory effect was apparent in the similar N balance among all the animals injected with AM 251 (at 1, 2, and 5 mg/kg) and the vehicle-injected control animals, as was a trend toward higher indices of retained-to-absorbed N in the AM 251-injected animals, thus reflecting an acceptable degree of utilization of absorbed N.

The results obtained lead us to conclude that there exists an interaction between the hypothalamic orexin neuronal system and the cannabinoid system. In addition to the hypophagic effect of AM 251, the subchronic administration of this inverse agonist also produces significant effects on the digestive utilization of protein. This suggests that AM 251 could be applied to the treatment of diseases caused by excessive food intake and impaired energy metabolism. This is a novel finding, since for the first time data are provided concerning the effect of CB1 receptor inverse agonists on nutrient utilization for a particular diet, beyond those related to daily food intake.

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