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Original Article

Opioids in the medial nucleus of the solitary tract are not involved in feeding disorder in activity-based anorexia in rats

Eriko Ishihara*, Yoshio Sakurai

Laboratory of Neural Information, Graduate School of Brain Science, Doshisha University, Kyotanabe, Japan

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SUMMARY

Background & aims: Activity-based anorexia (ABA) is an abnormal behavior caused by mealtime restriction and excessive running. A previous study revealed that running causes the secretion of opioids in rats, and excessive opioids are known to decrease food intake. Thus, we hypothesized that the suppression of food intake in ABA rats that experienced mealtime restriction and excessive wheel running was due to an increase in opioids caused by running. Our previous study showed that ABA rats consumed more food than the control group after an intraperitoneal injection of naloxone. This result indicates that opioids are involved in the regulation of food intake in ABA rats. Here, we queried the involvement of the medial nucleus of the solitary tract (mNST) of the brain that mediates satiety information from the internal organs in rats' ABA and also examined whether opioids in the mNST were involved in feeding regulation in them.

Methods: Each ABA rat was given a running wheel and received feeding time restriction, followed by microinjection of naloxone or saline into the mNST. Each control rat was subjected to only feeding time restriction and then microinjection of naloxone or saline into the mNST.

Results: The results showed that food intake of the ABA group was not increased by naloxone injection into the mNST.

Conclusions: Our results suggest that the mNST is not involved in the regulation of food intake by opioids. Regulation of food intake

* Corresponding author.
E-mail address: dddb1002@mail4.doshisha.ac.jp (E. Ishihara).

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by opioids may occur in other parts of the brain, such as the parabrachial nucleus and/or hypothalamus.

1. Introduction

Activity-based anorexia (ABA) is an abnormal behavior in rats, similar to anorexia nervosa. When rats are placed in an environment where feeding time is limited and they can run on the running wheel during the rest of the feeding time, they tend to starve themselves by running excessively and eating very little [1]. It has been reported that animal ABA could be a model for human ABA, as seen in ballet dancers who restrict their diet and train excessively [1].

Changes in opioid secretion may be related to ABA. Running increases opioids in the rat's body [2]; the amount of beta-endorphin, a type of opioid, in the plasma and hypothalamus was found to increase with high or long-term exercise load in the rat.

In addition, the administration of excessive opioids is known to decrease food intake in rats. When morphine was injected intraperitoneally into rats after starvation for 24 h, the amount of food intake for 1 or 2 h after injection was less than that of the control group [3]. Moreover, food intake decreases in proportion to morphine dosage [4]. These studies used a standard diet, not a high-fat or high-reward diet. Therefore, these studies indicate that increased opioid activation decreases the food intake of a standard diet.

In contrast, antagonization of opioids also decreases food intake [5]; they injected naloxone, an opioid antagonist into rats after starvation periods and measured the amount of food intake of a standard diet. The result also showed a significant decrease in food intake in proportion to the dose of naloxone. This indicates that naloxone above a certain dose also suppresses the standard diet intake.

From the above cited studies [3–5], we suggest that opioids within the appropriate concentration ranges are necessary to maintain normal food intake. As the level of opioids increases, the amount of food intake also increases; however, after the proper opioid concentration range has been reached, the amount of food intake starts to decrease. Previously, starved rats showed small increases in food intake when administered 1 mg/kg of morphine [3]. We assumed that the food intake in rats starved only was near the maximal. On the other hand, the food intake in ABA rats decreased because their opioids levels increased by wheel running and exceeded the level that caused the maximal food intake.

In our previous study, we examined the hypothesis that a decrease in food intake in ABA rats was due to an excess of opioids released by running [6]. The rats were divided into two groups: the control group, in which rats were subjected to feeding-time restriction only, and the ABA group, in which rats were subjected to feeding-time restriction and running periods. The amount of food intake was measured after suppression of opioids in both groups by intraperitoneal injection of naloxone. The amount of food intake after naloxone injection in the ABA group was significantly higher than that in the control group. This suggests that the decrease in food intake in ABA rats was caused by excessive opioid secretion due to running, and suppression of excessive opioids could restore the decreased feeding. In this previous study, however, naloxone was injected intraperitoneally. At present, it is unclear whether the effects of naloxone are central or peripheral, and if central, which brain region is the primary site of action. Therefore, in this study, we examined whether naloxone acts in the brain.

