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Research report

Activity-based anorexia alters hypothalamic POMC and orexin populations in male rats

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ABSTRACT

The objective of this study was to investigate the orexin and POMC populations in the hypothalamic nuclei of male Wistar rats after the activity-based anorexia (ABA) procedure. Four groups were established based on food restriction and activity: activity (A), ABA, diet (D) and control (C). The ABA protocol consisted of free access to a running wheel for a period of 22 h and access to food for 1 h. When the animals in the ABA group reached the ABA criterion, were sacrificed, and their brains were collected and serially sectioned. The free-floating sections were processed for orexin and POMC immunostaining. The number of orexin A-ir cells in the perifornical-dorsomedial-hypothalamus continuum (PFD) and lateral hypothalamus (LH) and the number of POMC-ir cells in the arcuate nucleus (Arc) were estimated. Data on food intake, body weight and wheel turns were also analyzed. The ABA procedure caused a significant decrease in body weight along with a significant increase in activity. Moreover, at the end of the ABA procedure, the number of POMC-ir cells decreased in the Arc in the A group, and significantly more in the ABA group, and the number of orexin A-ir positive cells decreased in the LH in D and ABA groups. The differential decrease in POMC in the ABA group emphasizes the importance of the melanocortin system in the maintenance of ABA, but more research is needed to elucidate the involvement of this peptide in the mechanism that promotes and maintains anorexia nervosa and how increased activity may interact with all these processes.

1. Introduction

Experimental studies have found that rats subjected to food restriction and free access to a running wheel show an increase in activity coupled with a high mortality risk [6,15]. The critical characteristics of the procedure were established decades ago [24,44], and the procedure was later termed activity-based anorexia (ABA) [13].

Activity seems to play a crucial role in the development of ABA in rats [42,50], and a similar importance of physical activity has also been observed in humans suffering from anorexia nervosa (AN). Holtkamp et al. [28] found that food restriction contributes to increased exercise levels in patients with AN, and up to 80% of the anorexic population participate in excessive levels of exercise. These results suggest that there are relevant similarities between ABA in rodents and AN in humans, and ABA is widely accepted as an animal model of this human pathology [26].

In humans, the cause and maintenance of AN have been analyzed mainly from cognitive, emotional and social points of view, focusing on symptoms [25,32]. Although studying the neurobiological determinants in humans that predispose an individual to the onset and maintenance of AN is more complex, some studies have shown alterations in the balance between orexigenic and anorexigenic peptides that regulate food intake in patients suffering from AN. Increases in leptin, peptide YY (PYY) and α-melanocyte stimulating hormone (α-MSH), along with a significant

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decrease in ghrelin plasma levels have been described [19,20,49]. The role that these changes play in the onset and maintenance of the disorder and the mechanisms that trigger them are still unclear. In this regard, the ABA model allows us to address the effects that fasting and activity have on the neurohormonal system, which regulates energy metabolism. Several nuclei of the hypothalamus participate in this regulation. Signaling from the periphery about the energy status of the body reaches the proopiomelanocortin (POMC) and neuropeptide Y/Agouti-related peptide (NPY/AgRP) neurons in the arcuate nucleus (Arc). Second order neurons located in the perifornical area (PFA) and lateral (LH), dorsomedial (DMH) and paraventricular (PVH) nuclei of the hypothalamus receive anorexigenic signals throughout α-MSH, and/or orexigenic input throughout NPY and AgRP neuropeptides [34,38,46,45].

Studies carried out with female ABA model rats have characterized alterations in some neuropeptides involved in food intake regulation. Specifically, on day four of the ABA procedure, in the Arc, the expression of the anorexigenic peptide POMC decreases and the expression of NPY and AgRP increases. Moreover, in addition to the changes in the Arc, the expression of melanin-concentrating hormone (MCH) is selectively increased in the lateral hypothalamus in ABA animals [11]. Other studies of prenatally stressed female rats subjected to the ABA procedure in adulthood also reported changes in the mRNA expression of POMC on day 4 or 6 of the ABA procedure [5,27]. Moreover, works by Gutiérrez et al. [22] and Daimon and Hentges [9] have also shown the involvement of melanocortin system in the development of ABA. The former demonstrated that an increase in the temperature (from 21 to 32 ºC) when ABA rats lost 20% of body weight decreased hypothalamic melanocortin 4 receptor (MC4R) and POMC expression in ABA rats. The latter revealed that the increase of POMC mRNA observed during ABA procedure may be the cause of μ opioid receptor (MOR) increases and β-endorphin activation that promote ABA [9,22].

Considering the data above, it seems that, during the first 4–6 days of the development of ABA, an imbalance in the hypothalamic expression of some feeding regulatory neuropeptides may occur. However, the process of anorexia usually lasts more than 4 or 6 days, extending to at least one more week. During the ABA process, wheel activity increases, which leads to a significant decrease in the body weight of the animals. Therefore, while it is important to understand the alterations in the neuropeptides that are related to the control of food intake at the beginning of ABA, it is even more important to identify the alterations that occur in these neuropeptides at the end of the process when the severity of the pathology is greater, specifically at the critical point at which, if the animals are not removed from the experimental procedure, they would probably die.

