Doctoral Dissertation

The role of the nucleus of the solitary tract in rats with activity-based anorexia

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Abstract

Activity-based anorexia (ABA) is an abnormal behavior caused by mealtime restrictions and excessive running. A previous study revealed that running results in the secretion of opioids in rats, and excessive opioids are known to decrease food intake. Thus, it is hypothesized that the suppression of food intake in rats with ABA, which experienced mealtime restriction and excessive wheel running, is due to an increase in opioids caused by running. Our previous study showed that ABA rats consumed more food than those in the control group after an intraperitoneal injection of naloxone, suggesting that opioids are involved in the regulation of food intake in ABA rats.

Here, the first experiment investigated whether opioids received in the medial nucleus of the solitary tract (mNST) of the brain, which mediates satiety signals from the digestive organs, were involved in the regulation of eating in ABA rats. For these objectives, each ABA rat was given a running wheel and received a feeding time restriction, followed by the microinjection of naloxone or saline into the mNST. Each control rat was subjected to feeding time restriction only, followed by the microinjection of naloxone or saline into the mNST. The results showed that food intake in the ABA group was not increased by naloxone injection into the mNST, indicating that it is unlikely that the mNST is involved in regulating food intake by

opioids. The regulation of food intake by opioids may be underlain by other parts of the brain, such as the parabrachial nucleus and/or hypothalamus.

The second experiment investigated whether the mNST was involved in the neural mechanism of feeding in ABA rats. The mNST of each rat was lesioned with direct current by an electrode, and the ABA procedure was the same as that in the first experiment. The results showed that the body weights of ABA rats did not differ from those in the control group, indicating that ABA rats with the mNST lesions did not develop ABA. The amount of food intake in the ABA rats did not differ from that in the control group, as in the first experiment. However, the increase in the running distance, observed in the first experiment, was not significant, indicating a difference in ABA progression from that in the first experiment. These results indicate that the mNST is related not to the regulation of food intake but rather to the regulation of running in ABA rats. Because some previous studies have shown that the NST regulates respiration and exercise tachycardia, the NST may be involved in increase in the amount of exercise performed by ABA rats.

Acknowledgments

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Chapter 1. General introduction

1.1. Activity-based anorexia

Activity-based anorexia (ABA) is abnormal behavior in rats, similar to anorexia nervosa (AN). ABA was first reported by Routtenberg in 1967 [1]. When rats were placed in an environment where feeding time was limited and they could run on the running wheel during the remainder of the feeding time, they tended to starve by running excessively and eating very little. Compared with the ABA rats that lost their weights and sometime finally died, a previous study found that rats subjected to food restriction alone without the running wheel lost their weights but also maintained stable weights to a certain degree [2]. A previous study reported that approximately three-fourths of rats exposed to ABA procedures developed severe ABA and died [3]. Some breeds have been considered to be too hyperactive for ABA experiments, and the most commonly used breeds have been Sprague-Dawley and Wistar rats. [3][4]. It has been reported that such animal ABA could be a model for human ABA, observed in some ballet dancers who restrict their diet and train excessively [2]. In recent years, ABA has been increasingly investigated; however, contradictory reports have been published. For example, a previous study found that ABA rats had significantly reduced fat mass, decreased circulating leptin levels, markedly increased ghrelin levels, and increased insulin sensitivity [5]. However, another study reported a decrease in insulin levels [6].

Although only the specific case of insulin is mentioned here, previous studies on ABA have reported contradictory results, and its mechanism is still unclear. Further research in particular of its neural mechanism is needed to learn more regarding ABA. The present study followed our former one [7] and focused on opioids in the brain to reveal the neural mechanism of ABA.

1.2. Opioids and their effects on diet

Opioids are widely known to provide analgesia and a sense of relief and euphoria. The main endogenous opioids are endorphins, enkephalins, and dynorphins. Among them, beta-endorphin is known as the substance involved in runner's high [8]. In the field of feeding research, opioids have been studied in relation to hedonic foods such as sweet and high-fat foods [9].

Some previous studies have explored the relationship between opioids and normal eating, and one of them reported that antagonizing opioids with naloxone reduced eating in healthy individuals who were not suffering from eating disorders [10]. In that study, carbohydrate intake did not change significantly, but protein and fat intake decreased significantly. However, it should be noted that naloxone did not decrease appetite itself.

In addition, there are some previous studies relating opioids to eating disorders in people. For example, one previous study found that opioid antagonism with naloxone improved AN in people [11]. In that study, AN patients receiving a constant intravenous infusion of naloxone with doses ranging from 3.2 to 6.4 mg/day showed significant increases in body weight during the treatment period. Although naloxone did not increase the amount of food intake, it had no lipolytic or other effects. Therefore, the authors suggested that naloxone might have altered metabolism by acting on the central nervous system. However, the AN patients in this previous study were also treated with the antidepressant amitriptyline. It should be noted that, despite the anorexic side effect of this drug, they did not lose any amount of food intake.

Thus, the relationship between eating disorders and opioids is still unclear. Therefore, more research is needed.

1.3. Previous studies on increased feeding in ABA rats

Because ABA is also a model for AN, some studies have investigated its association with anxiety and mother-infant separation; however, the primary causes of ABA are feeding suppression and running. Of these, only a few studies have specifically focused on feeding suppression.

For example, previous studies showed that high-fat chow or sweetened high-fat chow, but not standard chow with sucrose or saccharin, prevented the progression of ABA [12]. These results suggest that conditioned taste aversion does not occur in ABA when the novel diet is highly palatable. Highly palatable diets, such as high-fat chow, released opioids and dopamine; however, the sweet taste did not alter the amount of food intake in ABA rats and thus did not result in weight recovery, suggesting that specific opioids or dopamine in the body are essential to the recovery of food intake in ABA rats.

Another study showed that dopamine antagonists increased food intake in ABA rats. In a previous study, cis-flupenthixol, a nonselective dopaminergic D1/D2 receptor antagonist, was chronically injected intraperitoneally using osmotic pumps [13]. However, only the 0.1 mg/day dose increased food intake and prevented weight loss rather than dose-proportional recovery. The 1.0 mg/day dose also decreased locomotor activity but suppressed food intake and accelerated weight loss. These results mean that only a limited number of doses increase food intake, and incorrect doses accelerate ABA progression.

Furthermore, the cannabinoid agonist, Δ9-tetrahydrocannabinol (THC), increased food intake in ABA rats [14]. Among the three doses (0.1, 0.5, and 2.0 mg/kg/day), only 2.0 mg/kg/day caused an increase in food intake and body weight. However, food

intake was significantly higher than that in the control group only during the first 2 days of the experiment, suggesting that the increase in food intake during these 2 days in the ABA group delayed weight loss as a temporary coping strategy rather than preventing weight loss. Another previous study reported that, although the dose was 10 mg/kg and the dosing schedule was different (twice daily), THC produced drug tolerance in 1.5 days, and potency was reduced to one-twelfth to one-thirty-sixth on 3.5 days [15].

All these previous studies that examined feeding involved the reward system. These studies used high-reward chows or enhanced reward values for feeding. To the best of our knowledge, only a few studies have reported increased food intake without enhancing the reward system. Further studies are needed to understand the mechanism of ABA in rats.

Chapter 2. Experiment 1

2.1. Introduction

Changes in opioid secretion may be related to ABA. Running increases opioids in the rat's body [16]; 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 [17]. Moreover, food intake decreases in proportion to morphine dosage [18]. 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 [19]; 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, 4, 5], opioids within the appropriate concentration ranges are necessary to maintain normal food intake. Figure 1. shows an assumed

relationship between endogenous opioids and food intake. Although the specific amounts are unknown, the vertical axis indicates the amount of food intake and the horizontal axis indicates the amount of endogenous opioids.

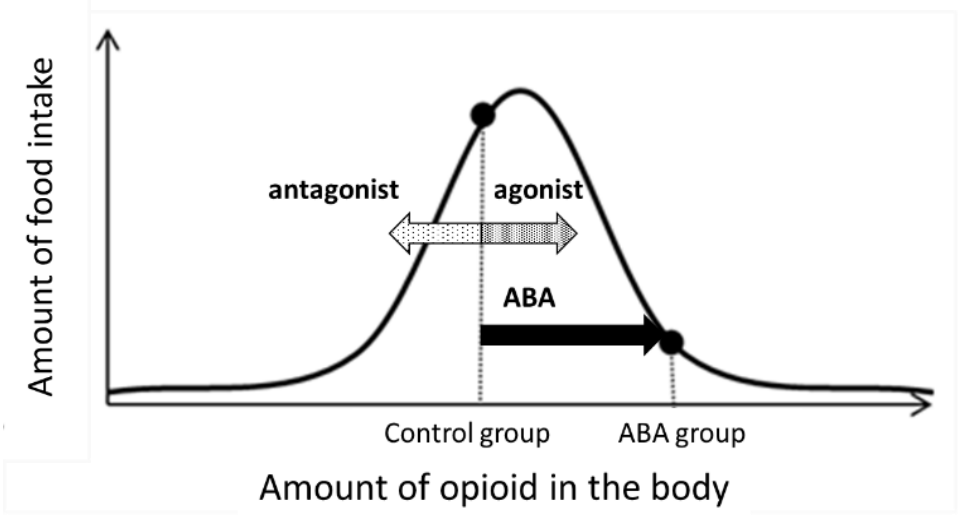


Figure1. Assumed relationship between endogenous opioids and food intake.