The nucleus of the solitary tract (NST) is the primary central nervous nucleus that receives afferent input from the visceral organs of the digestive system via the vagus nerve [7]. There seems to be no previous study showing that opioids in the NST interact with satiety information from the internal organs. However, the NST has inhibitory circuits with feeding regulators such as Cholecystokinin (CCK) [8] and Peptide YY (PYY) [9], which convey satiety information from the internal organs, and the NST has opioid receptors [10]. Therefore, it may be possible that opioids in the NST modify the functions of feeding regulators and processing satiety information from the viscera is interrupted by increased

opioids in the NST. As this study was the first experiment in which naloxone was microinjected into the NST of ABA rats, we targeted the medial NST (mNST) for the microinjection. The reason is that the mNST has the largest coronal surface in the NST and is located slightly caudal it in the upper part of the medulla oblongata [11]. This was favorable for the reliable microinjection into the NST without damaging the medulla oblongata. In addition, there are more neurons in the mNST than the other regions of NST [12]. Therefore, this study did the microinjection of naloxone into the mNST and investigated whether such microinjection could alter the amount of food intake in ABA rats.

2. Materials and methods

2.1. Animals

Sixteen male Long-Evans hooded rats (Shimizu Laboratory Supplies, Japan) were individually housed under a 12-h/12-h light/dark cycle. They were randomly divided into two groups (ABA and control). Rats were approximately 11 weeks old and weighed 318–402 g at the start of the experiment and were allowed free access to water and food (MF, Oriental Yeast, Japan) until the start of the experiment. The day immediately prior to the experiment, we did not provide the rats with food. All procedures were performed following the Guidelines for Animal Experiments at Doshisha University and with the approval of the Doshisha University Animal Research Committee.

2.2. Surgery

Under 1%–3% isoflurane, a hole was drilled into the skull for implantation of the single guide cannula (26G, Plastic One, USA) into the mNST (-13.30 mm from the bregma and 0.6 mm from the midline). The depth of the guide cannula was 6.8 mm from the skull surface. The target region was based on an atlas [11]. The cannula was anchored to the skull using small steel screws and dental cement. A 33-gauge single internal cannula was inserted into the guide cannula to set its tip 1 mm below the end of the guide to reach the mNST. The guide cannula was covered with a single dummy cannula, except during drug administration. The length of the dummy cannula was the same as that of the guide cannula. After surgery, the rats were allowed at least 7 days to recover before experiments began.

2.3. Apparatus

The rats were housed for the ABA procedure in an operant chamber measuring 45 × 31 × 33 cm. The front wall had a lever (4.5 × 2 cm), 5.5 cm above the floor and 2 cm from the right wall. The LED light was located near the lever. At the start of the feeding time, the LED lit up, and only one food pellet was ejected into the food magazine. The LED turned off after the end of the feeding period. During the feeding time, when the lever was pressed, the food dispenser delivered a 25 mg food pellet to the food magazine. The food magazine was 2 cm above the floor and 1.5 cm from the lever. The front wall contained a water bottle. A hole (1 cm diameter) through which the rats could access the water bottle was located 11.5 cm above the floor and 5.5 mm from the left wall. Each operant chamber had a built-in wheel located opposite to the front wall, to which the rats had free access. The diameter of the wheel was 30 cm, and the width was 13 cm (Sanko, Japan). The wheel had a small hole that was drilled, and the metal rod was passed through the hole when the wheel was locked. The number of wheel rotations was recorded using a handmade counter. Each chamber was enclosed in a soundproof box (Brain Science Idea, Japan), and the temperature in the soundproof box was maintained at approximately 22 ± 2 °C. The task was controlled and behavioral data were recorded using a personal computer (NEC, Japan).

2.4. Procedure

Following a previous study [13], the ABA procedure for this study was designed with a 1-h feeding and non-runnable period and a runnable period of 22.5 h. This was done to observe the changes in food

intake and running separately. The remaining 30 min per day was used for cleaning the apparatus and weighing the rats. Water was available at all times. The details of the procedure are shown in Fig. 1.

The first two days were for the Free feeding & Locked wheel period (Fig. 1A). The rats were maintained in the operant chamber for 23.5 h for training and were allowed to press the lever to eat pellets at any time during this period. The wheels were locked during this period. The rats were then returned to their home cages for 30 min.