Although some studies address the effects of the ABA process in its final period on different brain parameters [8,18,29], to our knowledge, there are no reports in which POMC or orexin changes in the brain during this final period of the ABA process are shown, in protocols in which food restriction and increase of exercise levels are implemented at the beginning of the procedure. For this reason, the aim of this work was to investigate the changes that occur in two neuropeptides involved in food intake regulation, POMC and orexin, when an animal has reached the criterion of AN as defined by ABA. POMC is expressed in the Arc, brain stem and pituitary, but anorexigenic effects are mainly produced through active peptide α-MSH from POMC neurons in the Arc [48]. Orexin is synthetized and released from neurons in the LH, that mainly regulate arousal, feeding and reward related behaviors. This peptide promotes food intake in response to NPY secreted by Arc neurons [45,46]. Alterations in the expression of these two peptides, POMC and orexin, have been reported in the development of ABA [5,11], and it is important to investigate whether the alterations that underlie the development of anorexia are maintained at the end of the process or, on the contrary, whether the expression of the neuropeptides changes. In this study, we use male rats to investigate alterations in orexin and POMC hypothalamic populations that are due exclusively to a restricted diet or to physical activity and those due to ABA. The prevalence of anorexia nervosa is higher in women than in men, but recent data point to an increase in the number of cases of AN in adolescent males [21]. Moreover, sex differences in the symptom presentation, neurocognitive inefficiencies or cardiac alterations have been shown [30,47]. For these reasons, it is also becoming relevant to understand the mechanisms underlying AN in males so as to advance in the knowledge of this disorder and to find out the specificities in each sex when they exist.

2. Methods

2.1. Animals

24 male Wistar rats (8 weeks old) (Charles River Laboratories, Lyon, France) were used. They were housed in groups of four for two weeks and were then individually housed beginning at the start of the behavioral procedure, with food (normal chow diet: 20% proteins, 5% fat, 55% carbohydrates, 3438 kcal/kg) and water freely available. The ambient conditions of the room were rigorously controlled, (temperature of 22 ± 2 °C; 55 ± 10% relative humidity; light–on from 8:00 to 20:00).

The rats were randomly divided into three groups of 8 subjects each: ABA group, which were exposed to the ABA procedure; D (diet) group, which had the same food restrictions as the ABA group; and C (control) group, which had free access to food. The rats in the D and C groups were paired with the rats in the ABA group according to their initial weights.

In an additional experiment, an activity (A) group with access to the running wheel as ABA group but with no food restriction was studied (n = 8). Animals of this group were paired in the initial weight with the animals of the other three groups.

All procedures were performed according to the European Union legislation (Council Directives 86/609/EEC and 2010/63/UE) and the Spanish Government Directive (R.D. 1201/2005) and approved by our Institutional Bioethical Committee (UNED, Madrid). Special care was taken to minimize animal suffering and to keep the number of animals used to the minimum necessary.

2.2. Apparatus

All subjects were housed in individual transparent Plexiglas chambers measuring 21x35x24 cm. The cages for the rats in the ABA and A groups had a hole on the left-hand side accessible by a manual flap to provide/prevent access to a running wheel. Each running wheel was 9 cm wide and had a diameter of 34 cm. All running wheels were equipped with a brake mechanism to stop them from rolling during feeding times. The number of completed laps was measured by equipment and software (MED-PC for Windows, MED Associates Inc., Georgia, VT, USA) placed in a separate room.

2.3. Behavioral procedure

All animals were weighed daily at the beginning of the feeding period (9:00–9:15). Food was available for 1 h (from 9:15 to 10:15) for the ABA and D groups. The C and A animals had *ad libitum* food access during their respective experiment. Unlike other ABA studies that have had the animals acclimate to the wheel before beginning food restriction, in the present study we have followed the same protocol as in our previous works, in which access to the wheel began at the same time as dietary restriction. In this way the differences between the subjects are not modulated by the adaptation period, which has been shown to increase activity when access to the wheel is prior to dietary restriction (e.g., [4]). All models produce interesting results of the ABA process, because they allow us to establish potential differences due to these details of the procedure. In this case, the protocol allows us to compare the results of the present work with those of our previous studies [7,40,41,50]. From the first day of the experiment, free access to a running wheel was provided to the ABA and A animals for a period of 22 h

(11:00–9:00 the next day) at the beginning of the experiment at the same time as food restriction protocol started in ABA and D groups. Food anticipatory activity (FAA) was measured during the two-hour period prior to the wheel stop, from 7 a.m. to 9 a.m. During the feeding period, the brake was activated for the A and ABA rats, and the manual flap was closed to prevent access to the running wheel. Individual food consumption, wheel turns, and body weight were measured daily. A rat was considered to reach the ABA criterion when its weight dropped below 75% of the free-feeding value for two consecutive days, a commonly accepted starvation criterion [12].

2.4. Tissue preparation

Each ABA group rat (and its respective paired D and C animals) was removed from the procedure when it reached the ABA criterion. All animals were maintained in their specified nutritional and/or activity conditions until they were sacrificed. Sacrifice days of the animals in the A group were paired with those of the animals in the previous experiment. Animals were deeply anesthetized with an overdose of tribromoethanol (1 ml/kg). Then, the animals were transcardially perfused with saline followed by 4% paraformaldehyde (PAF). Their brains were removed, stored in a freshly prepared PAF solution for two hours at 4 °C, and then stored in a 30% sucrose solution in PBS at 4 °C until they were examined. Then they were frozen on dry ice and serially sectioned along the coronal plane at a thickness of 40 μm. Serial sections were collected in four series, two of which were used in this study processed as free-floating sections for orexin A and POMC immunostaining.