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 [17]. The food intake in rats starved only might have been 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, the hypothesis that a decrease in food intake in ABA rats was due to an excess of opioids released by running [7] was examined. 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, whether naloxone acts in the brain was examined.

The nucleus of the solitary tract (NST) is the primary central nervous nucleus that receives afferent input from the digestive organs of the digestive system via the vagus nerve [20]. 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) [21] and Peptide YY (PYY) [22], which convey satiety information from the internal organs, and the NST has

opioid receptors [23]. Although opioids are not directly involved in regulation of feeding, it may be possible that opioids in the NST modify the functions of feeding regulators and processing of satiety information from the digestive organs. As this study was the first experiment in which naloxone was microinjected into the NST of ABA rats, the medial NST (mNST) was targeted 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 [24]. 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 the NST [25]. 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.

As a supplementary experiment, electroencephalography (EEG) was recorded from the parietal cortex and hippocampus following microinjection of naloxone into the NST. The purpose of this supplemental experiment was to determine whether the naloxone used in Experiment 1 had enough volume and concentration to affect the brain activity. If the EEG was altered by the naloxone to the NST, the injected naloxone could be considered to be effective.

2.2. Material and methods

2.2.1. Animals

Seventeen male Long-Evans hooded rats (Shimizu Laboratory Supplies, Japan) were individually housed under a 12-h/12-h light/dark cycle. The rats were divided into 16 rats and 1 rat, the former for the main experiment and the latter for the supplementary experiment, respectively. The sixteen rats were randomly divided into two groups (ABA and control). They 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, the rats could not have any food. The one rat for the supplemental experiment had not experienced the procedures of the main experiment. At the beginning of the experiment, the rat was 23 weeks old and weighed 451g. Before and during the supplemental experiment, the rat was kept in an environment with free access to food and water.

All procedures were performed following the Guidelines for Animal Experiments at Doshisha University and with the approval of the Doshisha University Animal Research Committee.

2.2.2. 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).

2.2.3. Electrode

Bipolar electrodes for recording EEG were created by placing two 0.2 mm diameter stainless steel soft wires isolated by polyurethane coating in parallel. Each wire of the bipolar electrode was placed in two polyimide tubes 1 mm apart and fixed with instant glue and dental cement. This ensured that the soft wires remained stable and were fixed in the targeted position during and after implant surgery. All tips of the wire in the polyimide tubing were cut, leaving a wire tip of 1 mm out. The coating on the wire tips was removed to expose 0.5 mm of metal. The ground electrode was a 1 mm diameter stainless steel screw soldered with the same wire used for the bipolar electrodes.

2.2.4. Surgery

Under anesthesia with 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) in the main experiment. The depth of the guide cannula was 6.8 mm from the skull surface. The target region was based on an atlas [24]. 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.

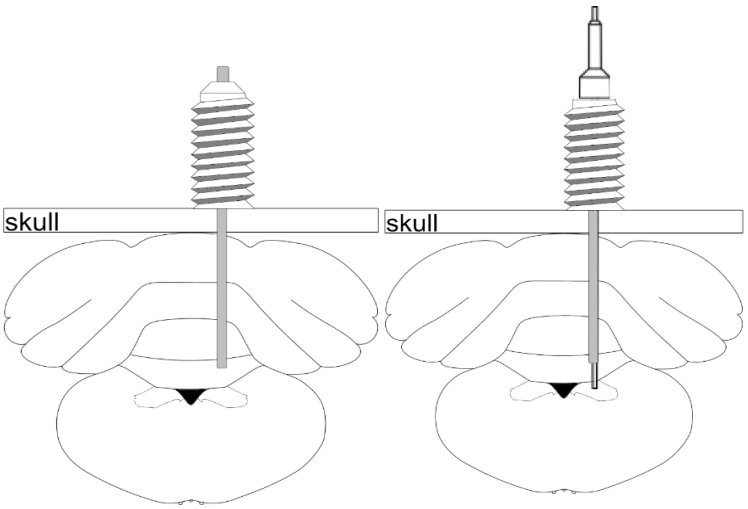


Figure2. Images of cannulas implanted in the NST.
The left figure is an image of the guide cannula permanently fixed to the rat, and the right figure is an image of the internal cannula inserted.

In addition to the main experiment, electrodes and guide cannulas were implanted to measure EEG in one rat based on a previous study [26]. Under anesthesia with 1% – 3% isoflurane, each hole was drilled into the skull for implanting the bipolar recording electrodes (each comprising a stainless wire 0.2 mm in diameter and having a vertical tip separation of approximately 1 mm) into the parietal cortex (-3.6 mm from the bregma and 3.0 mm from the midline; the depth was 1.4 mm from the skull surface) and hippocampus (-3.6 mm from the bregma and 2.4 mm from the midline; the depth was 3.0 mm from the skull surface). A ground electrode was implanted into the skull above the cerebellum, such that the tip of the screw was in contact with the surface of the brain. The guide cannula was implanted in the mNST following the same procedure as in the main experiment. The target region was based on an atlas [24]. After surgery, the rats were allowed to recover for at least 7 days before the experiments began.

2.2.5. Procedure

Following a previous study [27], 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. 3.

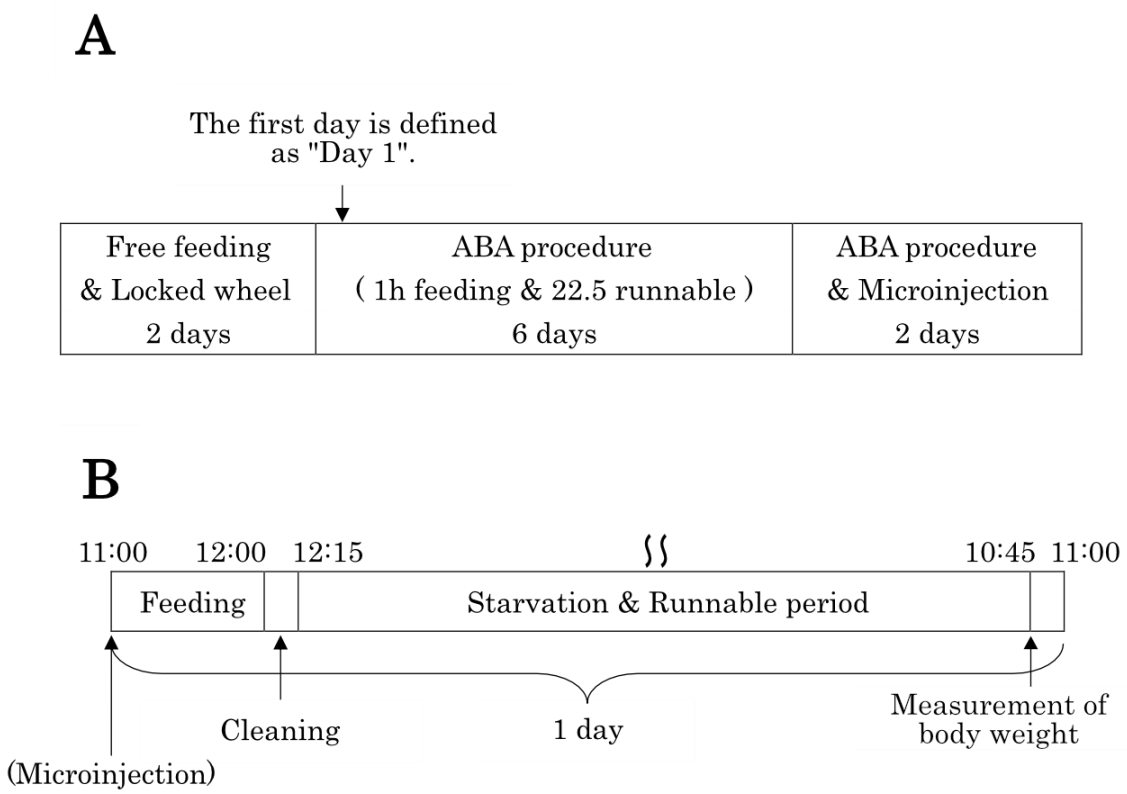


Figure 3. Procedure for ABA and control groups.
(A) Outline of experiment. **(B)** Details of ABA procedure (1 day).

The first two days were for the Free feeding & Locked wheel period (Fig. 3A.). 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. 3A.). 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. 3A.). 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.

In the supplementary experiment, EEG was recorded for 30 min from the parietal cortex and dorsal hippocampus 4 h after urethane injection. Naloxone was then microinjected in the same manner as in the main experiment, and EEG was recorded for 1h.