The next 6 days were for the ABA procedure period (Fig. 1A). The first day of this procedure is defined as “Day 1”. The rats were placed in the operant chambers for 1 h during the feeding period. After the feeding period, the rats were returned to their home cages for 30 min. Subsequently, the rats were placed in the operant chambers for a runnable period of 22.5 h. Feeding was not performed on Day 1, because the period just before the restricted feeding period (Day 0) was the free feeding period that strongly influenced feeding on Day 1. Therefore, the restricted feeding period started on “Day 2”, the second day of the ABA procedure.

The last two days (Days 7 and 8) were for the microinjection (Fig. 1A). Just before feeding time, rats were microinjected with naloxone before the last day and with saline on the last day. Other procedures are the same as for the ABA period.

2.5. Pharmacological treatment

To observe the effect of opioid suppression on feeding, we injected rats with naloxone hydrochloride before feeding on Day 7 and saline as a control on Day 8. Naloxone hydrochloride was dissolved at 2.0 μg in 0.1 μl of saline solution. The dosage of naloxone was based on a previous study [14]. In a previous study, naloxone was injected intraperitoneally, causing withdrawal symptoms in rats with ABA [15]. Each solution was contained in a microliter syringe (Hamilton, USA) with a polyethylene tube (Plastic One, USA) and an internal cannula. One minute after the cannula was set, the entire

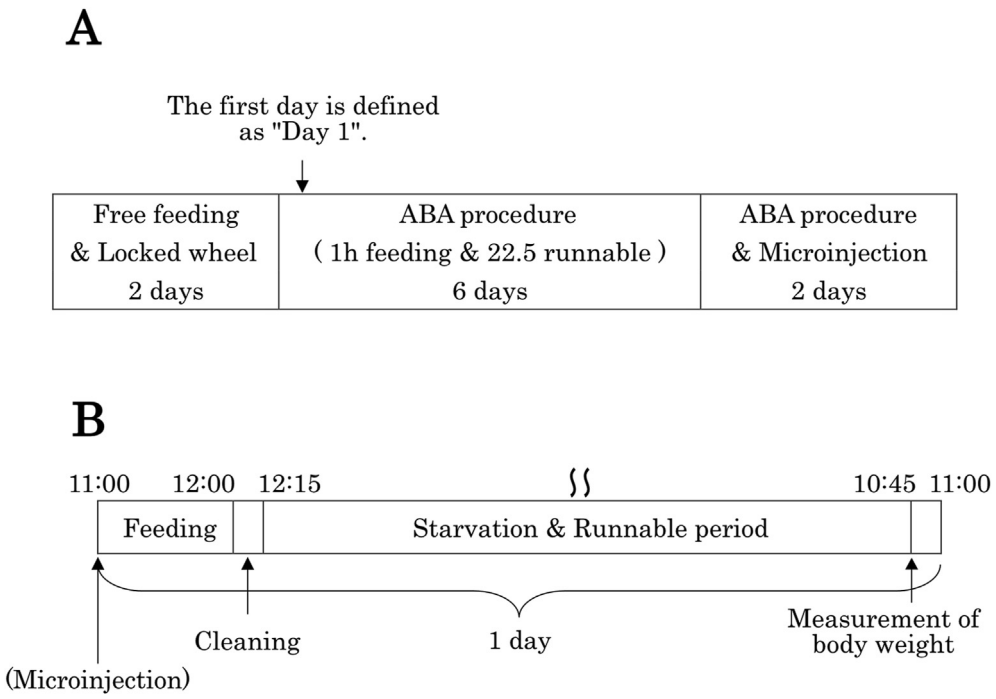

Fig. 1. Procedure for ABA and control groups. (**A**) Outline of experiment. (**B**) Details of ABA procedure (1 day).

volume (0.1 μl) was injected over 1 min. An automatic syringe pump (Fusion Touch 100, ISIS, Japan) controlled the injections. The solution was injected into the rats while they were awake. One minute after the injection, the rats were placed in the operant chambers.