2.5. Orexin A and POMC immunostaining

We used the protocols published elsewhere in which optimal dilutions and incubations times had been determined [14,43]. Briefly, the sections were incubated in PBS overnight. Endogenous peroxidase activity was blocked by incubation with H2O2 in 0.5% Triton X-100 in PBS for 30 min. After a brief wash in PBS, the sections were incubated in normal goat serum (diluted 1:5 in PBS; Vector, California, USA) for 30 min at room temperature. Then, the sections were incubated for 48 h at 4 °C in a rabbit anti-orexin A primary antibody (Calbiochem, San Diego, USA) or in a rabbit anti-POMC primary antibody (Phoenix Pharmaceuticals Inc., Burlingame, USA);1:2000 in both cases. This step was omitted in control sections. After several brief washes in PBS, the sections were incubated with biotinylated anti-rabbit IgG serum (Vector,1:200) for 90 min and then an avidin-peroxidase complex (Immunopure ABC Vector) for 60 min at room temperature. Finally, the presence of peroxidase activity was visualized with a solution containing 0.02 g/ml diaminobenzidine (DAB; Aldrich, Madrid, Spain) and 0.025% hydrogen peroxidase in Tris–HCl, pH 7.6. The sections were mounted on gelatin-coated slides, dehydrated in ethanol, washed in xylene and coverslipped with DPX (Surgipath Europe Ltd., Peterborough, UK).

2.6. Morphometrical analysis

Sections were positioned in the anteroposterior axis around bregma (−1,72 mm for the Arc; −2,28 for DMH, PF and LH) [39]. The number of orexin A-ir cells in the DMH, PF and LH and the number of POMC-ir cells in the anterior subdivisions of the Arc (the dorsal (ArcD), medial (ArcM), lateral (ArcL), subdivisions) were estimated. The person conducting the counting of cells was blinded about the condition of the animals. Briefly, a microphotograph (x20) of each section was acquired using a scanner (Nikon Collscope Eclipse Net-VSL, Tokyo, Japan) with a monitor (Digital Sight DS-L1, Tokyo, Japan). Hypothalamic POMC and orexin A-ir cell bodies were clearly visible and all cells expressing POMC or orexin A, were included in the counting, regardless the intensity of staining, and the number of orexin A-ir or POMC-ir cells in each section was estimated using the "cell counting" tool of the ImageJ (ImageJ bundled with 64-bit Java 1.8.0; National Institutes of Health, USA). The orexin-ir or POMC-ir cells included within the boundaries of the different nuclei studied were counted. Since the resulting count was the total cell number of one out of 4 series, the total number of orexinA-ir or POMC-ir cells counted was multiplied by four to estimate the entire cell population in each case [33], and a correction for counting split units was implemented [1].

2.7. Statistical analysis

For initial weight and weight at perfusion, one-way ANOVA was implemented, followed by Student-Newman-Keuls tests for post-hoc comparisons. The significance level was set at $p < 0.05$.

The evolution of body weight, food intake, activity in the wheel, and food anticipatory activity (FAA) (in the ABA and A groups), during the experimental procedure was analyzed using repeated-measures ANOVA with treatment as the within-subject factor, and body weight, food intake or running wheel turns as the between-subject factor. To determine the differences between the groups, one-way ANOVA was performed when appropriate. Post-hoc comparisons were carried out by Student-Newman-Keuls tests. The significance level was set at $p < 0.05$. Additionally, Pearson correlations between body weight and running wheel turns in ABA and A groups were implemented. The significance level was set at $p < 0.001$.

The number of orexin A-ir cells in the perifornical-dorsomedial hypothalamic nuclei (PFD) continuum and LH and of the number of POMC-ir cells in the anterior subdivisions or the arcuate nuclei described by Paxinos and Watson [39] (ArcD, ArcM, ArcL) in both hemispheres were estimated. The data were submitted to one-way ANOVA with the hemisphere as a factor to determine the potential differences between the right and left hemispheres. Once the effect of the hemisphere was discarded, the mean value of the two hemispheres was used for statistical analysis performed by one-way ANOVA followed by Student–Newman–Keuls tests when appropriate, and the significance level was $p < 0.05$.

3. Results

3.1. ABA procedure

Two animals (and their paired D and C controls) were excluded from the experiment after the behavioral procedure because one of them did not reach the ABA criterion after 30 days and the other reached it on day 26, which suggests a resistance to ABA acquisition. The rest of the animals in the ABA group reached the ABA criterion, according to the following distribution: one animal on day 11; one animal on day 13; one animal on day 14; 3 animals on day 15. To compare the obtained results and to follow previous studies of the ABA procedure [41], the food intake, body weight and wheel turns were analyzed until day 11, when the first ABA rat was removed from the procedure and the results of all of the animals could be analyzed.

3.2. Initial and final body weight

On initial body weight ($F3,22 = 0.263; p = 0.851$) no differences were observed at the beginning of the experiment. However, a main effect of group was detected on final body weight at sacrifice ($F3,22 = 36.562; p < 0.001$). Post-hoc analysis showed a decrease in body weight in the ABA, D and A groups compared to the C group. Moreover, the D and A groups are significantly heavier than the ABA group ($p < 0.05$ for all comparisons) (Fig. 1).

3.3. Body weight and food intake during ABA induction

A main effect of group was found ($F3,36 = 24; p < 0.024$). Post-hoc comparisons showed significant differences among the C and the other groups ($p < 0.05$ for all comparisons), with the C group rats being heavier than the rats from the other three groups beginning on day 1.

