

RESEARCH PAPER

Cannabinoid CB₁/CB₂ receptor agonists attenuate hyperactivity and body weight loss in a rat model of activity-based anorexia

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Received 31 January 2017; **Revised** 27 April 2017; **Accepted** 23 May 2017

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BACKGROUND AND PURPOSE

Anorexia nervosa (AN) is a serious psychiatric condition characterized by excessive body weight loss and disturbed perceptions of body shape and size, often associated with excessive physical activity. There is currently no effective drug-related therapy of this disease and this leads to high relapse rate. Clinical data suggest that a promising therapy to treat and reduce reoccurrence of AN may be based on the use of drugs that target the endocannabinoid (EC) system, which appears dysregulated in AN patients.

EXPERIMENTAL APPROACH

The activity-based anorexia (ABA) rodent model mimics the severe body weight loss and increased physical activity, as well as the neuroendocrine disturbances (i.e. hypoleptinaemia and hypercortisolaemia) in AN. This study investigated whether cannabinoid agonists can effectively modify anorexic-like behaviours and neuroendocrine changes in rats subjected to a repeated ABA regime that mimics the human condition in which patients repeatedly undergo a recovery and illness cycle.

KEY RESULTS

Our data show that subchronic treatment with both the natural CB₁/CB₂ receptor agonist Δ^9 -tetrahydrocannabinol and the synthetic CB₁/CB₂ receptor agonist CP-55,940 significantly reduced body weight loss and running wheel activity in ABA rats. These behavioural effects were accompanied by an increase in leptin signalling and a decrease in plasma levels of corticosterone.

CONCLUSION AND IMPLICATIONS

Taken together, our results further demonstrate the involvement of the EC system in AN pathophysiology and that strategies which modulate EC signalling are useful to treat this disorder, specifically in patients where physical hyperactivity plays a central role in its progression and maintenance.

Abbreviations

AN, anorexia nervosa; CB₁ receptor, cannabinoid type-1 receptor; CP, CP-55,940; EC, endocannabinoid system; THC, Δ^9 -tetrahydrocannabinol

Introduction

Anorexia nervosa (AN) is a serious psychiatric condition characterized by a lower normal body weight as a consequence of rigid dietary restriction associated with an intense fear of weight gain due to disturbed perceptions of body shape and size (APA, 2013). In 80% of AN patients, physical hyperactivity also occurs in response to the need to control and maintain a reduced body weight, thereby contributing to the progression of the disorder (Davis *et al.*, 1997; Gümmer *et al.*, 2015; Achamrah *et al.*, 2016). AN onset is typically in adolescence, affects more women than men and often is accompanied by significant psychiatric comorbidities, such as depression, anxiety and/or obsessive-compulsive disorder (Hudson *et al.*, 2007; Mattar *et al.*, 2012; Smink *et al.*, 2012). Since there is currently no effective drug-related therapy, AN has a low rate of recovery as a large percentage of patients relapse within 1 year of terminating a specialized treatment programme (Carter *et al.*, 2004; Bergh *et al.*, 2013). Consequently, AN tends to be chronic and disabling, with considerable tolls on physical and mental health (Marzola and Kaye, 2015; Mehler and Brown, 2015). Furthermore, AN has the highest mortality rate (~5.6%) of any mental illness (Arcelus *et al.*, 2011; Keshaviah *et al.*, 2014). Thus, discovery of molecular targets for the development of specific medications to treat and reduce its reoccurrence is of critical importance.

Clinical data suggest that a promising pharmacotherapy may be based on the use of drugs that target the endocannabinoid (EC) system, which appears dysregulated in AN patients. For example, elevated plasma levels of the EC **anandamide**, as well as increased cannabinoid **CB₁ receptor** availability, were found in women with AN compared with healthy controls (Monteleone *et al.*, 2005; Gérard *et al.*, 2011). Moreover, heightened levels of CB₁ receptor mRNA in the blood of AN patients further support the notion of impaired EC signalling in this disorder (Frieling *et al.*, 2009). These effects are likely to represent a compensatory up-regulation in order to compensate for a hypoactivity of the EC system in anorexic states (van der Stelt and Di Marzo, 2003; Di Marzo, 2008). The EC system has numerous physiological functions, including a crucial role for **leptin** in the regulation of appetite and energy balance by controlling both homeostatic and hedonic aspects of food intake mainly in the CNS (Di Marzo and Matias, 2005). Specifically, by activating the CB₁ receptors expressed in brain regions involved in energy modulation (i.e. hypothalamus and mesocorticolimbic system), both endogenous and exogenous cannabinoids are capable of promoting food intake, modifying the release of orexigenic and anorexic mediators, as well as reinforcing hedonic valuation of food (Di Marzo and Matias, 2005; Jager and Witkamp, 2014). In contrast, specific antagonists at the CB₁ receptor exert an opposite effect: they suppress food intake and reduce body weight in laboratory animals (Carai *et al.*, 2006). Given the EC system's role in controlling feeding behaviour, it is not surprising that its dysregulation could be connected to the pathophysiology of AN, and that symptoms and progression can be attenuated by normalizing EC signalling through pharmacological treatments based on specific cannabinoids (Di Marzo, 2009).

Animal models are very useful for investigating neurobiological substrates and pharmacological determinants of

human disorders, like AN (Casper *et al.*, 2008). The activity-based anorexia (ABA) rodent model is among the most commonly used for AN studies; animals undergo restricted feeding schedules (1–2 h·day⁻¹) with free access to a running wheel (Routtenberg and Kuznesof, 1967; Routtenberg, 1968). These two factors, when applied simultaneously, model key aspects of human AN, specifically hyperactivity and reduced body weight as well as neuroendocrine disturbances such as dysregulation of appetite-regulating hormones (i.e. decreased basal plasma levels of leptin) and activation of the hypothalamic–pituitary–adrenal axis (HPA; i.e. increased basal plasma levels of **corticosterone**) (Burden *et al.*, 1993; Davis *et al.*, 1997; Adan *et al.*, 2011). However, animal models present some limitations since they can reproduce only some traits of the pathology. For example, psychological components such as obsessing over body weight and shape cannot be easily assessed in animals (Casper *et al.*, 2008). Using this model, exposure to the natural CB₁/CB₂ receptor partial agonist **Δ⁹-tetrahydrocannabinol** (THC) has been previously shown to attenuate the weight loss associated with the development of ABA (Verty *et al.*, 2011). Moreover, ABA development has been associated with an *in vivo* increase in CB₁ receptor availability in different brain areas (Casteels *et al.*, 2014).

As mentioned before, AN tends to be chronic and patients repeatedly undergo a recovery and illness cycle, and the ABA model can also be used to investigate this occurrence by subjecting the animals to the effect of repeated ABA regime (Chowdhury *et al.*, 2015; Aoki *et al.*, 2017). The present study was performed to extend data published by Verty *et al.* (2011), investigating the effects of THC on weight loss and hyperactivity in rats exposed to a repeated ABA regime, to better assess the effectiveness of pharmacological treatments (Chowdhury *et al.*, 2015; Aoki *et al.*, 2017). For the first time, we evaluated the effects of the synthetic CB₁/CB₂ receptor agonist **CP-55,940** (CP) to compare the effects of a full cannabinoid receptor agonist in comparison with the partial agonist THC (Castaneto *et al.*, 2014). In clinical reports neuroendocrine disturbances, such as hypoleptinaemia and hypercortisolaemia, have been proposed as diagnostic markers for AN (Misra and Klibanski, 2014), therefore, we decided to evaluate the effect of CB₁/CB₂ receptor agonists on plasma levels of both leptin and corticosterone.