2.2.6. Recording system

EEG was recorded using a 16-channel bipolar ADC headstage (Intan Tech). The headstage was controlled by an Aduino Uno, and each channel (two channels in total) recorded the EEG at a sampling rate of 1 kHz. The recorded EEG (potential

differences) data were stored on a personal computer via serial communication and analyzed offline.

2.2.7. Pharmacological treatment

To observe the effect of opioid suppression on feeding, rats was injected 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 [26]. In a previous study, naloxone was injected intraperitoneally, causing withdrawal symptoms in rats with ABA [28]. 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 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.

A previous study used morphine [26]; however, urethane was used in the supplementary experiment. In the supplementary experiment, the urethane concentration was 1.5 g/kg, which was considered lethal based on previous studies [29]. Therefore, approximately half of that concentration was used, i.e., 0.7 g/kg of

urethane dissolved in 2.5 ml of saline solution. Naloxone dose in the supplementary experiment was also the same as that in the ABA experiment.

2.2.8. 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 [24].

2.2.9. Statistical Analysis

Data were analyzed using SPSS26 (IBM, USA) 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.

2.2.10. EEG data analysis

The recorded EEG data were compared 30 min before and after the naloxone injection. Fast Fourier transform (FFT) was used to separate the data recorded from each channel using a frequency band. MATLAB R2020b software (The Mathworks, Inc., MA) was used for the analysis. The low-amplitude-high-frequency, and high-amplitude-low-frequency activities were focused on.

2.3. Results

Our histological examination confirmed that the cannulas were inserted into the targeted area in 16 rats. Fig. 4. shows the locations of the tips of the cannulas for all the rats.

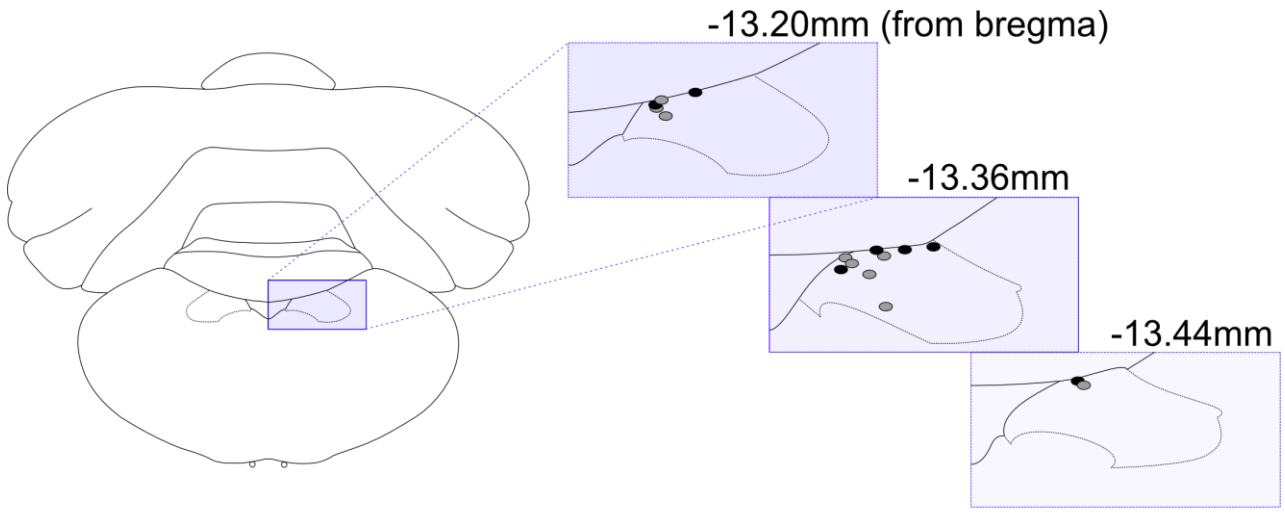


Figure 4. Cannula tips in the NST. Gray circles indicate injection sites for the ABA group, and black circles indicate injection sites for the control group.

2.3.1. 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. 5A. 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: $F=9.786$, $df=2.457$, $P<0.01$). The distance of wheel running was gradually increased, except on the last day, but Bonferroni's post-hoc analysis indicated that the distance on Day 8 was significantly larger than that on Day 1 ($p < 0.05$).

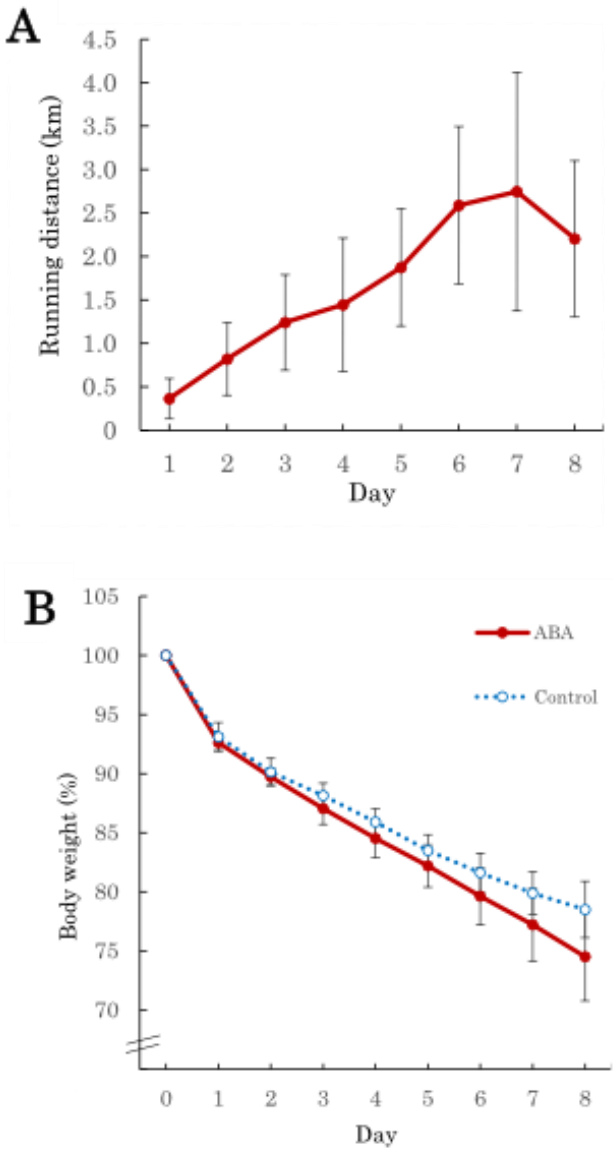


Figure 5. **(A)** Mean running distance from Days 1 to 8. **(B)** Mean percentages of body weight from Days 0 to 8. Percentages were calculated by dividing the body weight of each day by the body weight of Day 0. Error bars indicate standard errors of the mean.

2.3.2. 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. 5B. 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 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: $F=119.129$, $df=1.274$, $P<.001$). Although the main effect of group was not significant (Greenhouse–Geisser corrected: $F=4.152$, $df=1$, ns), the interaction between day and group was significant (Greenhouse–Geisser corrected: $F=7.425$, $df=1.274$, $P<0.05$).

2.3.3. 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 0 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. 6. 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).