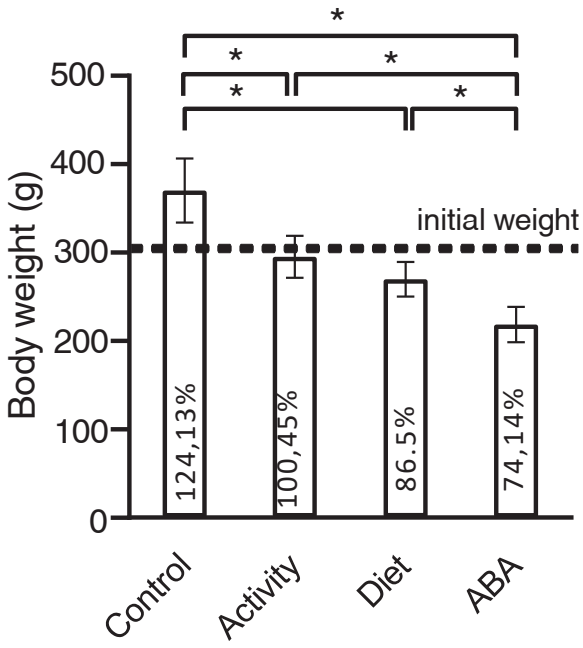

Fig. 1. This figure shows the differences in body weight (in grams and as a percentage) at sacrifice (One-way ANOVA). *: significant differences between groups ($p < 0.05$ in all cases). All values are expressed as means ± S.D.

From day 5 onwards, the ABA group showed significantly less body weight than the A group, and from day 8 onwards than the D group ($p < 0.05$ in all cases). Moreover, the A group rats were significantly heavier than the D group rats beginning on day 9 ($p < 0.05$ in all cases)

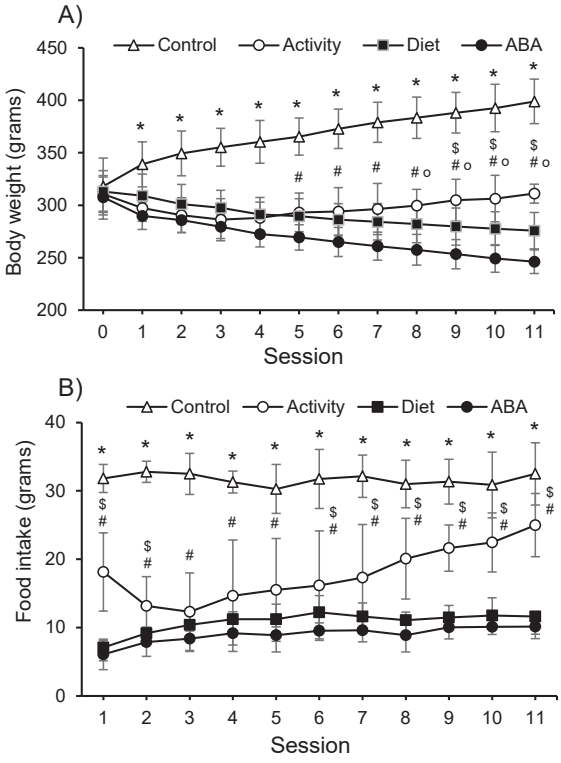

Fig. 2. A) Body weight evolution in all groups during ABA procedure. B) Food ingested in all groups during ABA procedure. Repeated measured ANOVA). Statistically significant differences ($p < 0.05$) are labeled as follows: * = C different from D, A and ABA; # = A different from ABA 0 = D different from ABA, $=A different from D. All values are expressed as means ± S.D.

(Fig. 2A).

Similar results were found for food intake, a main effect of group was detected ($F3,36 = 4.805; p < 0.024$), and post-hoc analyses showed that the C group rats ate significantly more than the D, A and ABA group rats beginning on day 1 ($p < 0.05$ in all cases). With respect to the A group, a significant difference between this and the ABA group was detected during the whole period with the ABA rats eating less ($p < 0.05$ in all cases). On the other hand, the A group ate significantly more than the D group during the whole period except from day 3–5 in which no differences were observed. From day 1 to day 11, no differences were detected between the D and ABA groups ($p < 0.05$ in all comparisons) (Fig. 2B).

3.3.1. *Food anticipatory activity and running wheel activity in the A and ABA group*

In the A and ABA group, activity was measured as the number of running wheel turns and was analyzed. Data showed an increase in the number of wheel turns ($F1,10 = 25.39; p < 0.001$) from days 1–11. No significant differences in activity were observed from day 1 to day 4. From day 5 onwards, the ABA group experimented a significant increase in the number of wheels turns with respect to the A group (Fig. 3A). An inverse correlation between body weight and number of wheel turns was detected in the first ($r = 0.159$; $p < 0001$) and the last day of the procedure ($r = 0.655; p < 0.001$) as can be seen in Fig. 3B and C, in which individuals measures of activity are represented.

Food anticipatory activity (FAA) has been analyzed in these two groups. As in activity, an increase in FAA has been detected from day 1–11 ($F1,10 = 49.54; p < 0.001$) (Fig. 4A). Significant differences between groups can be detected from day 6 onwards showing animals in the ABA group higher levels of FAA than animals in the A group ($p < 0.05$) in all comparisons (Fig. 4B).