Methods

Animals

Female Sprague Dawley rats (Envigo, Italy) weighing 125–150 g at the start of the study were used. Animals were housed in a climate-controlled animal room (21 ± 2°C; 60% humidity) under a reversed 12 h light/12 h dark cycle (lights on at 12:00 h) and fed standard rat chow and water *ad libitum*. Young female rats were chosen because >90% of human anorexic patients are adolescent young women (Hudson *et al.*, 2007). All procedures and experiments were carried out in an animal facility according to Italian (D.L. 26/2014) and European Council directives (63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments at the University of Cagliari.

(Sardinia, Italy) and the Italian Department of Health (286/2016). All possible efforts were made to minimize animal pain and discomfort, as well as reduce the number of experimental subjects. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Apparatus

Standard polycarbonate cages [48(h) × 32 (w) × 47(d) cm] or polycarbonate cages equipped with running wheels (35 cm in diameter) served as experimental chambers. Each activity wheel cage had a magnetic switch and LCD revolution counter; the switch continuously counted whole revolutions of the activity wheel (Ugo Basile, Varese, Italy).

Experimental design

After 1 week of acclimatization, rats were divided into four groups matched for initial body weight and then housed individually in standard home cages (sedentary rats) or home cages equipped with a running wheel (running rats): (1) 'Control' rats received 24 h food access but no access to the activity wheel; (2) 'Restricted' rats were allowed food access for 1.5 h·day⁻¹ but no access to the activity wheel; (3) 'Exercise' rats received 24 h food and activity wheel access; (4) 'ABA' rats were allowed food for 1.5 h·day⁻¹ and had 24 h activity wheel access.

Animals were adapted to their housing conditions for 7 days with *ad libitum* food and running wheel access (adaptation period). Body weight, food intake and running wheel activity (RWA) (where applicable) were recorded daily within 30 min of the start of the 12 h dark cycle in order to obtain a stable baseline (BL). After adaptation, ABA rats were exposed to two bouts of the ABA regime separated by 10 days of recovery (Figure 1) (Chowdhury *et al.*, 2014; Aoki *et al.*, 2017).

First ABA induction. On the last day of the adaptation period, at the onset of the 12 h dark cycle, food was removed from the cages of ABA rats and restricted access to food commenced. For ethical reasons, rats were not allowed to lose more than 22–25% of their initial body weight (Gutierrez, 2013). Therefore, in order to avoid a drop larger than 25%, rats were maintained on this restricted scheduled feeding for a maximum of 6 days (Verty *et al.*, 2011). During each day of the restricted feeding schedule, rats were given

unlimited access to food for 1.5 h at the onset of the 12 h dark phase. Access to running wheels was blocked during the 1.5 h feeding period to prevent RWA from competing with eating. Food consumption was measured by weighing the food before and after the 1.5 h access period. The same restricted-feeding schedule was imposed on the Restricted group of rats, while the Control and Exercise groups of rats continued to receive *ad libitum* access to food for 24 h. In all four experimental groups, body weight and RWA (where applicable) were recorded daily within 30 min of the start of the 12 h dark cycle. At the same time, food intake was measured for Control and Exercise rats by weighing it every 24 h.

Recovery from ABA. On day 6 of the restricted feeding schedule and after the 1.5 h access to food, ABA and Restricted rats were allowed food *ad libitum* for 24 h for 10 days for weight restoration, while Control and Exercise rats continued to receive 24 h food access. During this recovery phase, Exercise and ABA rats had unlimited access to running wheels (Dixon *et al.*, 2003; Ratnovsky and Neuman, 2011). In all experimental groups, body weight, food intake and RWA (where applicable) were recorded daily within 30 min of the start of the 12 h dark cycle.

Second ABA induction. At the end of the recovery phase (day 10), before the start of the 12 h dark cycle, animals from each experimental group ($n = 42$ rats per group) were randomly divided into subgroups according to their pharmacological treatment: (i) THC-treated rats received either 0.0 (vehicle), 0.5 or 0.75 mg·kg⁻¹ THC ($n = 7$ rats per dose); (ii) CP-treated rats received either 0.0 (vehicle) 0.03 or 0.06 mg·kg⁻¹ CP ($n = 7$ rats per dose). Drug doses were chosen based on preliminary experiments (we discarded doses that resulted in a decrease in spontaneous locomotor activity; data not shown) and according to the literature (Järbe and DiPatrizio, 2005; Dodd *et al.*, 2009). Day 1 of drug treatment represented the start of the second ABA induction in which ABA rats were exposed to the ABA paradigm described above. During the second ABA induction, all rats from all experimental groups were injected daily for 6 days with either vehicle or drug 30 min before the onset of the 12 h dark phase. At the onset of the 12 h dark phase, ABA and Restricted rats were given unlimited access to food for 1.5 h,

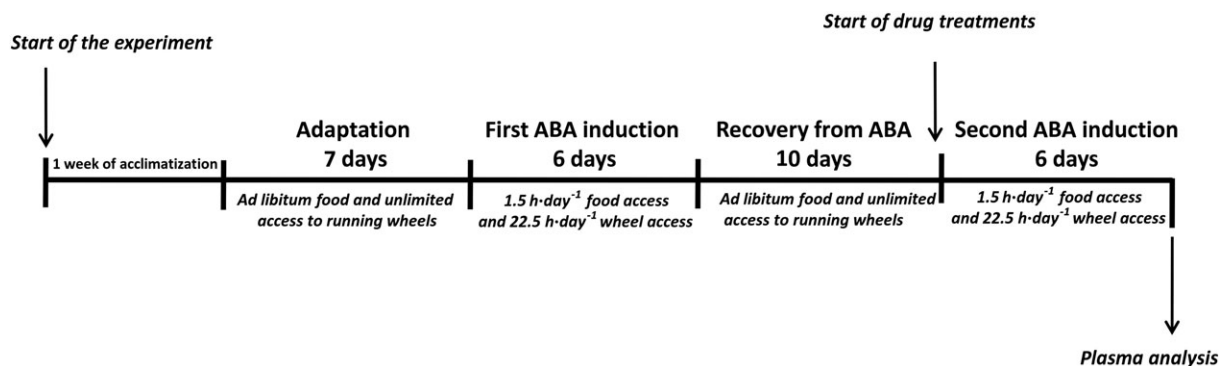


Figure 1

Schematic representation of the experimental design.

and food consumption was measured by weighing the food before and after the access period. Control and Exercise rats continued to receive 24 h food access. Running wheel access was blocked (where appropriate) during the feeding period as described above; otherwise, animals had unlimited access. Body weights and RWA were recorded daily just prior to drug injections. At the same time, food intake was measured for *ad libitum*-fed rats.

Plasma analysis

All rats were killed at the end of the 12 h light phase on day 6 of the second ABA induction. Plasma leptin and corticosterone levels were measured using a commercially available ELISA kit according to the manufacturer's protocols (EZRL-83K/Rat Leptin ELISA, EMD Millipore, St. Charles, Missouri, USA; Corticosterone Elisa Kit ADI-900-097, Enzo Life Sciences, Lausen, Switzerland). Trunk blood was collected into K₃EDTA tubes, centrifuged at 3000× *g* for 15 min at 4 ± 2°C and then plasma was stored at -20°C for hormone analysis.