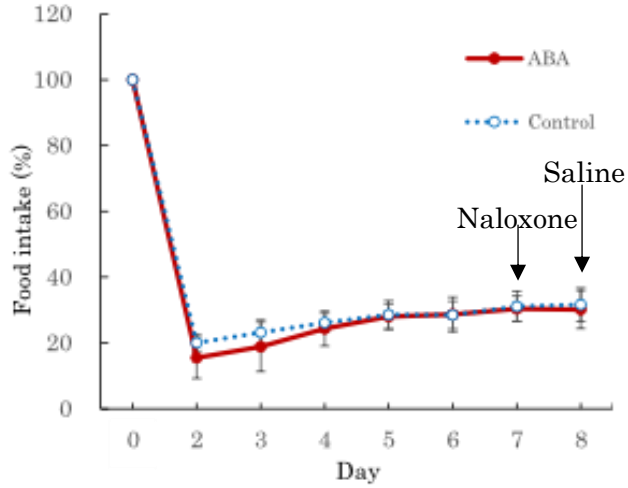
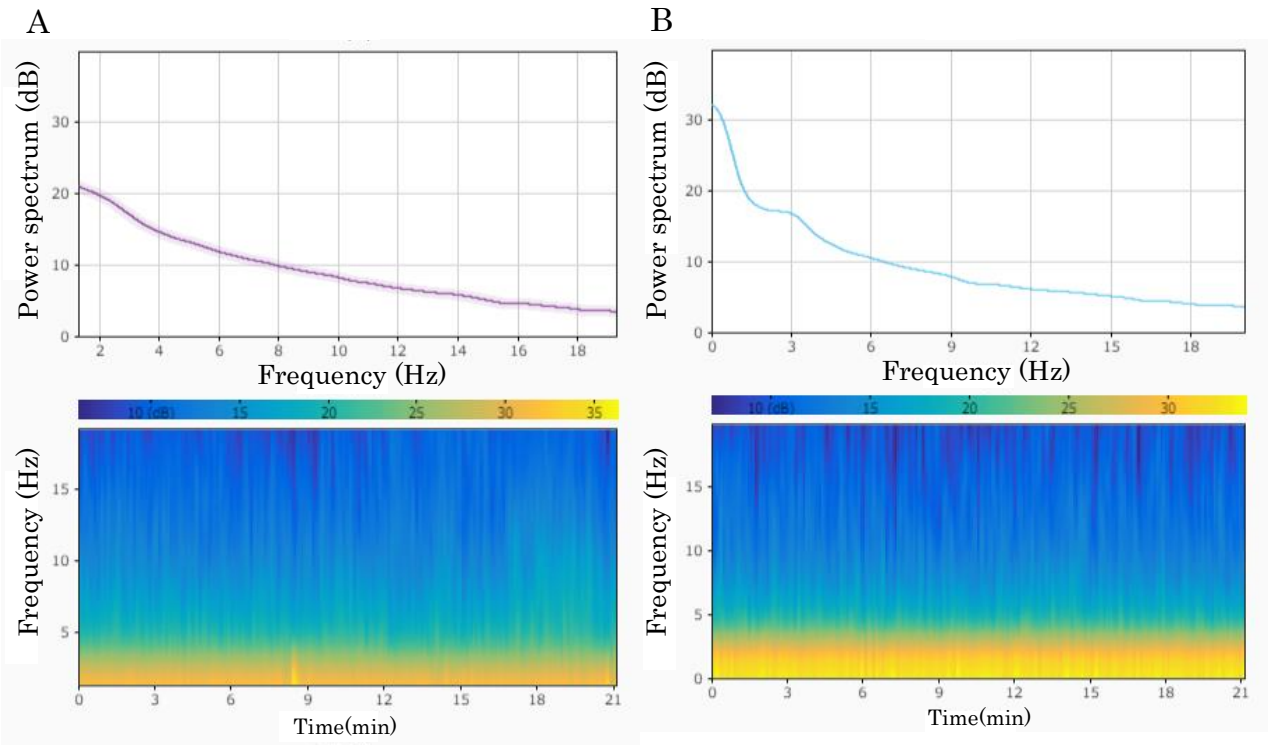


Figure 6. Mean percentages of food intake from Day 0 to Day 8 except Day 1 (see Materials and Methods). Percentages were calculated by dividing the amount of each day by the amount of Day 0. Error bars indicate standard errors of the mean.

2.3.4. Effect of naloxone on EEG

Figure 7. shows the results of the analysis of EEG recorded from the parietal cortex and hippocampus. EEG in the parietal cortex slightly changed after naloxone injection and the low frequency of EEG became somewhat prominent. On the other hand, hippocampal EEG clearly changed after naloxone injection.

The frequencies of 4.3 Hz and 8.7 Hz increased after the injection. In addition, the activity after naloxone injection showed higher frequencies compared to that at the baseline.



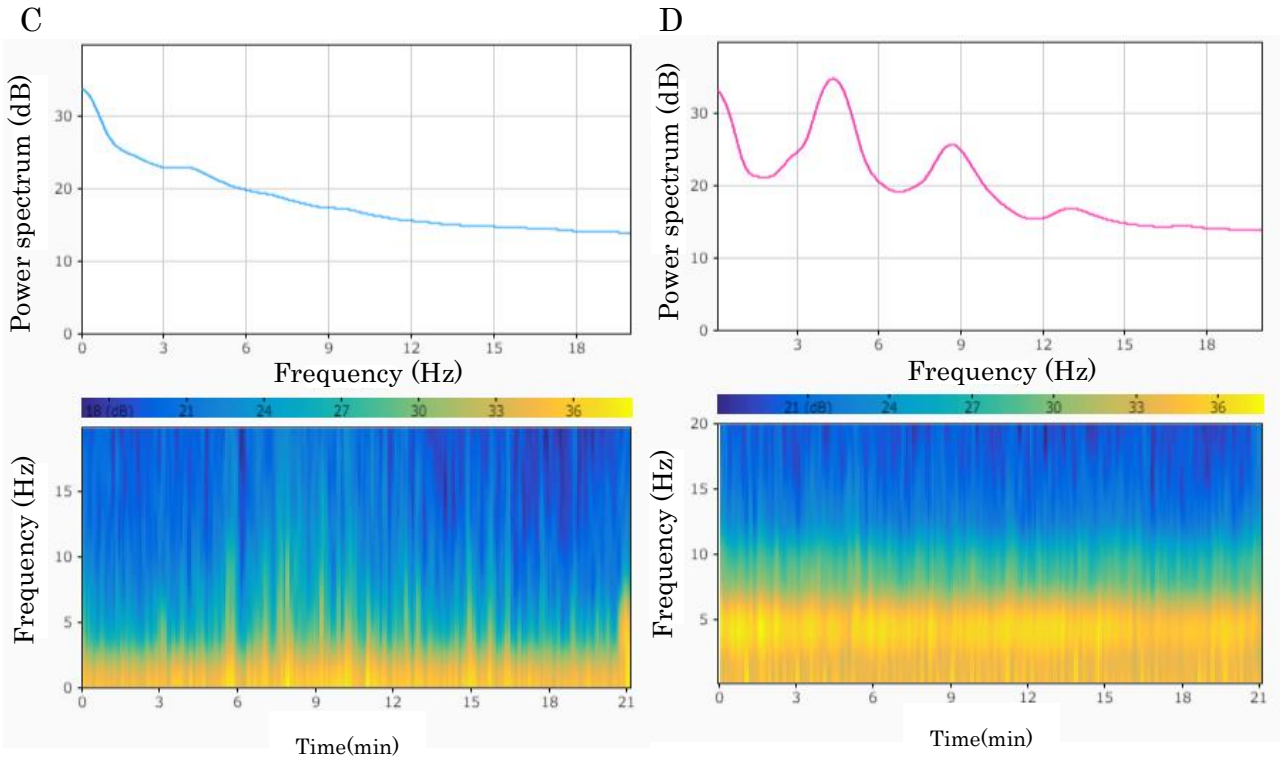


Figure 7. Results of the analysis of EEG in the parietal cortex and hippocampus. (A) EEG in the parietal cortex before naloxone injection as baseline, (B) EEG in the parietal cortex about 20 minutes after naloxone injection, (C) EEG in the hippocampus before naloxone injection as baseline and (D) EEG in the hippocampus about 20 minutes after naloxone injection. The upper and lower figures in each of A-D are, multitaper spectrums and colored power spectrograms, respectively.

2.4. Discussion

The purpose of this experiment was to investigate whether the mNST altered food intake in ABA rats. To determine the rats in the ABA group will develop ABA, their running and body weights were compared at first. The volume of wheel running in the ABA group significantly increased during the Days (Fig. 5A.). 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. 5B.). 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 [30]. In the preliminary experiment that used 8-week-old rats in the ABA procedure, the rats ate very little on the second day of the procedure and ate no pellet from the third day thereafter. As a result, the weight was lost too rapidly and too unstable for the experimental schedule that included the naloxone and saline injection dates. Therefore, 11-week-old rats were used 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, it is unlikely that opioids in the mNST are involved in the feeding symptoms in the ABA rats.

In this experiment, only one dose of naloxone (2.0μg in 0.1μl saline) was administered. This volume was considered based on the following previous studies. One of the studies examined the changes in parietal cortex and hippocampal EEG induced by the injection of naloxone into the NST [26]. In this experiment, two groups of naloxone doses were used: a low dose of 1.0 or 2.5 μg and a high dose of 10 or 30 μg, resulting in a dose-dependent suppression of EEG. And the other previous study showed that intraperitoneal administration of naloxone to ABA rats caused withdrawal symptoms [28]. The NST is associated with the occurrence of withdrawal symptoms and naloxone is able to cross the blood-brain barrier [31][32]. Therefore, excessive doses of naloxone could have caused withdrawal symptoms in ABA rats, resulting in a failure of the experiment. Therefore, the dose of naloxone injected in this study could be considered to be a concentration that could affect the NST and not too much to antagonize the opioid receptors.

The results of the EEG in the supplemental experiment showed that the naloxone injection had some effect on the brain activity. The effect was observed in both the parietal cortex and hippocampus, but it was clearer in the hippocampus than in the parietal cortex. Although the data was preliminary and obtained from only one rat, the

result indicated that the injected naloxone induced changes in EEG in the neocortex and limbic structure. The EEG in this experiment is not the same as in the previous study. This is because the previous study used different methods of recording and focused only on the changes in the parietal cortex EEG. However, the previous study also suggested that naloxone injection into the NST could affect the EEG in wide areas in the anterior part of the brain. The EEG results in the present study are consistent with the suggestion by the previous study. Therefore, it can be said that the result of Experiment 1, that is, injecting naloxone into the NST had no effect on the amount of food intake, was not due to too little injected naloxone to alter EEG in the brain.