3.4. *Orexin A and POMC cell numbers*

3.4.1. *Orexin A-ir*

No orexin A-ir cells were detected in the ventromedial or paraventricular hypothalamic nuclei. Orexin A-ir cells were observed in the medial–ventral area of the LH nucleus. Likewise, a small population of orexin A-ir cells was detected in the lateral edge of the dorsomedial hypothalamic nucleus (DMH) adjacent to the orexin A-ir cells in the PF, which is why the data are shown and were analyzed on the PFD continuum. In all nuclei studied, the cells that expressed orexin A were easily detectable because the cell body was heavily labeled, as can be seen in Fig. 5.

No differences between the hemispheres were found for the PFD continuum ($F1,39 = 0.34; p = 0.56$) or LH ($F1,39 = 0.218; p = 0.643$).

In the PFD continuum, no main effect of group was found ($F3,17 = 2.180; p = 0.128$), and no differences were detected in this parameter among the groups. However, in the LH, a main effect of group was found ($F3,17 = 9.845; p < 0.001$), with significant differences in the number of orexin A-ir cells among the groups. Post-hoc analyses detected a greater number of orexin A-ir cells in the C group than in the ABA and D groups ($p < 0.05$ in all cases). A significant difference was found between the A and ABA groups ($p < 0.05$), with ABA animals displaying a decrease in the number of orexin A-ir cells detected. Moreover, no differences were found in this parameter between the D and ABA or D and A groups (Fig. 6).

3.5. *POMC-ir*

POMC-ir cells were easily distinguishable because the cell body was heavily labeled (Fig. 5). Cells expressing POMC were detected in the ArcM, and ArcL, subdivisions of the Arc but not in the ArcD.

No differences between the hemispheres were found in the ArcM ($F1,39 = 0.50; p = 0.480$) or ArcL ($F1,39 = 0.033; p = 0.856$).

No main effect of group was detected in the ArcM ($F3,17 = 2.388$;

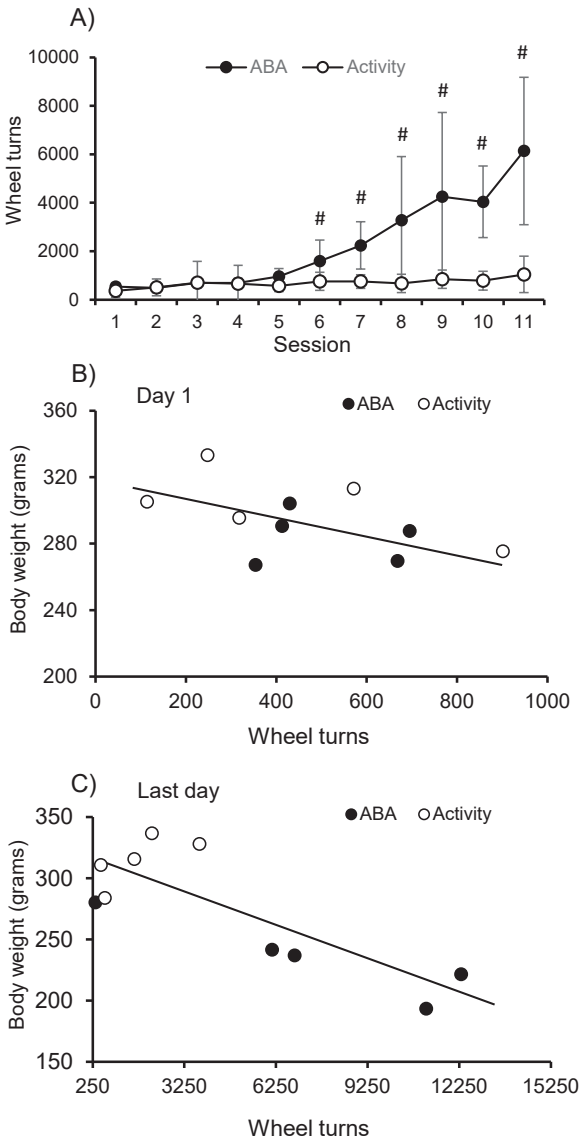

Fig. 3. A): Running wheel activity in the A and ABA groups measured as the number of running wheel turns during ABA procedure. (Repeated measured ANOVA). Statistically significant differences ($p < 0.05$). # = A different from ABA 0 = D different from ABA. All values are expressed as means ± S.D. B) representation of individual values on day 1; C) representation of individual values in the last day.

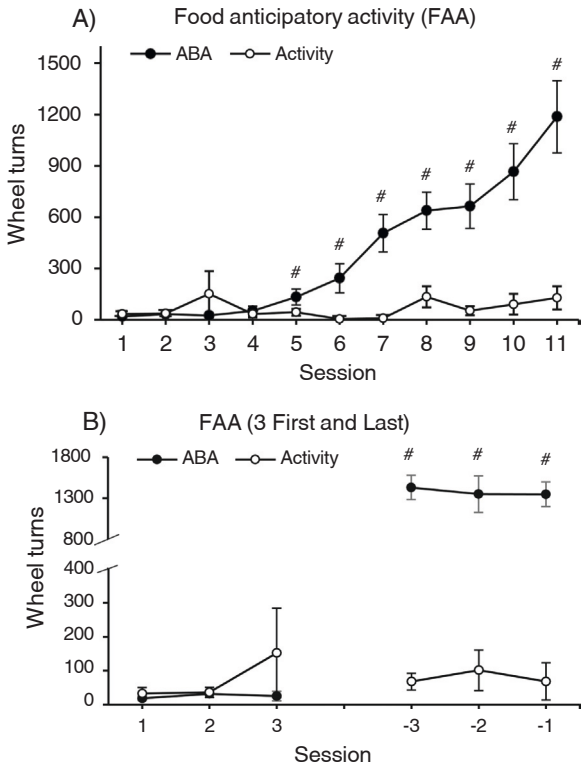

Fig. 4. Food anticipatory activity in the A and ABA groups measured as the number of running wheel turns during ABA experiment (Repeated measured ANOVA). Statistically significant differences ($p < 0.05$). # = A different from ABA 0 = D different from ABA. All values are expressed as means ± S.D. B) representation of individuals during the first 3 days; C) representation of individual values during the last 3 days.