Statistical analysis

Body weight, food intake and RWA are presented as mean ± SEM and were analysed by two-way ANOVA for repeated measures with two factors being groups as a between-subjects factor and time (days) as within-subjects factor and a repeated factor. Within each experimental group, body weight, food intake and RWA from pharmacologically-treated rats are presented as the mean ± SEM and were analysed either by repeated measures two-way ANOVA with treatment as a between-subjects factor and time (days) as a within-subjects factor, or one-way ANOVA with groups as a between-subject factor. Plasma levels are presented as the mean ± SEM and were analysed by one-way ANOVA with groups as a between-subject factor. *Post hoc* comparisons were made with Newman-Keuls multiple comparison or Bonferroni tests, where appropriate. Analysis of results was carried out using Graph Pad Prism® 5 for Windows (Graph Pad software, USA). In all cases, differences with a *P* < 0.05

were considered significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Drugs

The natural CB₁/CB₂ receptor partial agonist THC (THC Pharm, Frankfurt am Main, Germany, 1 g·5 mL⁻¹ in ethanol solution) and the synthetic CB₁/CB₂ receptor agonist CP (Tocris Bioscience, Bristol, UK) were dissolved in 2% Tween-80, 2% ethanol and 96% saline. All drugs were injected i.p. in a volume of 1 mL·kg⁻¹.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/2016 (Alexander *et al.*, 2015).

Results

First ABA induction

With the restricted feeding schedule, body weight significantly decreased in both ABA and Restricted rats compared with Control and Exercise groups (Figure 2A). Two-way ANOVA revealed a significant main effect of group × time interaction [*F*(18,984) = 269.91, *P* < 0.05]. Moreover, weight loss was more pronounced in ABA compared with Restricted rats starting at day 2 and progressively decreased to a total of ~21% loss. Restricted rats exhibited a significant but stable weight loss of 10%. Both Control and Exercise rats presented an increase in body weight with no significant difference between them. Due to the restricted-feeding schedule, daily food intake was lower in the ABA and Restricted rats compared with *ad libitum*-fed rats, and two-way ANOVA revealed

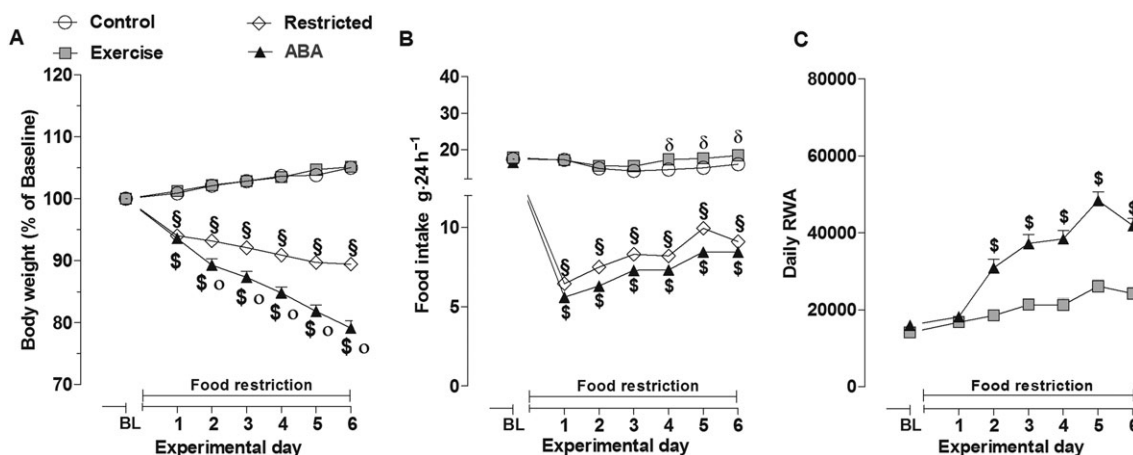


Figure 2

Body weight (A), food intake (B) and RWA (C) in Control, Exercise, Restricted and ABA groups during the 6 days of the first ABA induction. Results are presented as the mean ± SEM (*n* = 42 rats per group). Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post hoc* test (body weight: °*P* < 0.05 vs Restricted rats, \$*P* < 0.05 and \$*P* < 0.05 vs Exercise and Control rats; Food intake: \$*P* < 0.05 and \$*P* < 0.05 vs Exercise and Control rats; RWA: \$*P* < 0.05 vs Exercise rats).

a significant main effect of group \times time interaction [$F(18,984) = 32.45, P < 0.05$; Figure 2B]. Moreover, Exercise rats consumed significantly more food starting from day 4 than Control rats. The weight loss in ABA rats was accompanied by a concomitant and progressive increase in RWA which became significant from day 2 of the ABA protocol when compared with that of Exercise rats (Figure 2C). Two-way ANOVA revealed a significant main effect of group \times time interaction [$F(6,492) = 23.56, P < 0.05$]. On day 6, ABA rats displayed a 161% increase in RWA relative to the BL. In contrast, Exercise rats exposed to the running wheel without any food restriction showed only a modest but stable increase in RWA (+71% from BL).

Recovery from ABA

ABA rats recovered to baseline body weight within the first 4 days of the recovery phase (Figure 3A). Nonetheless, they weighed significantly less than the other main experimental groups at the end of this period, and two-way ANOVA revealed a significant main effect of group \times time interaction [$F(30,1640) = 23.29, P < 0.05$]. However, Restricted rats recovered their 10% weight loss within the first day of recovery, and by day 4, their body weight was not statistically different from that of *ad libitum*-fed rats. There were no significant differences in body weight between Control and Exercise rats. With regard to daily food intake, two-way ANOVA also revealed a significant main effect of group \times time interaction [$F(27,1476) = 7.09, P < 0.05$; Figure 3B]. Food intake was significantly higher in ABA rats than the other groups. In contrast, Restricted rats returned to baseline levels of food intake by day 8 of recovery. Moreover, Exercise rats continued to consume significantly more food compared with Control rats. During the first 3 days of recovery, the RWA of ABA rats was significantly lower compared with Exercise rats, and two-way ANOVA revealed a significant main

effect of group \times time interaction [$F(9,738) = 14.45, P < 0.05$; Figure 3C].

Second ABA induction

Effect of THC administration. As shown in Figure 4, no significant main effect of treatment \times time interaction was observed for the body weight of the Control, Exercise and Restricted groups [two-way ANOVA Control: $F(12,108) = 0.90, P > 0.05$; Exercise: $F(12,108) = 0.35, P > 0.05$; Restricted: $F(12,108) = 0.95, P > 0.05$; Figure 4A–C]. In contrast, the body weight of ABA rats was modified by THC treatment (0.5 and 0.75 mg·kg⁻¹). Two-way ANOVA revealed a significant main effect of treatment \times time interaction [$F(12,108) = 1.94, P < 0.05$]; compared with vehicle-treated ABA rats, *post hoc* analysis showed that 0.75 mg·kg⁻¹ of THC significantly reduced body weight loss on days 6 and 7 (–20 vs –15.2% and –21.8 vs –16.72% respectively). THC administration did not change food intake in *ad libitum*-fed rats [two-way ANOVA, main effect of treatment \times time interaction: Exercise, $F(10,90) = 1.76, P > 0.05$; Control, $F(10,90) = 0.89, P > 0.05$; Figure 4E, F]. However, a significant increase in feeding was observed in both Restricted and ABA rats only on day 1 of treatment [two-way ANOVA: main effect of treatment \times time interaction Restricted $F(10,90) = 2.80, P < 0.05$; ABA $F(10,90) = 3.89, P < 0.05$] (Figure 4G, H). Compared with vehicle-treated rats, *post hoc* analysis revealed that only the dose of 0.5 mg·kg⁻¹ was effective in the restricted rats (+105%) and that both doses of THC effectively increased food intake in ABA rats (0.5 mg·kg⁻¹: +125%; 0.75 mg·kg⁻¹: +134%). THC treatment did not affect the RWA of Exercise rats according to two-way ANOVA of the treatment \times time interaction [$F(12,108) = 0.71, P > 0.05$] and treatment alone [$F(2,108) = 0.19, P > 0.05$; Figure 5A]. However, with regard to THC impact on RWA of ABA rats, two-way ANOVA