A previous study has shown that increasing or antagonizing opioids suppresses food intake [19]. Therefore, in this study, the 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 [7]. However, it is not clear how the intraperitoneally injected naloxone was effective. The reason is that opioid receptors are distributed throughout the body [33][34], and the naloxone crosses the blood-brain barrier [32] and reaches the brain. Therefore, intraperitoneal injection of naloxone could affect on the whole body and brain, and it makes difficult to determine which regions the naloxone targets on. There are two main pathways of satiety signaling from

digestive 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 the mNST may not be 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 [35]. In addition, a previous study have shown that another substance in the NST, oxytocin, plays a role in suppressing exercise-induced tachycardia [36]. 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 [37],[38]. 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.

Chapter 3. Experiment 2

3.1. Introduction

The purpose of Experiment 1 was to determine whether adjusting the opioid dose of the mNST would alter food intake in rats with ABA. However, no significant differences were found between the ABA and control groups on the day of microinjection, indicating that the microinjection of naloxone into the mNST did not alter food intake in the ABA group. Therefore, Experiment 1 suggested that the effect of endogenous opioids on the mNST may not be associated with feeding disruption (suppression) in ABA rats. ABA rats tend to stop eating early and/or their food intake is insufficient to match their locomotor activity per meal [27] [5]. Therefore, the NST, which is one of the pathways involved in and the first place to reach the satiety center, did not appear to be involved in the etiology of ABA.

Previous studies that investigated the relationship between the NST and feeding inhibition included studies that destroyed the NST and those that destroyed the vagus nerve [39]. In both cases, the destruction of the NST resulted in the loss of feeding inhibition.

Despite the previous studies mentioned above and the characteristic suppression of that increased feeding in ABA rats, no studies have attempted to disrupt the NST in

these rats. Therefore, disruption experiment was conducted to understand the function of the NST in ABA rats.

If the NST itself enhances satiety in ABA or that the pathway through the NST is the cause of increasing satiety in ABA, then destroying the NST may eliminate appetite suppression and increase the amount of food intake in the ABA rat. Increased food intake would be sufficient to match the amount of food intake with the running volume, and it is expected that the body weight of ABA rats would not decrease. However, if disrupting the NST does not change the behavior of ABA rats, the NST is not associated with the incidence of ABA, although it is involved in satiety.

3.2. Material and methods

3.2.1. Animals

Eleven male Long-Evans hooded rats (Shimizu Laboratory Supplies, Japan) were approximately 11 weeks old and weighed 319-350 g at the start of the experiment. The other conditions were similar to those in Experiment 1. All procedures were performed following the Guidelines for Animal Experiments at Doshisha University and with the approval of the Doshisha University Animal Research Committee.

3.2.2. Apparatus

In addition to the devices used in Experiment 1, other devices were used, such as an operant chamber measuring 15.5 × 17.0 × 21.0 cm. The front wall had a nose-poke operandum (2.5 cm diameter) on the right side, which was located 4.5 cm above the floor and 2.5 cm from the right wall. An infrared sensor was placed 0.6 cm from the front edge of the nose-poke opening. The light bulb was located near the nose-poke operandum. At the start of the feeding period, the light bulb lit up, and only one food pellet was ejected through the food opening. A square food opening (5.0 × 5.0 cm) was present on the left side of the front wall, located 1.5 cm from the floor and 1.0 cm from the wall. The light bulb was 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 through the food opening. The back wall contained a water bottle. A hole (1.0 cm diameter) through which the rats could access the water bottle was located 5 cm above the floor and 3.7 mm from the right wall. Rats could move freely between the operant chamber and the wheel through a square hole (8.0 × 10.5 cm). The square hole was located on the left wall, 1.0 cm from the floor and 4.5 cm from the front wall. The diameter and width of the wheels were 36.0 cm and 11.0 cm, respectively. The wheel floor was made of 0.5 cm metal rods spaced 1.5 cm apart. A solenoid-operated brake was attached to the wheel. Wheel turns were electronically counted using a computer.

The task was controlled, and behavioral data were recorded using a personal computer (EPSON, Japan) connected to the apparatus via a LabVIEW interface. 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.

3.2.3. Surgery

Under anesthesia with 1%-3% isoflurane, lesions were made by passing an anodal direct current (1 mA, 10s) using the Lesion Making Device (53500, UGO BASILE SRL, Gemonio, VA, Italy) and a stainless bipolar electrode (150 μm diameter, UB-9007, UNIQUE MEDICAL Co. Ltd., Tokyo, Japan). The electrode was inserted into the mNST (-13.30 mm from the bregma and 0.6 mm from the midline). The depth of the guide cannula was 8.3 mm from the skull's surface. The target region was based on an atlas [24]. After surgery, the rats were allowed to recover for at least 7 days before the experiments began.

3.2.4. Procedure

The procedure was almost the same as that in Experiment 1 but was different in the last 2 days. In Experiment 2, the ABA procedure was performed without microinjection. The details of this procedure are shown in Fig. 8.

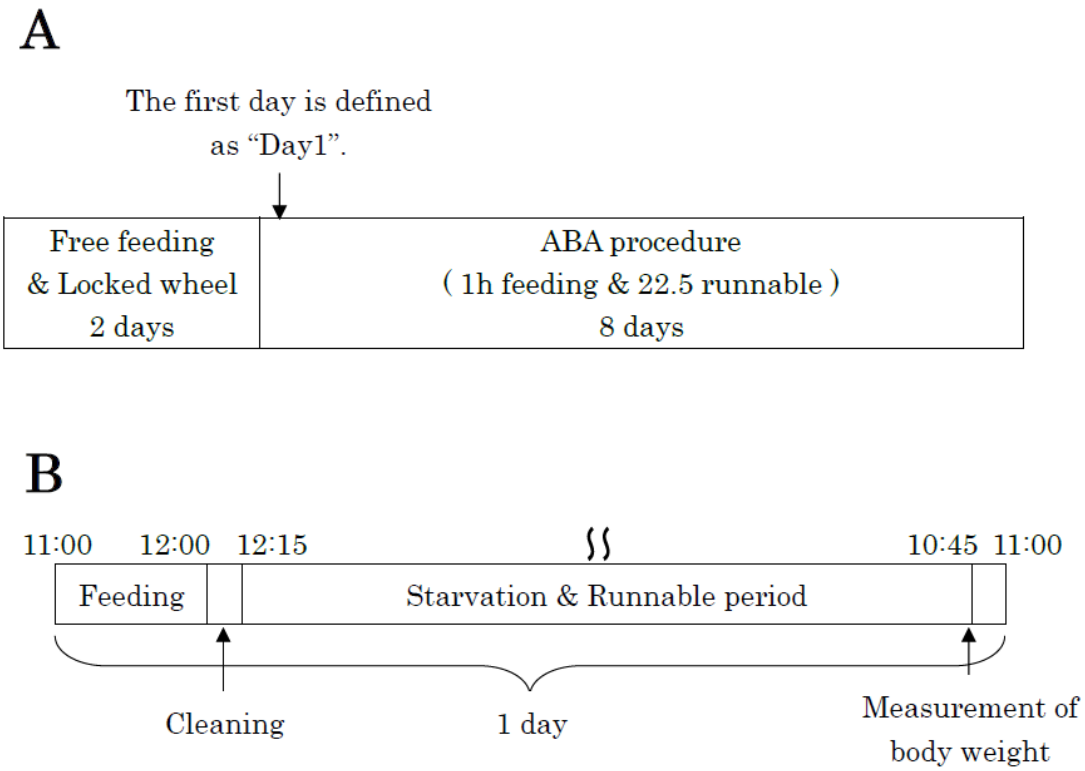


Figure 8. Procedure for ABA and control groups.
(A) Outline of experiment. **(B)** Details of ABA procedure (1 day).

3.2.5. Histology

After the experiment was completed, the rats were deeply anesthetized with sodium pentobarbital and perfused with PBS and 4% paraformaldehyde. The brains were removed, post-fixed in 4% paraformaldehyde, and sliced into 50 μm coronal sections. The locations of cannulas in the brain were identified using a stereotaxic atlas [24].

3.2.6. Statistical Analysis

The data were analyzed using MATLAB R2020b (The Mathworks, Inc., MA) and SPSS28 (IBM, USA) software. Body weight and food intake were calculated with Day 0 set at 100%. On the last day (Day8), the body weight of one ABA rat decreased below 70%; thus, the experiment was terminated for the corresponding rat. Therefore, data of the corresponding rat on Day 8 were analyzed using data from Day 7. This was performed to eliminate the mismatch in the analyzed values due to missing data. A one factor analysis of variance (ANOVA) was used to compare the differences in wheel running, and a two factor ANOVA was used to compare the differences in body weight. The analysis was performed on the last 3 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; however, the analysis was performed on the last 2 days.

3.3. Results

Histological examination confirmed the death of rat with the NST destruction. Figure 9. shows the area of destruction of the NST in the dead rat. The maximum area of the NST destruction in the dead rat was approximately 40% on one side. Therefore, even partial destruction of the NST on one side was considered potentially life-threatening

to rats. Therefore, the data of rats in whom the area of the NST destruction was more than 20% was used, which was half of that of the dead rat.