$p = 0.105$). However, a main effect of group was found in the ArcL ($F3,17 = 11.061; p < 0.001$), with significant differences in the number of POMC-ir cells among the groups. Significant differences were detected among C and the two activity (A and ABA) groups ($p < 0.05$ in all cases) having C the greatest number of cells. Moreover, the D and A groups exhibited a greater number of POMC-ir cells than the ABA group due to a strong decrease in the number of POMC-ir neurons in this group ($p < 0.05$), No significant differences were found between the A and D groups (Fig. 7).

4. Discussion

In the ABA group, exposure to the ABA procedure resulted in a considerable loss of body weight that occurred quickly, with the first animal reaching 75% of its initial body weight for two consecutive days on day 11 and the final one reaching this criterion on day 15. The weight loss of the D and A animals was less pronounced and for the C animals, their weights gradually increased, as expected due to normal developmental changes. These results replicate those found in other ABA experiments [7]. The differences in the body weight of the C group and the rest of the groups, were significant on the first day of the experimental procedure and increased each day until the last day of the experiment, when weight loss and hyperactivity were evident in ABA group. It is important to note that the body weight decrease was significantly greater in the ABA group than in the A group from the fifth experimental day onwards and also greater than in the D group from the eighth experimental day onwards. This suggests that this period from 5 to 8 day of procedure represents a pivotal point in the development of AN in which changes in energy metabolism likely determine a progression towards a point of no return.

Regarding activity, in the ABA group an increase in wheel turns was observed throughout the experiment, and these data are in line with results found in other ABA experiments. Thus, the results of the present study agree with those of other studies and reinforce the use of the ABA model as an anorexia model [23,35,41,50]. The fact that the activity group without food restriction did not reach the activity level of the ABA group demonstrates that activity alone does not produce the onset of ABA.

The data showed that the A animals ate more than the ABA animals during the whole procedure, and this result is in line with studies that suggest that exercise can, as in our study, interfere with food intake initially due to an activity-induced satiation signal [42] or to taste-conditioned aversion produced by the nausea associated with intense exercise [36]. It is interesting to note that, beginning on day 7, food intake was almost maintained while body weight decreased and activity on the running wheel increased in the ABA group. This suggests that hyperactivity causes the observed body weight loss and that the regulatory activity of metabolic energy cannot respond to these changes,