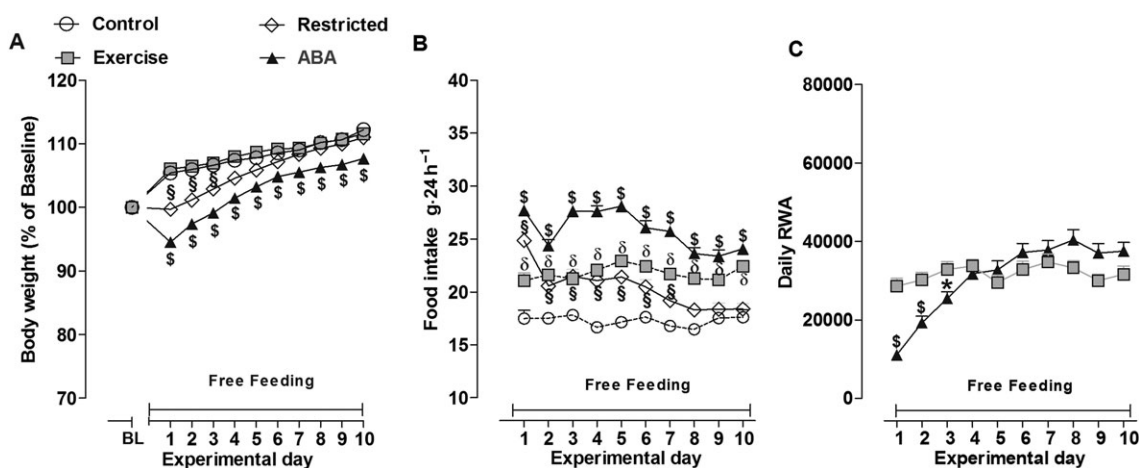


Figure 3

Body weight (A), food intake (B) and RWA (C) in Control, Exercise, Restricted and ABA groups during the 10 days of recovery from ABA. Results are presented as the mean \pm SEM ($n = 42$ rats per group). Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post hoc* test (Body weight: $\$P < 0.05$ vs Restricted, Exercise and Control rats, $\$P < 0.05$ vs Exercise and Control rats; food intake: $\$P < 0.05$ vs Restricted, Exercise and Control rats, $\$P < 0.05$ and $\delta P < 0.05$ vs Control rats; RWA: $\$P < 0.05$ vs Exercise rats).

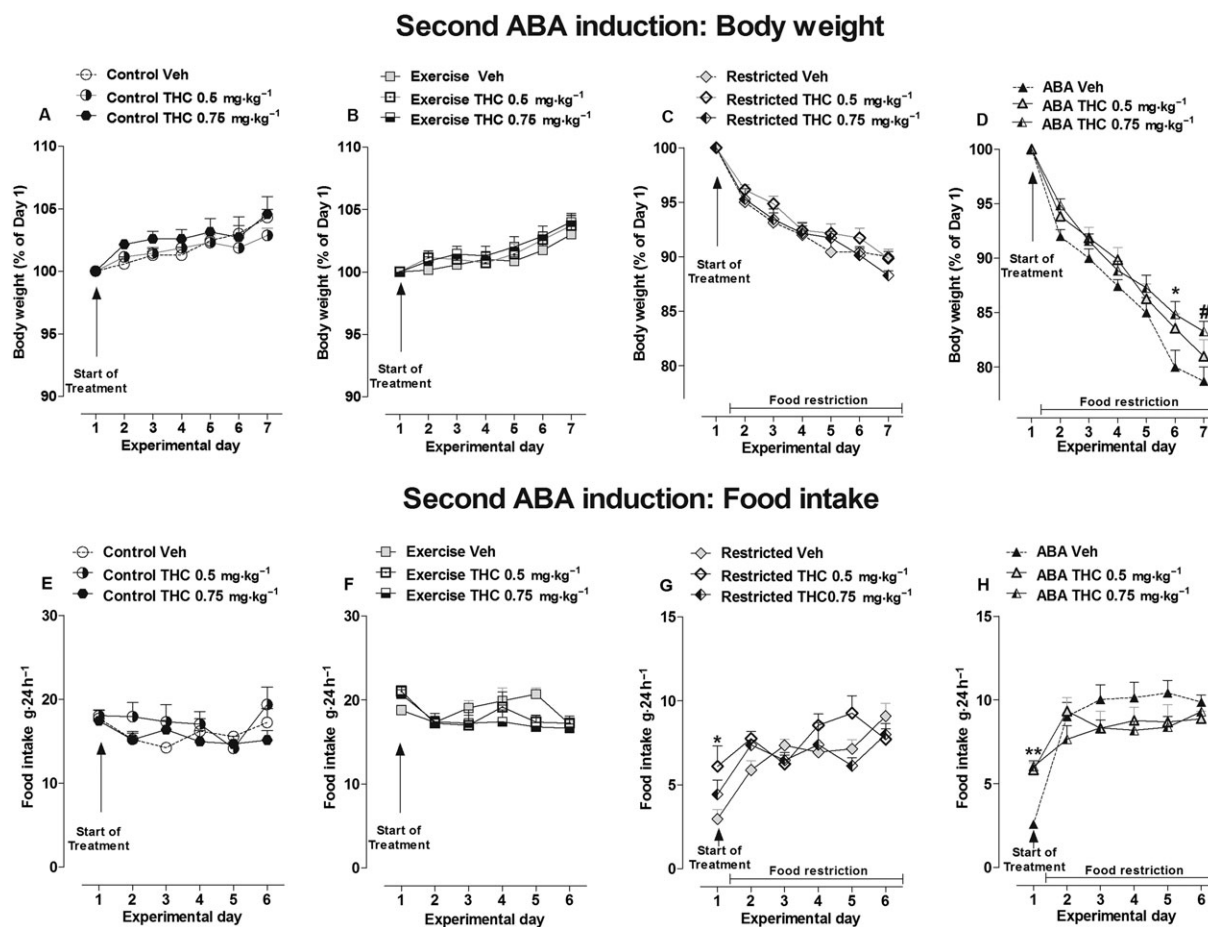


Figure 4

Effect of THC administration (0.5 and 0.75 mg·kg⁻¹) on body weight (A–C) and food intake (E–H) in Control, Exercise, Restricted and ABA groups during the 6 days of the second ABA induction. Results are presented as the mean ± SEM ($n = 7$ rats per dose). Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post hoc* test (body weight: * $P < 0.05$ vs vehicle; food intake: * $P < 0.05$ vs vehicle).

revealed a significant effect of treatment [$F(2,108) = 6.20$, $P < 0.05$] and time [$F(6,108) = 4.87$, $P < 0.05$], but the interaction between treatment and time did not have a significant effect [$F(12,108) = 1.41$, $P > 0.05$]. Subsequent individual one-way ANOVAs showed that both doses of THC effectively reduced RWA on days 2 [$F(2,18) = 8.40$, $P < 0.05$] and 3 [$F(2,18) = 7.470$, $P < 0.05$] compared with vehicle-treated rats, and 0.75 mg·kg⁻¹ of THC was also effective on days 4 [$F(2,18) = 4.695$, $P < 0.05$] and 5 [$F(2,18) = 3.529$, $P < 0.05$; Figure 5B].