CONTROL210906-

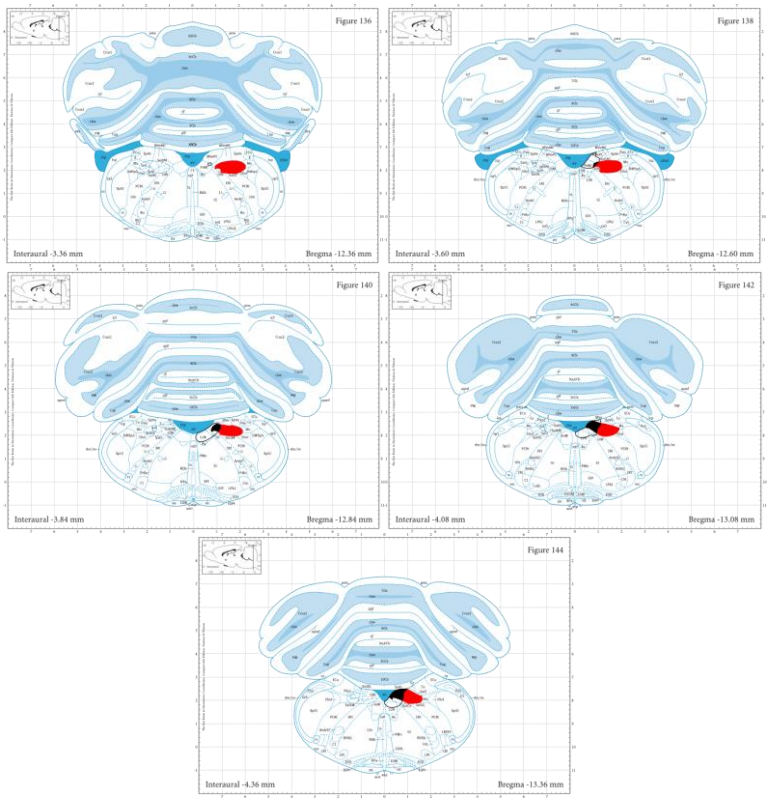
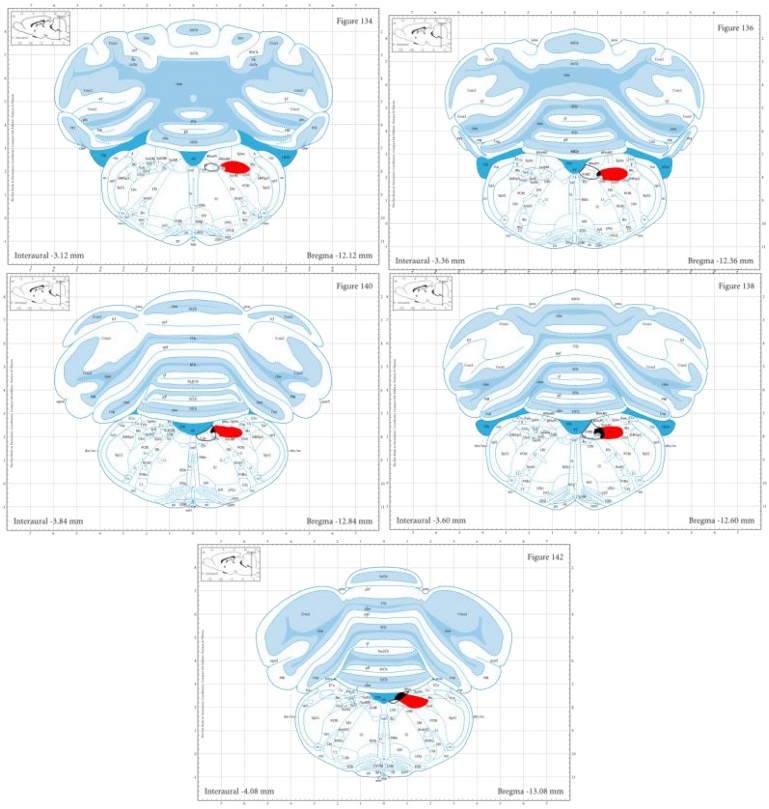


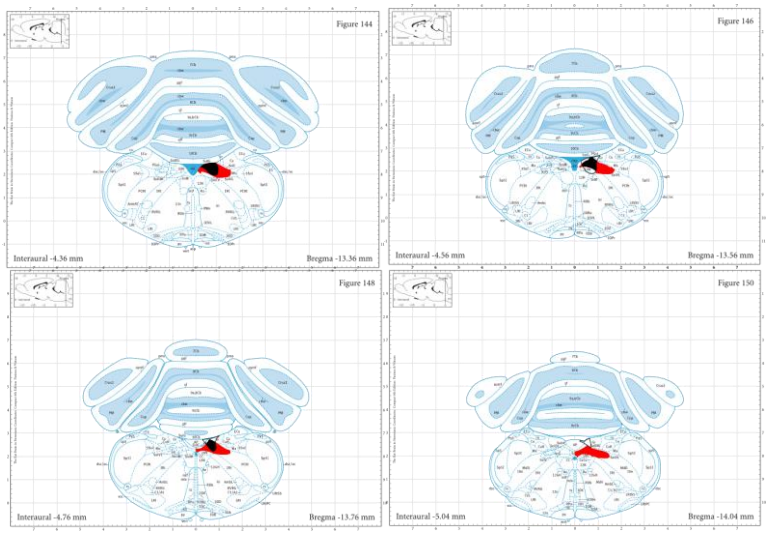
Figure 9. Coronal section of the extent of the NST lesions in the dead rat.
The red area indicates the area of the NST (one side). The black line indicates the area of the lesion, of which the area of overlap with the NST is filled in black.

Histological examination confirmed that the NST could be destroyed in 10 rats. Figure 10. shows the areas of the NST destruction in all rats. Figures of the area of destruction of the NST are shown at 200 μm intervals, based on the maximum extent of destruction in the coronal sections.

ABA210920-



ABA211129-



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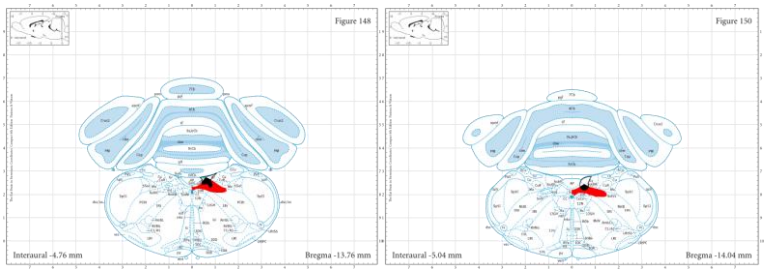

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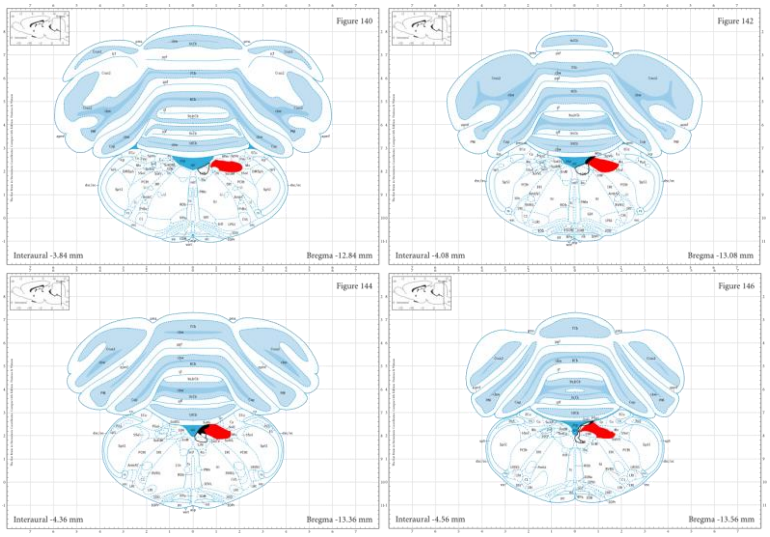

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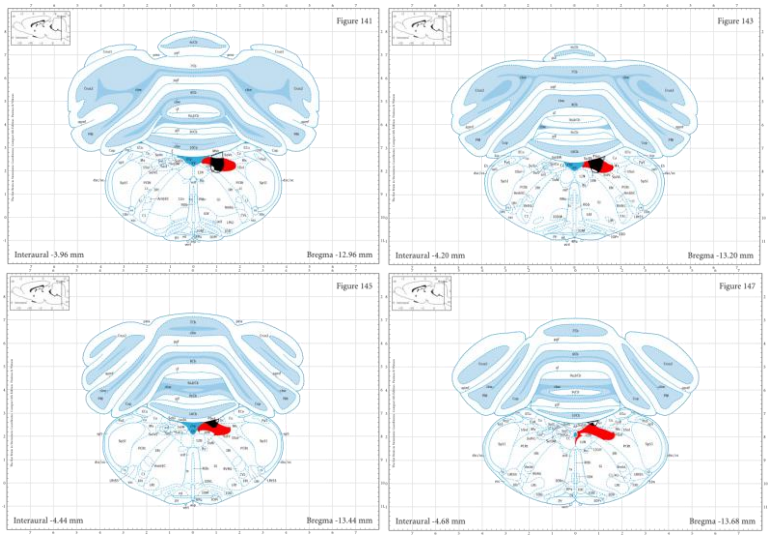