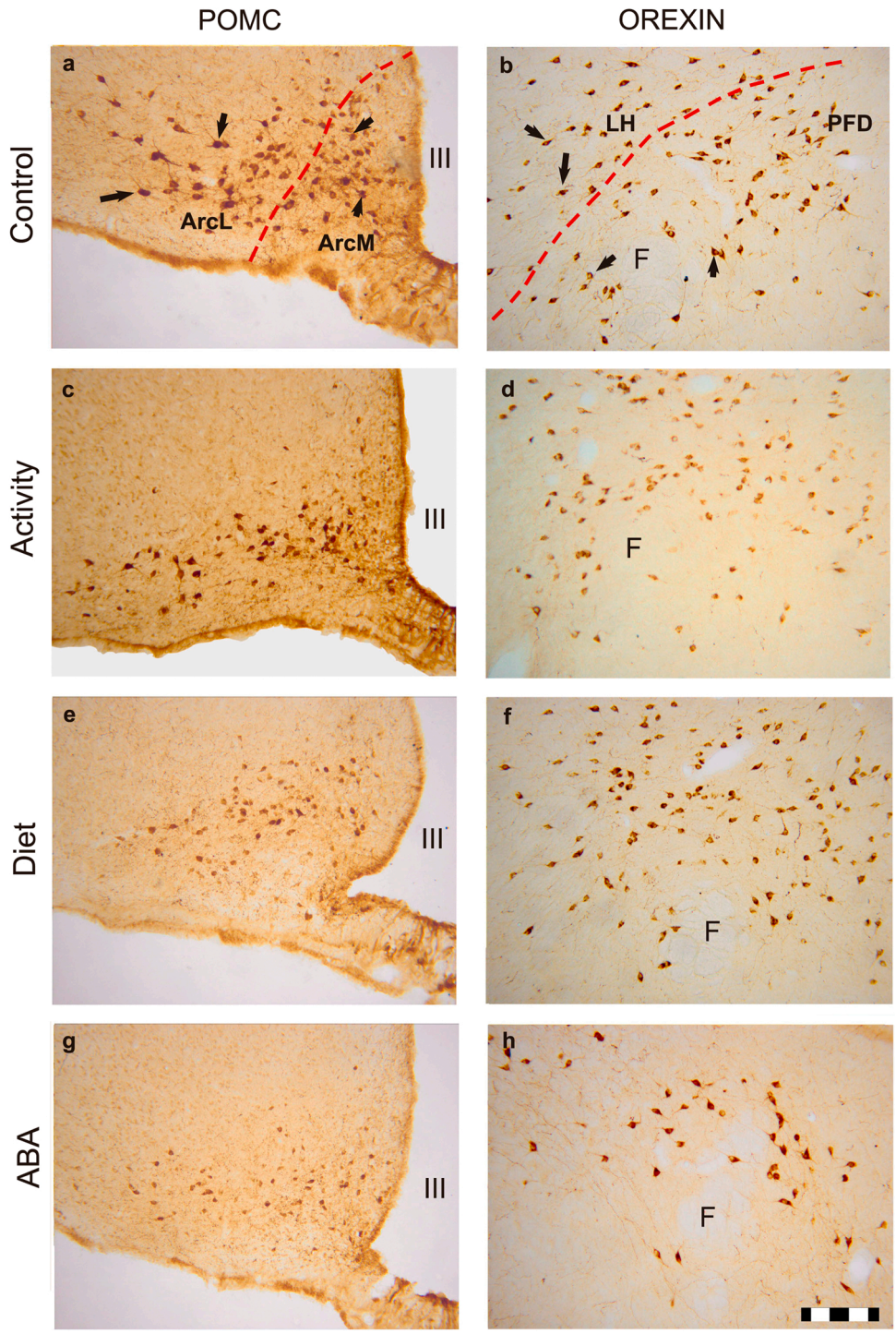

Fig. 5. Photomicrographs (x25) showing the distribution of immunostaining in orexin-ir positive cells in the LH and POMC-ir positive cells in Arc nucleus in the experimental groups. ArcL: arcuate nucleus, lateral subdivision; ArcM: arcuate nucleus, medial subdivision; F: fornix; LH: lateral nucleus of the hypothalamus; PFD: perifornical-dorsommedial hypothalamic nuclei (PFD) continuum. Black arrows shows an example of cell counted. Bar = 200 μm. [39].

therefore, the mechanism that triggers the increase in activity on the running wheel likely involves other systems. Some authors have reported that the dopamine system, which also seems to be altered in this procedure, may be responsible for the hyperactivity of these animals [3]. Considering all these data, the integrative action of both the homeostatic and reward systems in the onset and maintenance of ABA cannot be excluded.

The present results showed alterations in the number of orexin A and POMC cell in some of the hypothalamic nuclei studied differentially in the A, D and ABA rats. Activity significantly decreases POMC population in the ArcL and food restriction produces a significant reduction of orexin A cells in LH. But when these two requisites, activity and diet coincide in the same experimental condition a dramatic decrease in the number of POMC cells in ArcL and orexin in LH is observed and might be determinant to reach the ABA criterion. Regarding orexigenic peptides, diet restriction causes a decrease in the orexin A-ir cells just in the LH, which points to the specific involvement of this neuropeptide in the LH independent of the activity of the animals in situations in which energy

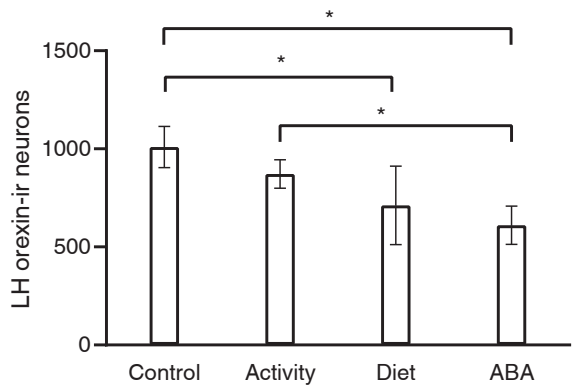

Fig. 6. Graph of the number of orexin-ir LH neurons in all experimental groups (Two-way ANOVA). * Indicates differences between groups ($p < 0.05$ in all cases). All values are expressed as means ± S.D.

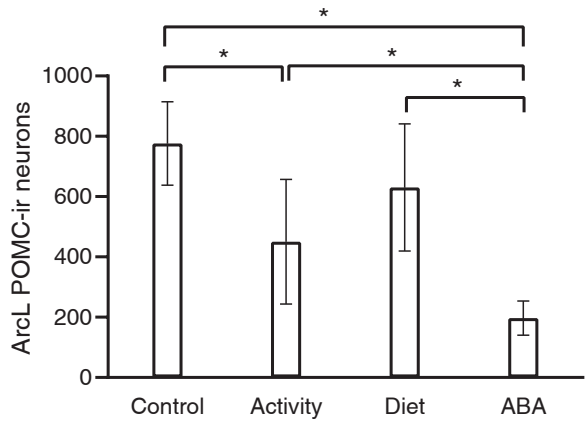

Fig. 7. Graph of the number of POMC-ir ArcL neurons in all experimental groups (Two-way ANOVA). * Indicates differences between groups ($p < 0.05$ in all cases). All values are expressed as means ± S.D.

metabolism is compromised. It is important to note that similar results have been reported when a severely restricted diet was implemented from gestational day 6 to postnatal day 60 [43]. This result is surprising, taking into account that when dietary restriction is implemented, orexigenic peptides decrease. The contrary is expected, as it has been reported on the day four of the ABA procedure by other authors [11]. However, since, in the present work, the orexin A population was measured at the end of the anorexia procedure, it may be that, as diet restriction is maintained as in the diet groups, or even intensifies, as in the ABA group, an adaptive mechanism similar to that which occurs during long-term fasting takes place [43].