Effect of CP administration. Over the CP (0.03 and 0.06 mg·kg⁻¹) treatment period, there were no significant differences in body weight between vehicle- and CP-treated rats in the Control, Exercise and Restricted groups [two-way ANOVA, main effect of treatment × time interaction: Control: $F(12,108) = 0.78$, $P > 0.05$; Exercise: $F(12,108) = 1.63$, $P > 0.05$; Restricted $F(12,108) = 1.01$, $P > 0.05$; Figure 6A–C]. In contrast, the body weight of ABA rats was modified. Two-way ANOVA showed that interaction between treatment and time had a significant effect [$F(12,108) = 2.15$, $P < 0.05$]; *post hoc* analysis showed that while only 0.06 mg·kg⁻¹ of CP prevented weight loss relative

to the vehicle on day 6 (–13.74 vs –17.46% respectively), both doses prevented weight loss on day 7 (vehicle: –21.11%, CP 0.03 mg·kg⁻¹: –17.17%, CP 0.06 mg·kg⁻¹: –14.68%; Figure 6D). Similar to THC, CP-treatment did not modify food intake in *ad libitum*-fed rats since two-way ANOVA did not detect any main effect of treatment × time interaction: Exercise: $F(10,90) = 1.28$, $P > 0.05$; Control: $F(10,90) = 1.72$, $P > 0.05$; (Figure 6E, F). On day 1 of treatment, both doses of CP affected food intake in Restricted rats by 103 and 107%, respectively, versus vehicle-treated rats [two-way ANOVA of treatment × day interaction: $F(10,90) = 1.96$, $P < 0.05$; Figure 6G]. At the same time point (day 1), both doses of CP also significantly increased food intake of ABA rats [two-way ANOVA treatment × time interaction: $F(10,90) = 2.82$, $P < 0.05$]. As shown in Figure 6H, *post hoc* analysis showed that CP increased food intake in ABA rats at both doses tested compared with vehicle (0.03 mg·kg⁻¹: +82%, $P < 0.05$; 0.06 mg·kg⁻¹: +88%, $P < 0.05$). Finally, no consequence of CP-treatment was found on the RWA of Exercise rats [two-way ANOVA of treatment × day interaction: $F(12,108) = 0.76$, $P > 0.05$; treatment: $F(2,108) = 0.31$, $P > 0.05$; Figure 7A]. However, CP

Second ABA induction: RWA

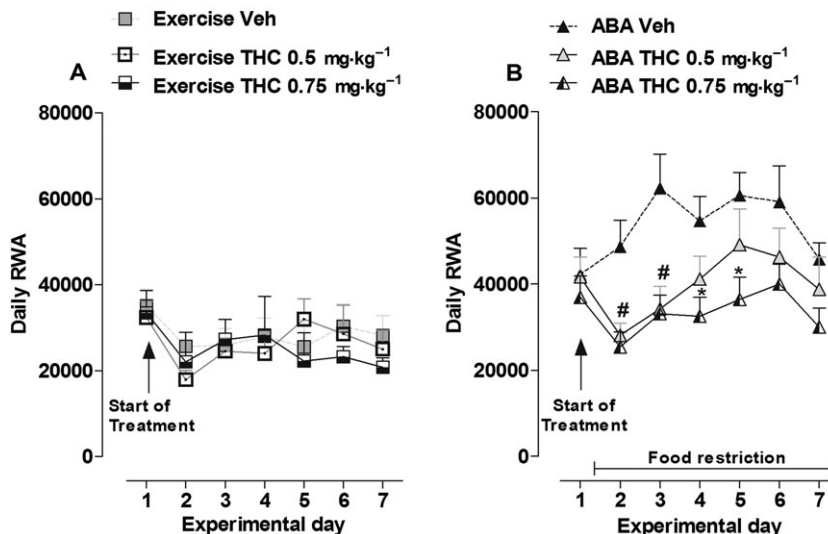
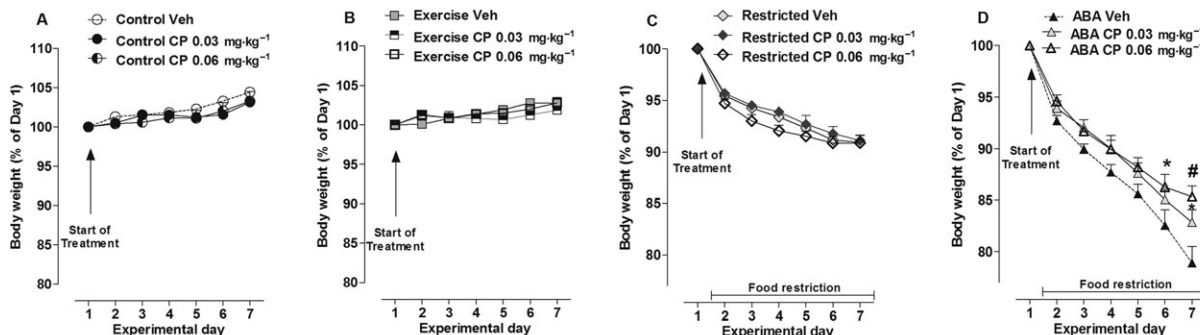


Figure 5

Effect of THC administration (0.5 and 0.75 mg·kg⁻¹) on RWA in Exercise (A) and ABA (B) rats during the 6 days of the second ABA induction. Results are presented as the mean ± SEM (n = 7 rats per dose). Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls post hoc test (*P < 0.05 vs vehicle).

Second ABA induction: Body weight



Second ABA induction: Food intake

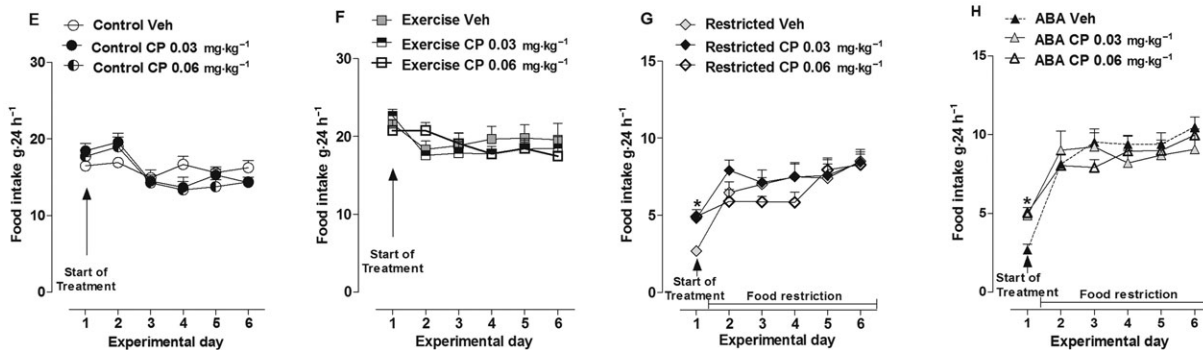


Figure 6

Effect of CP administration (0.03 and 0.06 mg·kg⁻¹) on body weight (A–C) and food intake (E–H) in Control, Exercise, Restricted and ABA groups during the 6 days of the second ABA induction. Results are presented as the mean ± SEM (n = 7 rats per dose). Statistical analysis was performed by two-way ANOVA followed by Bonferroni post hoc test (body weight: *P < 0.05 vs vehicle; food intake: *P < 0.05 vs vehicle).