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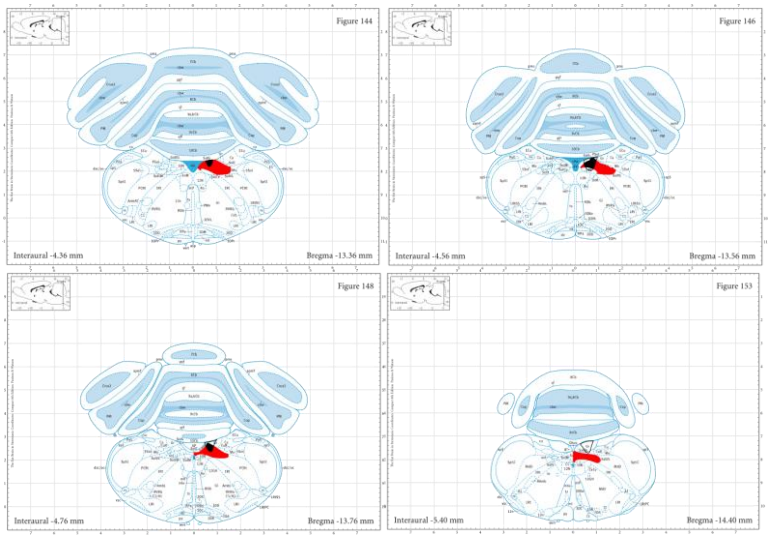
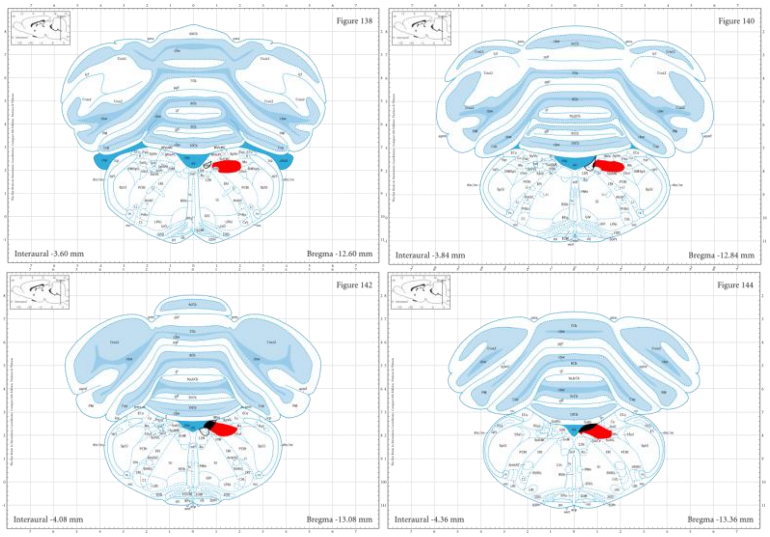


CONTROL210823-



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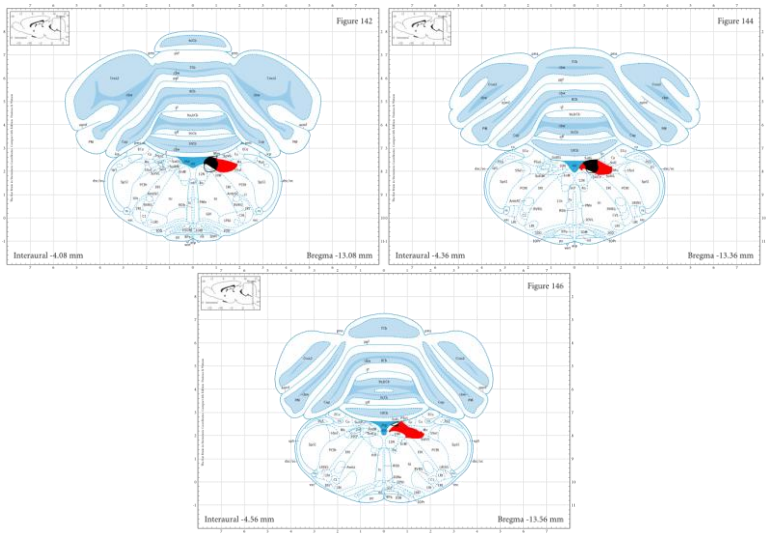

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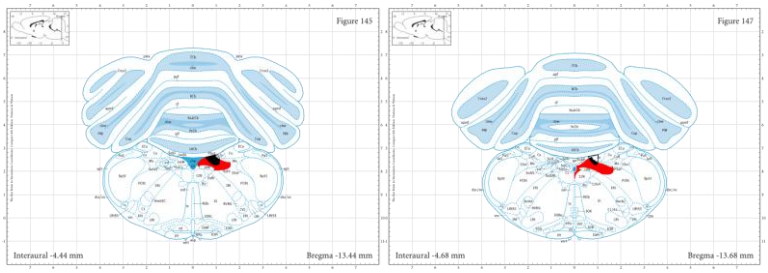

CONTROLn220117-

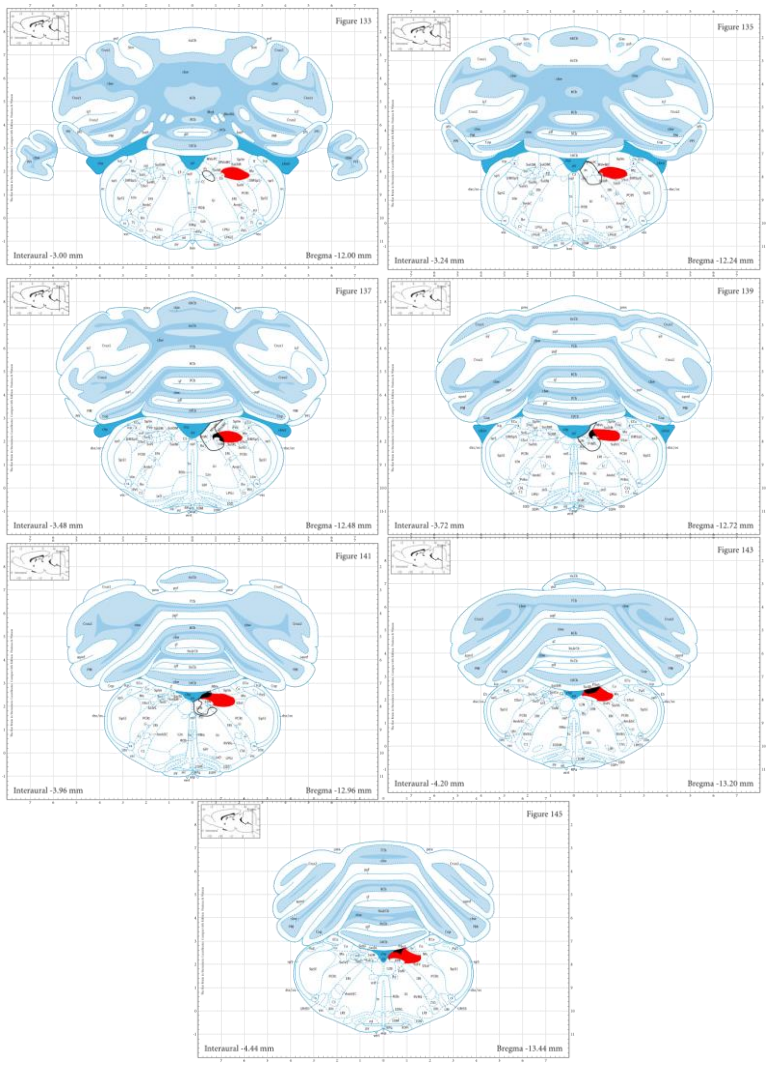

Figure 10. Coronal section of the extent of the NST lesions in 10 rats.
The red area indicates the area of the NST (one side). The black line indicates the area of the lesion, of which the area of overlap with the NST is filled in black.

3.3.1. Body weight

The body weight data measured immediately before the start of starvation (weights immediately after free feeding) were labeled as the data for Day 0. The data for the last day were labeled as Day 8. The average weight of rats on Day 0 was 365 g (SD = 17.21 g) in the ABA group and 364.4 g (SD = 10.09 g) in the control group. The t-test indicated no significant difference between the two groups (t[9] = 0.062, ns).