While the orexin responds to the metabolic situation, POMC reacts to the activity levels of the animals. The most relevant result related to the onset and maintenance of AN is the differential decrease in the number of hypothalamic POMC-ir cells observed in the ABA group. The number of POMC-positive neurons in the ArcL of the diet group was similar to that in the control group and although the activity group showed a significant decrease in this parameter with respect to the control group, POMC population decreased significantly in this same hypothalamic structure in the ABA animals compared to the animals of the rest of the groups. This result is what is expected during fasting, in which satiety signals decrease as food intake is more necessary; however, no decrease in the number of POMC-ir cells was observed in the diet group, even though these animals were exposed to the same restricted diet as the ABA group. These data agree with those reported by other authors since the POMC mRNA levels decreased to baseline levels on the fourth day of the ABA procedure [27]. The fact that, at the end of the ABA procedure, the number of POMC-positive neurons decreased compared to that in the control group, even though the diet group did not show any decrease in this measure, and that the activity group decreased, but not to the same extent as the ABA group, may indicate an association between POMC and the maintenance of AN. Our study showed that the number of POMC-positive cells diminished specifically in the ArcL when AN was established, which points to a specific function of this subdivision and suggests that the melanocortin system may be a determinant of the onset and/or maintenance of AN.

With the procedure used in this experiment, it is not possible to confirm whether the decrease in the number of POMC-ir cells is a cause or an effect of ABA itself, but our results indicate a possible involvement of this peptide in the maintenance of AN, and are in addition to those reported by other authors showing that changes in POMC during the ABA procedure may contribute to the appearance and maintenance of some ABA characteristics [9,10,22]. There is evidence showing that hyperactivity is rewarding in ABA rats [2], and that some hypothalamic neuropeptides involved in food intake regulation are related to this aspect of AN. Specifically, an increase in ghrelin promotes food-anticipatory behavior, and a decrease in leptin signaling increases locomotor activity observed in ABA animals. The authors suggest that these changes can be modulated by dopamine neurons in the ventral tegmental area (VTA) [3]. On the other hand, and in relation to the POMC, it has been shown that the inhibition of POMC neurons by chemogenetic Designer Receptors Exclusively Activated by Designer Drugs (DREADD) produced a decrease in food anticipatory activity, although the rodents did not lose body weight or food intake [10]. Moreover, some reports have demonstrated in mice that the anorexigenic effect produced by D-fenfluramine through an increase of serotonin is modulated by POMC-expressing neurons [52], and an overlapping and synergistic function has been proposed among the dopamine rewarding system, prefrontal impulse control network and hypothalamic feeding circuit (see [31], and [51] for review).

It is important to highlight all of the interactions reported between neuropeptides involved in metabolic energy regulation and neurotransmitters involved in the reward processes. It has been recently shown that activation of the reward circuitry increases food intake and food anticipatory activity, preventing ABA-associated weight loss in rats [16]. Moreover, inhibition of the medial prefrontal cortex-nucleus accumbens shell pathway also prevents body weight loss in ABA rats pointing to the interaction between the reward and homeostasis systems in the development of ABA [37]. A synergistic activity between these networks, including the prefrontal impulse control circuit, may explain the mechanism underlying the onset and maintenance of AN since fasting, the reward of this fasting and the reinforcement of increased activity are the crucial symptoms of the disorder.

The present results emphasize the importance of the POMC system in the maintenance of ABA, and more research is needed to elucidate the involvement of this peptide in the mechanism that promotes and maintains ABA. A new approach to the ABA model has been recently reported by Frintrop et al. [17], proposing a chronic ABA protocol, which represents many somatic aspects of AN that are not manifested in the acute model used in our study, therefore, studying changes in feeding-related peptides in this new model would provide relevant information on the neurohormonal mechanisms involved in this disorder. Likewise, to know the humoral levels of hormones such as leptin or ghrelin would be of great interest. It is important to note that although AN is a disorder that occurs in a significantly higher proportion in adolescent females, it also occurs in males, and therefore it is also important to know the characteristics of ABA in males in order to have a better understanding of the factors involved in its onset and maintenance. The restriction of diet is the main symptom of AN, regardless of the motivation for fasting. It is crucial to determine the reason that diet can become so restricted as to endanger the energy balance of the organism, reaching irreversible damage, and how increased activity may interact with all these processes. Thus, understanding the alterations of

the neurohormonal system that regulate homeostasis at the beginning of and throughout the disorder is fundamental to designing appropriate therapies to complement the behavioral treatments that have been developed to treat this disorder.

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Data Availability

The data presented in this study are available on request from the corresponding author.

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Conflicts of interest

All authors listed have contributed to the work and all authors have agreed to submit the manuscript. All authors read and approved the final manuscript that I am now submitting, and no portion of the work has been or is currently under consideration for publication elsewhere. All experiments were designed according to the guidelines published in the "NIH Guide for the care and use of laboratory animals", the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience, the European Union legislation [Council Directives 86/609/EEC and 2010/63/UE] and the Spanish Government Directive [R.D. 1201/2005]. Experimental procedures were approved by our Institutional Bioethical Committee [UNED, Madrid]. Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum necessary. Moreover, no portion other than the abstract has been published or posted on the Internet. The corresponding author can provide all original data for review. The authors declare no conflict of interest.

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