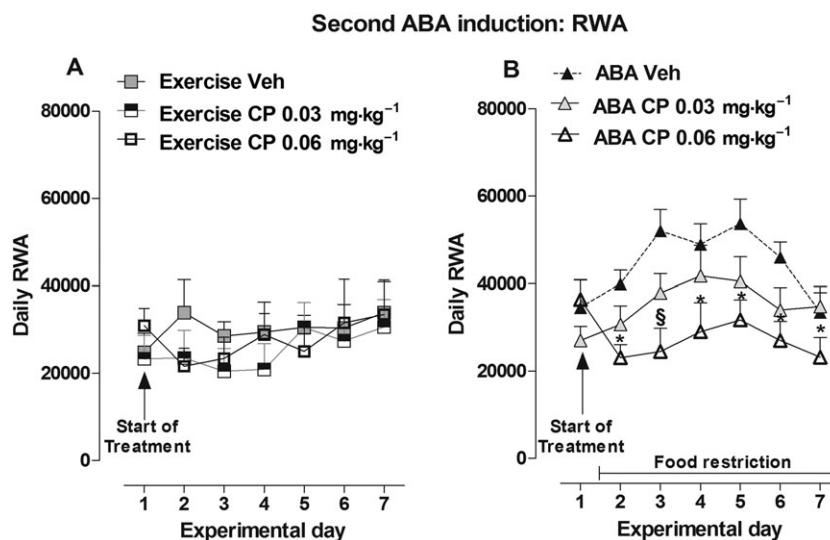


Figure 7

Effect of CP administration (0.03 and 0.06 mg·kg⁻¹) on RWA in Exercise (A) and ABA (B) rats during the 6 days of the second ABA induction. Results are presented as the mean ± SEM ($n = 7$ rats per dose). Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post hoc* test (*and § $P < 0.05$ vs vehicle).

treatment significantly affected RWA in ABA rats [two-way ANOVA, main effect of treatment × day interaction: $F(12,108) = 2.45$, $P < 0.05$; Figure 7B]. *Post hoc* analysis showed that the higher dose of CP effectively reduced RWA compared with vehicle-treated rats starting from the day 2 of treatment.

Plasma analysis of hormone levels

Effect of THC administration. As expected (de Rijke *et al.*, 2005), comparison of vehicle-treated rats of all four main groups showed significantly lower plasma leptin levels in ABA and Restricted rats with respect to Exercise and Control rats [one-way ANOVA: $F(3,24) = 124.1$, $P < 0.05$]. *Post hoc* analysis also showed leptin plasma levels in Exercise rats were significantly lower than Controls (Figure 8A). A similar effect was observed when animals were treated with THC at both doses [one-way ANOVA: THC 0.5 mg·kg⁻¹ $F(3,24) = 25.79$, $P < 0.05$; THC 0.75 mg·kg⁻¹ $F(3,24) = 31.33$, $P < 0.05$]. Moreover, intra-group analysis revealed that leptin plasma levels in ABA rats were significantly affected by THC treatment. Compared with vehicle, one-way ANOVA revealed a significant THC treatment effect between ABA subjects [$F(2,18) = 6.76$, $P < 0.05$], and *post hoc* analysis showed that THC 0.75 mg·kg⁻¹ produced a significant increase in leptin plasma levels. There were no significant differences in leptin plasma levels between vehicle- and THC-treated rats in the other main experimental groups [one-way ANOVA: Restricted, $F(2,18) = 1.80$, $P < 0.05$; Exercise: $F(2,18) = 0.25$, $P > 0.05$; Control: $F(2,18) = 1.69$, $P > 0.05$]. However, as already demonstrated (Boersma *et al.* 2016), corticosterone plasma levels were significantly increased in vehicle-treated ABA rats compared with the vehicle-treated rats of the other main experimental groups [one-way ANOVA: $F(3,24) = 4.201$, $P < 0.05$; *post hoc* analysis: $P < 0.05$;

Figure 8B]. Likewise, ABA rats showed higher corticosterone levels when treated with 0.5 mg·kg⁻¹ THC compared with the other main experimental groups [one-way ANOVA: $F(3,24) = 2.862$, $P < 0.05$; *post hoc* analysis: $P < 0.05$]. No significant statistical difference was detected between the main groups with 0.75 mg·kg⁻¹ of THC although there is a clear upward trend in ABA rats [one-way ANOVA: $F(3,24) = 1.141$, $P > 0.05$].

Effect of CP administration. Similar to THC, comparison of vehicle-treated rats of all four main experimental groups showed plasma leptin levels were significantly lower in ABA and Restricted rats versus Exercise and Control rats [one-way ANOVA: $F(3,24) = 49.02$, $P < 0.05$]. Additionally, leptin plasma levels in Exercise rats were significantly lower compared with Controls (Figure 8C). The same differences between main groups were also found with CP treatment by one-way ANOVA [CP0.03 mg·kg⁻¹: $F(3,24) = 50.15$, $P < 0.05$; CP0.06 mg·kg⁻¹: $F(3,24) = 40.25$, $P < 0.05$]. Intra-group analysis revealed that CP treatment increased plasma leptin levels in ABA rats which became significant when animals were treated with CP0.06 mg·kg⁻¹ versus the vehicle [one-way ANOVA: $F(2,18) = 4.141$, $P < 0.05$]. However, there were no significant differences in leptin plasma levels between the vehicle and CP-treated rats in the other main groups [one-way ANOVA: Restricted $F(2,18) = 2.881$, $P > 0.05$; Exercise: $F(2,18) = 1.181$, $P > 0.05$; Control: $F(2,1) = 2.22$, $P > 0.05$]. Moreover, vehicle-treated ABA rats had higher corticosterone plasma levels compared with vehicle-treated rats of the other main groups [one-way ANOVA: $F(2,18) = 6.984$, $P < 0.05$; Figure 8D]. Conversely, there were no significant differences in corticosterone plasma levels between groups when animals were treated with either dose of CP [one-way ANOVA: CP 0.03 mg·kg⁻¹, $F(3,24) = 0.5179$, $P > 0.05$; CP 0.06 mg·kg⁻¹,