2.6. Histology

After the experiment was completed, the rats were deeply anesthetized with sodium pentobarbital and perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde. The brains were removed and post-fixed in 4% paraformaldehyde, and 50-μm coronal sections of the brains were stained with 4′, 6-diamidino-2-phenylindole DAPI (Nacalai Tesque, Japan) diluted in PBS (1 μg/ml) for 5 min. After the excess DAPI was removed, the sections were mounted in 50% glycerol in PBS. The locations of cannulas in the brain were identified with the aid of a stereotaxic atlas [11].

2.7. Statistical analysis

Data were analyzed using SPSS26 software. Body weight and food intake were calculated with Day 0 set at 100%. A one factor analysis of variance (ANOVA) and Bonferroni's post-hoc analysis were used to compare differences in wheel running. A two factor ANOVA was used to compare differences in body weight. The analysis was performed on the last three days of the experiment because the rats in the ABA group were sufficiently affected at the end of the procedure. A two factor ANOVA was also performed to compare the differences in food intake, but the analysis was conducted for 2 days of microinjection.

3. Results

Our histological examination confirmed that the cannulas were inserted into the targeted area in 16 rats. Fig. 2 shows the locations of the tips of the cannulas for all the rats.

3.1. Wheel running

The wheel-running data measured after the first starvation were recorded as the data for Day 1. The results of naloxone HCl and saline injection were recorded as the data for Day 7 and Day 8, respectively.

Fig. 3A shows the mean wheel running (km) per day for the ABA group. A one factor ANOVA revealed a significant effect of day (Greenhouse–Geisser corrected: $F=9.786$, $df=2.457$, $P=0.001$). The wheel running gradually increased, except on the last day, but Bonferroni's post-hoc analysis indicated that the wheel running volume on Day 8 was significantly larger than that on Day 1 ($p < 0.05$).

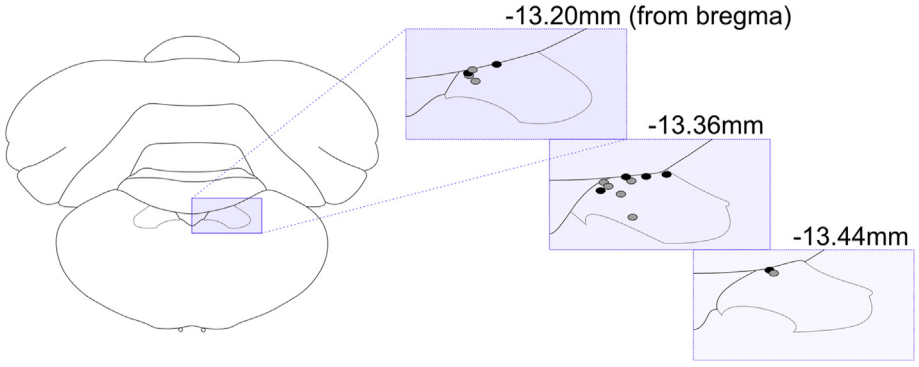

Fig. 2. Cannula tips in the NST. Gray circles indicate injection sites for the ABA group, and black circles indicate injection sites for the control group.

3.2. Body weight

The body weight data measured just before the start of starvation (weights immediately after free feeding) were labeled as the data for Day 0. The results of naloxone HCl injection and saline injection were labeled as the data for Day 7 and Day 8, respectively. The average weight in Day 0 was 381.44 g (SD = 14.58 g) for the ABA group and 386.71 g (SD = 16.91 g) for the control group. The t-test indicated no significant difference between the two groups (t[14] = -0.669, ns).

Fig. 3B shows the mean body weights as percentages for the ABA and control groups. Body weight on Day 0 was set as the base of 100%. Both the ABA group and the control group decreased their body