Second ABA induction: Plasma hormone levels

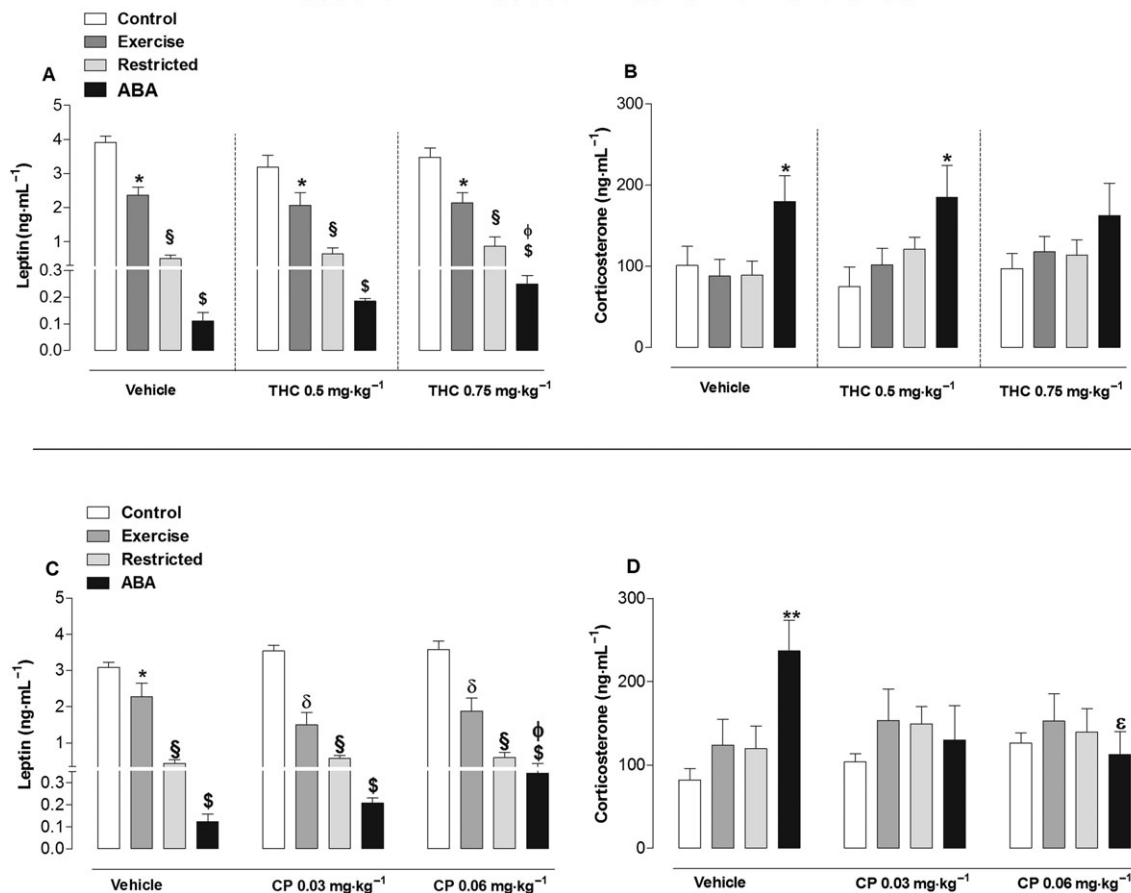


Figure 8

Effect of THC (0.5 and 0.75 mg·kg⁻¹) (A, B) and CP (0.03 and 0.06 mg·kg⁻¹) (C, D) administration on plasma leptin and corticosterone levels in Control, Exercise, Restricted and ABA groups. Results are presented as the mean ± SEM (*n* = 7 rats per dose). Statistical analysis was performed by one-way ANOVA followed by Newman–Keuls *post hoc* test (leptin: **P* < 0.05 vs Control, φ*P* < 0.05 vs vehicle; corticosterone: **P* < 0.05 vs Control, Exercise and Restricted rats; ε*P* < 0.05 vs vehicle).

F(3,24) = 0.0952, *P* > 0.05]. Accordingly, intra-group analysis of ABA rats treated with CP 0.06 mg·kg⁻¹ showed a significant effect compared with vehicle-treated ABA rats [one-way ANOVA: *F*(2,18) = 3.580, *P* < 0.05]. Intra-group analysis also revealed that there is no difference between vehicle- and CP-treated rats of the other experimental groups *via* one-way ANOVA [Restricted: *F*(2,18) = 0.347, *P* > 0.05; Exercise: *F*(2,18) = 0.8710, *P* = 0.4354; Control: *F*(2,18) = 3.40, *P* > 0.05].

Discussion

Using the ABA protocol, the present study evaluated whether positive pharmacological modulation of EC signalling could effectively modify weight loss, RWA and neuroendocrine changes in rats subjected to a repeated ABA regime (Chowdhury *et al.*, 2014; Aoki *et al.*, 2017). In agreement with previous reports, we confirmed that during ABA development (first ABA induction), rats subjected to a restricted feeding schedule in combination with free access to a running wheel presented a massive decline in body weight associated with a

progressive increase in RWA (Routtenberg and Kuznesof, 1967; Routtenberg, 1968). In contrast, control rats on a restricted feeding schedule without running wheel (Restricted rats) exhibited marginal weight loss compared with ABA rats. Moreover, control *ad libitum*-fed rats with continuous access to running wheel (Exercise rats) showed stable levels of RWA as well as an increase in food intake to compensate for their increased energy expenditure, compared with Control rats that did not have access to a running wheel and did not experience food restriction (Looy and Eikelboom, 1989; Scheurink *et al.*, 1999).

During the recovery period, during which restricted rats were allowed to feed *ad libitum*, ABA rats showed a transient decline in RWA and a hyperphagia that persisted throughout the entire recovery phase. Even though their body weight was restored to baseline within 4 days, ABA rats weighed less than those of the other main experimental groups at the end of the recovery period. It is important to note that during recovery, ABA rats had continuous access to running wheels so it is possible that exercise availability influenced body weight recovery (Dwyer and Boakes, 1997; Ratnovsky and Neuman, 2011). In agreement with this observation, women with AN

tend to maintain lower body mass indices than healthy women after recovery, and physical activity could be a possible contributing factor (Dellava *et al.*, 2011). Indeed, to get back to a healthy body weight, people with AN require even larger amounts of food than healthy controls, and physical activity could also contribute to this increased caloric requirement (Kaye *et al.*, 1988). Compared with ABA rats, weights of Restricted rats were restored to baseline levels within the first day of recovery, and their body weights were not statistically different from that of *ad libitum*-fed rats at the end of the recovery period.

After the refeeding period, we re-exposed the ABA rats to a second ABA protocol. Even though this fails to mimic a real relapse because there are no precipitating events that drive the animals into ABA (food restriction is imposed rather than voluntary), the double exposure to the ABA protocol might lead to a better understanding of the efficacy of pharmacological treatments on repeated ABA regimes and is reminiscent of the human condition, in which patients repeatedly undergo cycles of recovery and illness.

During the second ABA induction, we found that ABA rats treated with vehicle show a percentage of weight loss similar to that of the first induction, while RWA further increased. The excessive RWA of ABA model therefore mimics the physical hyperactivity that is frequently utilized by AN patients as a strategy to lose weight, and our results are in agreement to the notion that it appears to play a central role in the progression of the disorder (Gümmer *et al.*, 2015; Achamrah *et al.*, 2016). Physical hyperactivity also appears to be related to the higher relapse rates in AN following weight restoration and, thus, seems to be associated with a higher probability of chronic outcome of AN (Casper and Leslie, 1996; Strober *et al.*, 1997; Carter *et al.*, 2004).

Subchronic treatment with both the natural CB₁/CB₂ receptor partial agonist THC and the synthetic CB₁/CB₂ receptor agonist CP effectively prevented body weight loss in ABA compared with vehicle-treated rats. In contrast, neither THC nor CP had an effect on body weight of Restricted rats or *ad libitum*-fed rats, suggesting a specific effect on ABA rat body weight. Similar data were previously published by Verty *et al.* (2011), which showed the attenuating effect of THC on weight loss associated with ABA development in female rats. Thus, our results extend Verty's data confirming that THC is also effective after a repeated regime of ABA. In addition, we showed, for the first time, the efficacy of a full cannabinoid receptor agonist CP, in attenuating the body weight loss in ABA rats. Accordingly, a randomized controlled clinical trial showed that dronabinol (a synthetic form of THC) administration induced a small but significant weight gain in women with severe AN in the absence of adverse events (Andries *et al.*, 2014). In line with the orexigenic effects of cannabinoid agonists in humans and rodents (Williams and Kirkham, 1999; Hart *et al.*, 2002), both drugs increased food intake during the 1.5 h food access period in both ABA and Restricted rats, even if only on day 1 of the treatment that represented the start of ABA when the animals were no yet under the restricted feeding schedule. Again, Verty *et al.* (2011) also found that subchronic THC treatment significantly stimulated chow intake only on day 1 of treatment. Since neither THC nor CP modified chow consumption after the second day of treatment, we assume that food intake

during the restriction period was already at its highest; therefore, the CB₁/CB₂ receptor agonists were not able to increase it further. In addition, it is well-established that chronic cannabinoid administration can rapidly induce tolerance to behavioural and biochemical outcomes (Breivogel *et al.*, 1999; Maldonado and Rodríguez de Fonseca, 2002). As a consequence, it is possible that the lack of effect on food intake observed in our study may be interpreted as development of tolerance to the orexigenic properties of THC and CP following repeated administration (Järbe and DiPatrizio, 2005). However, neither drug influenced the 24 h food intake in *ad libitum*-fed rats, which is probably due to the fact that the 24 h interval between measurements was too long to observe an orexigenic effect.

In our study, both THC and CP were able to significantly reduce RWA in ABA rats without a tolerance effect. This suggests that attenuation of body weight loss in our ABA rats may be due to a decrease in physical activity. Notably, THC and CP treatments did not affect RWA in Exercise rats, suggesting this effect was specific for ABA rats rather than due to a general motor effect. As mentioned above, hyperactivity has been associated with higher relapse rates; therefore, reducing activity levels in AN patients might be crucial for therapeutic outcome (Kostrzewa *et al.*, 2013; Maestro *et al.*, 2014).

Herein, plasma analysis performed at the end of the second ABA induction showed, as also reported after a single exposure to the ABA protocol (Pardo *et al.*, 2010), that the ABA group of rats had very low levels of leptin compared with all other experimental groups.

Importantly, our results demonstrate that both THC and CP treatments were able to significantly increase plasma leptin levels compared with vehicle-treated ABA rats. To the best of our knowledge, this is the first report demonstrating the ability of cannabinoid agonist drugs to attenuate the effect of ABA induction on leptin levels. Leptin is a hormone mainly synthesized in adipocytes whose levels are highly correlated with body mass index and percent body fat (Heymsfield *et al.*, 1999; Cammisotto *et al.*, 2006). Indeed, serum leptin levels rise with increasing adiposity and drop as a result of loss of fat mass (Frederich *et al.*, 1995; Maffei *et al.*, 1995). Accordingly, low leptin levels are an endocrinological feature of acute AN (Hebebrand *et al.*, 1997). Different studies support a potential link between decreased leptin signalling and the presence of physical hyperactivity in AN patients and rats (Hebebrand *et al.*, 2003; Holtkamp *et al.*, 2003). Thus, the observed effects of THC and CP on RWA in ABA rats could be attributed to the capacity of both cannabinoids to increase leptin signalling. In agreement with this, leptin treatment has been shown to reduce hyperactivity in ABA rat models (Exner *et al.*, 2000; Hillebrand *et al.*, 2005). Moreover, Verhagen *et al.* (2011) showed that leptin reduces locomotor activity in ABA models by acting at the level of the ventral tegmental area, where CB₁ receptors are abundantly expressed as part of the mesolimbic reward system (Herkenham *et al.*, 1990). Plasma analysis of our animals shows a decrease in leptin levels also in Restricted and Exercise rats compared with *ad libitum*-fed rats without running wheel access. Thus, the two variables manipulated in the ABA model were able to alter leptin signalling when applied separately. In agreement with this result, leptin levels have been shown to decrease in response to starvation or exercise

in both humans and rats (Ahima *et al.*, 1996; Iwasa *et al.*, 2016). It is very important to underline that both THC and CP treatments are able to significantly modify the reduced plasma leptin level exclusively in ABA rats that are exposed concomitantly to food restriction and exercise and not in Restricted and Exercise rats in which each variable is applied independently. Moreover, our ABA rats showed higher corticosterone levels compared with the other main groups. As previously discussed, the ABA regimen used herein resulted in activation of the HPA axis, with a subsequent increase in plasma corticosterone levels (Burden *et al.*, 1993). As for leptin signalling, a link between higher HPA axis activation and increased RWA with food restriction has been postulated (Challet *et al.*, 1995; Duclos *et al.*, 2005). Likewise, wheel running induced by food restriction was shown to be absent in adrenalectomized, food-restricted rats but increased in a dose-related manner with corticosterone replacement (Duclos *et al.*, 2009). Moreover, excessive physical activity seems to reinforce HPA axis over activation, as part of a vicious cycle, in some patients with AN (Klein *et al.*, 2007). Our data show no significant effect on corticosterone levels of ABA rats after THC treatment even though with the higher dose we found a trend toward a decrease. On the contrary, we found that treatment with CP was able to significantly decrease corticosterone levels, suggesting that cannabinoid stimulation by CP may in part exert a moderating effect on RWA by reducing HPA axis activation. Accordingly, augmentation of the EC signalling has been shown to suppress HPA axis activity and reduce stress-induced elevations in corticosterone levels (Ganon-Elazar and Akirav, 2009). In addition, Andries *et al.* (2015) suggested that dronabinol treatment alleviates the increased HPA axis activity in women with severe and chronic AN. Moreover, both genetic disruption and pharmacological blockade of CB₁ receptor signalling resulted in an increase in HPA axis activity under basal conditions and following exposure to acute stress (Hill and Tasker, 2012). The significant effect of CP versus THC in reducing the corticosterone levels in ABA rats could be ascribed to the full cannabinoid receptor agonism of this synthetic compound leading to greater activation of CB₁ receptors (Castaneto *et al.*, 2014).

Despite the growing knowledge of AN neurobiology, therapeutic interventions are still lacking, and the incidence of relapse remains high. At present, no medication has been approved for the treatment of AN, and there is a clear need for new, more effective AN treatments in addition to standard psychotherapy. As already mentioned in the introduction, a promising therapy for AN may be based on the use of drugs that target the EC system, which appears dysregulated in AN patients with increased plasma levels of AEA (Monteleone *et al.*, 2005). The authors speculated that elevated AEA levels may represent an adaptive response aimed at counteracting their restrictive behaviour by elevating the drive to eat. However, the limitation of this work is that it is uncertain whether peripheral levels could reflect the ones found in the brain. However, it has been hypothesized that the EC system is hypoactive in anorexic conditions, which could explain the increased CB₁ receptor availability seen in both human patients and rats subject to the ABA protocol (Gérard *et al.*, 2011; Casteels *et al.*, 2014). To date, no direct evidence on endocannabinoid levels have been reported in animal models of AN. However, Hanus *et al.* (2003) reported decreased levels of 2-AG in the entire mouse brain, including

hypothalamic and hippocampal regions, upon 12 days of food restriction in mice. Our findings show that cannabinoid agonists were able to attenuate RWA and, consequently, reduce the body weight loss associated with ABA, probably by increasing leptin signalling and stabilizing HPA axis activity through the activation of the EC system. In contrast, Lewis and Brett (2010) reported that the CB₁ receptor antagonist AM251 did not restore body weight or food intake in the ABA model in C57/BL6 mice but rather decreased feeding and furthermore increased mortality at the highest dose. If analogous effects occur in humans, pharmacological manipulation of the EC system with cannabinoid agonists shows promise for treatment of AN, specifically in patients where physical hyperactivity plays a central role in the pathogenesis and progression of this disorder.

Acknowledgements

This work was funded by Ministero dell'Istruzione, dell'Università e della Ricerca (PRIN 2010), by 'Regione Autonoma della Sardegna, Assessorato alla Programmazione' grants for basic research (Legge Regionale 7/2007), by Fondazione Banco di Sardegna (Prot.U627.2013/Al.551MGB) and by the Department of Biomedical Sciences Project (RICDIP_2012_Fratta_01) at the University of Cagliari.

Author contributions

P.F. and W.F. conceived and designed this study. M.S., V.S., R.C. and M.F.B. performed the experiments. M.S., P.F., W.F. and P.U. were involved in the discussions of the data. M.S. and P.F. wrote the manuscript. M.S., W.F. and P.F. reviewed and edited this manuscript. All the authors read and approved this manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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