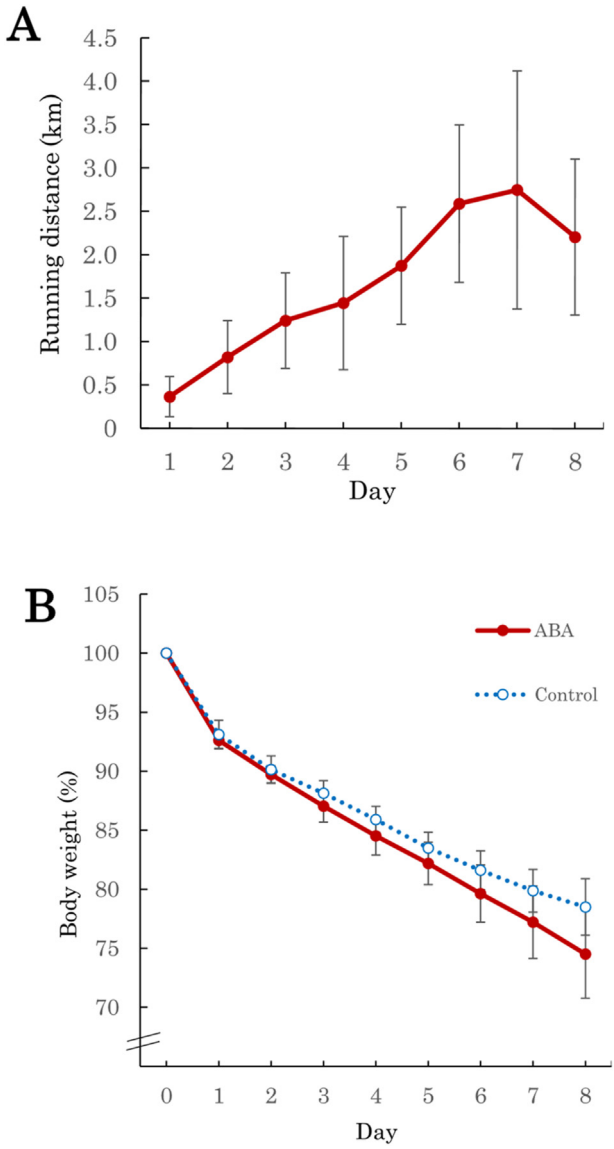

Fig. 3. (**A**) Mean running distance from Days 1 to 8. (**B**) Mean percentages of body weight from Days 0 to 8. Percentages were calculated by dividing the body weight of each day by the body weight of Day 0. Error bars indicate standard errors of the mean.

weight. However, the decrease of body weight of the control group was slower than that of the ABA group. To confirm whether the rats became ABA by the end of the day, a two factor ANOVA was performed for the body weights for the last 3 days of the experiment. The ANOVA revealed a significant main effect of day (Greenhouse–Geisser corrected: F=119.129, df=1.274, P=0). Although the main effect of group was not significant (Greenhouse–Geisser corrected: F=4.152, df=1, P=.061), the interaction between day and group was significant (Greenhouse–Geisser corrected: F=7.425, df=1.274, P=0.01).

3.3. Food intake

The amount of food intake just before the starvation period was labeled as the data for Day 0. The results of naloxone HCl injection and saline injection were labeled as the data for Day 7 and Day 8, respectively. There was no feeding on the first day in either group (first starvation period). The average food intake in Day 1 was 28.42 g (SD = 4.84 g) for the ABA group and 29.36 g (SD = 6.95 g) for the control group.

Fig. 4 shows the mean food intake percentages. The data for Day 0 were set at a base of 100%. There was no difference in food intake between the control group and the ABA group. The t-test showed no statistically significant difference between the two groups (t [14] = -0.317, ns). A two factor ANOVA, performed on the amount of food intake for the last 2 days (the days of the microinjection), revealed no significant main effect of day (F [1,14] = 0.187, ns), group (F [1,14] = 0.026, ns), or the interaction of day and group (F [1,14] = 0.155, ns).

4. Discussion

The purpose of this experiment was to investigate whether mNST altered food intake in ABA rats. To determine the rats in the ABA group will develop ABA, we first examine their running and body weights. The volume of wheel running in the ABA group significantly increased during the Days (Fig. 3A). In terms of body weight in Days 6, 7, and 8, the ABA group lost more weight than the control group as the days went by (Fig. 3B). These indicate that the rats in the ABA group became sufficiently ABA according to the present procedure.

The age of the rats could affect the results of this type of ABA experiment. In our preliminary experiment, where we used 8-week-old rats in the ABA procedure, weight losses were too rapid and

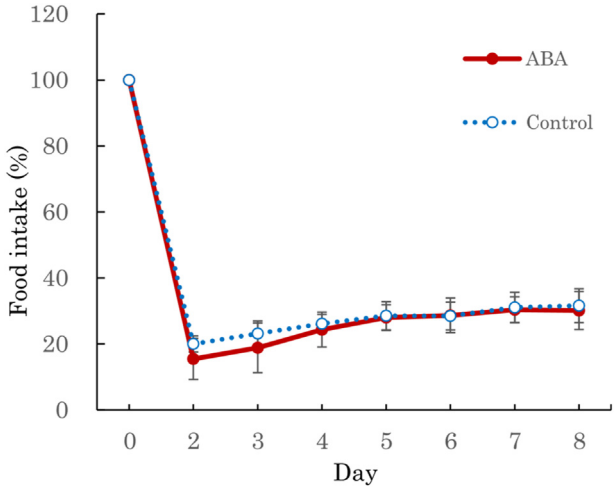

Fig. 4. Mean percentages of food intake from Day 0 to Day 8 except Day 1 (see Materials and Methods). Percentages were calculated by dividing the amount of each day by the amount of Day 0. Errors bars indicate standard errors of the mean.

very unsuitable for the total schedule of 10 days with the administration of naloxone and saline (Fig. 1). Therefore, we used 11-week-old rats in the present study to slowly decrease their body weight and to standardize the injection date for all rats.

Regarding food intake, there was no significant difference between the ABA and control groups in Days 7 and 8. This indicates that the microinjection of naloxone into the mNST did not change the food intake of the ABA group. In general, exercise should increase food intake. In spite of that, the present result showed that the amount of food intake in the ABA rats did not exceed that in the control group, meaning that the method for ABA model rats suppressed increment of their food intake induced by exercise. The result also showed that the food intake did not change after the microinjection of naloxone. If the naloxone injection into the mNST were effective, food intake in the ABA rats should have been greater than that in the control group. Therefore, we conclude that opioids in the mNST are not related to feeding disorder (suppression) in the ABA rats.

In this experiment, only one dose of naloxone (2.0μg in 0.1μl saline) was administered. This dose was selected based on: (1) In our former study [6], ABA was affected by intraperitoneal injection of naloxone at concentrations of 0.01 mg/kg and 0.1 mg/kg. (2) In our preliminary experiment, we injected 0.0364 pg of naloxone dissolved in 0.5 μl of saline into the mNST, referring to the study of the blood-brain barrier transport of naloxone [16], but this did not affect ABA. (3) Therefore, we adopted the present dose, which could affect the NST and was not too antagonistic [14]. This dose is thought to be maximum, and injection of a higher dose of naloxone to ABA rats might trigger withdrawal symptoms [15], which is related to the NST [17].

Previous studies have shown that increasing or antagonizing opioids suppresses food intake [5]. Therefore, in this study, naloxone was injected into the mNST of ABA rats to examine which brain regions are involved in opioid-induced feeding suppression in ABA rats. However, injection into the mNST had no effect, while intraperitoneal injection in our previous study did affect ABA [6]. However, it is not clear how the intraperitoneally injected naloxone was effective. There are two main pathways of satiety signaling from internal organs to the brain: (1) Substances secreted from the digestive organs are transported to the hypothalamus by the bloodstream. (2) Substances are received by the vagus nerve and transmitted to the hypothalamus through the NST via the parabrachial nucleus (PBN). The results of this study indicate that mNST is not related to the regulation of feeding by opioids. To determine which route is involved in feeding regulation by opioids, it will be necessary to examine the hypothalamus and PBN, the projection site of the NST.

According to relation between opioids in the NST and running, there is no previous research that confirmed such relation. However, besides regulating eating, the NST is involved in respiration and heart rate [18]. In addition, a previous study have shown that another substance in the NST, oxytocin, plays a role in suppressing exercise-induced tachycardia [19]. Therefore, there seems to be a possibility that the NST is related to running by playing a role in regulation of breathing and heart rate, though future studies are needed to examine the relationship between opioids in the NST and running.

In humans, athletes are at a higher risk of anorexia than non-athletes, and the risk of anorexia increases in professional athletes who are required to follow stricter dieting and exercise regimens [20,21]. Thus, investigating the biological and neural mechanisms of ABA in relation to opioids will lead to a better understanding of anorexia in humans, especially in athletes.

Ethics statement

The animal study was reviewed and approved by Animal Ethics Committee of Doshisha University.

Statement of authorship

E.I. performed the experiment and analyzed the data. E.I. and Y.S. designed the experiment and wrote the manuscript.

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Data availability statement

Data is available on request.

Declaration of competing interest

We have no conflict of interest.

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