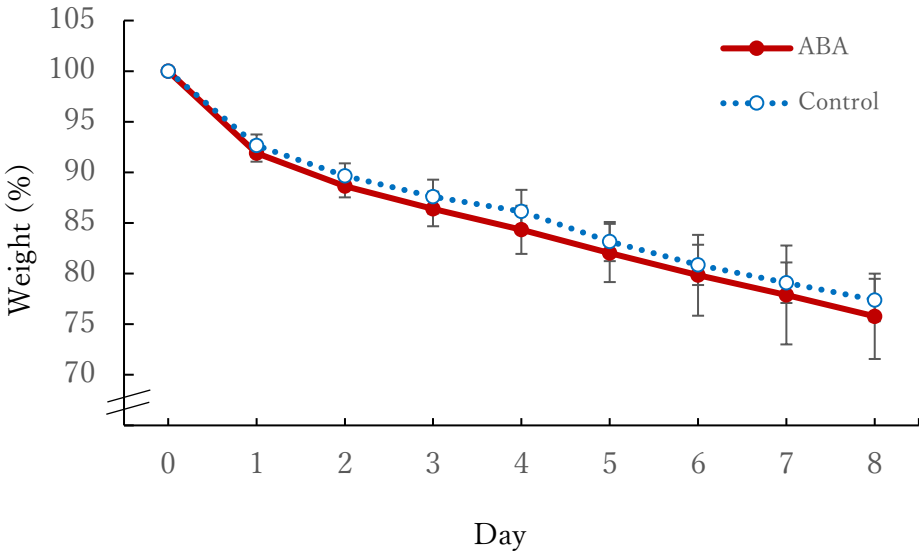

Figure 11. Mean percentages of body weight from Days 0 to 8. Percentages were calculated by dividing the body weight each day by the body weight of Day0. Error bars indicate standard errors of mean.

Figure 11. shows the mean body weights as percentages of the rats in ABA and control groups. Body weight on Day 0 was set as the base of 100%. Rats in both ABA and control groups showed decreased body weight. The body weights of rats in the

ABA group were slightly lower than those in the control group. As in Experiment 1, a two way ANOVA was performed using data on body weights of all rats on the last 3 days of the experiment to determine whether the rats in the ABA group had ABA. ANOVA revealed a significant main effect of day ($F[2,18] = 49.494$, $p<.001$). However, there was no significant main effect of group ($F[1,9] = 0.308$, ns) and no significant interaction between the day and group ($F[2,18] = 0.311$, ns).

3.3.2. Wheel running

The wheel running data measured after the first starvation were recorded as data for Day 1. The data for the last day were labeled as Day 8. Figure 12. shows the mean wheel running (km) per day for the ABA group. Although the running volume on Day 7 and Day 8 was nearly twice that on Day 1, the one factor ANOVA revealed no significant effect on the number of days ($F[7,40]=0.77$, ns).

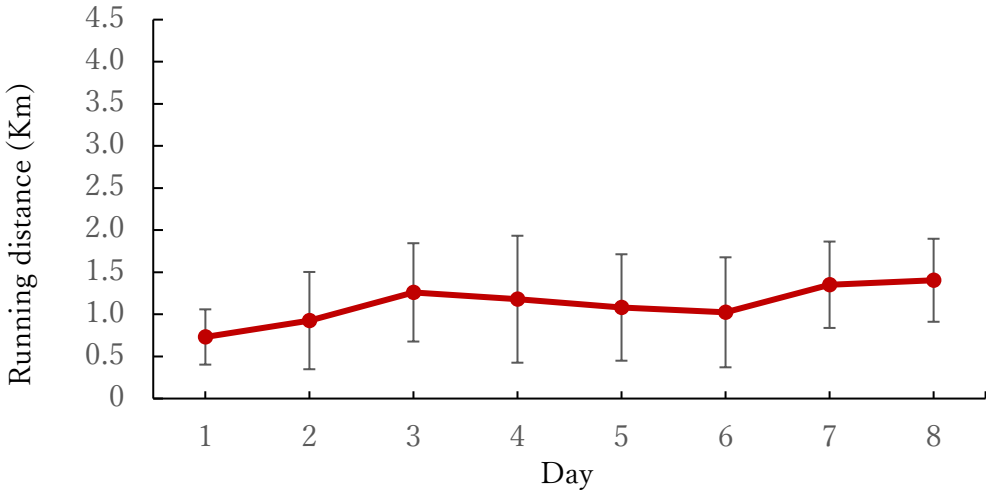

Figure 12. Mean running distance from Day 1 to 8.
Error bars indicate standard errors of mean.

In addition, the running distances of each ABA group in Experiments 1 and 2 were examined. Figure 13 shows the mean running distance per day for each ABA group in Experiments 1 and 2. A two way ANOVA showed significant main effects of group and day, and interaction between group and day ($F[1,104]=14.72$, $P<.001$, $F[7,104]=5.85$, $P<.001$, and $F[7,104]=2.88$, $P<.01$, respectively).

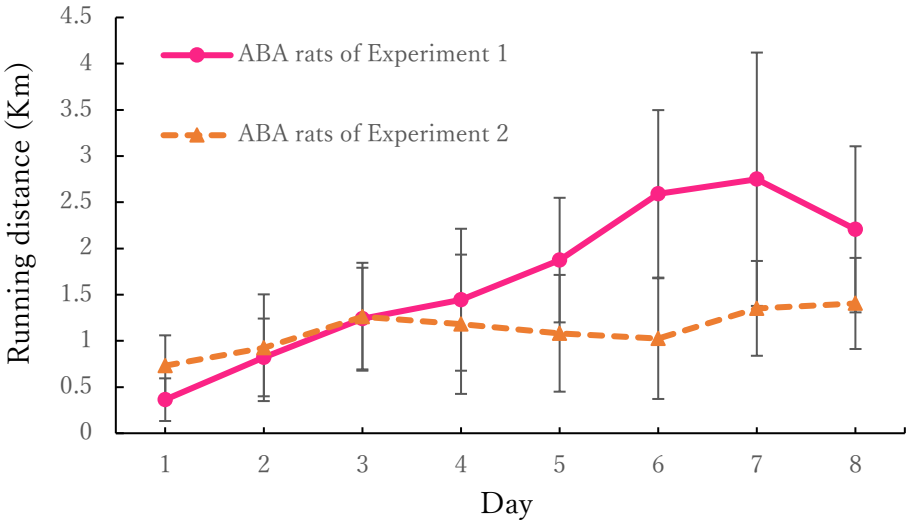

Figure 13. Mean running distance from Day 1 to 8. Error bars indicate standard errors of mean.

3.3.3. Food intake

The amount of food intake immediately before the starvation period was labeled as data for Day 0. The data for the last day were labeled as Day 8. There was no feeding on the first day in either group (the first starvation period). The average food intake on in Day 0 was 25.27 g (SD = 3.04 g) for rats in the ABA group and 27.59 g (SD = 4.47

g) for those in the control group. The t-test indicated no significant difference between the two groups (t[9] = -0.919, ns).

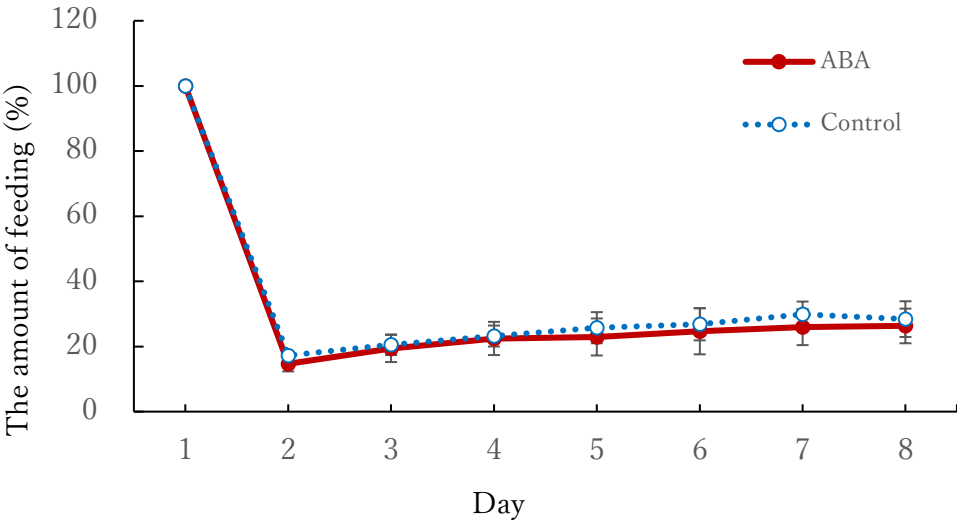

Figure 14. Mean percentages of food intake from Dya0 to Day8 except Day1 (see Materials and Methods). Percentages were calculated by dividing the amount of each day by the amount of Day0. Error bars indicate standard errors of mean.

Figure 14. shows the mean food intake percentages. The data for Day 0 were set at a base of 100%. As in Experiment 1, a two way ANOVA was performed on the amount of food intake of all rats to reveal the main effects of group and day, and the interaction between the groups and day on the last 2 days of the experiment. The ANOVA revealed that the main effects of group and day, and the interaction between group and day were all non-significant (F[1,9]=1.042, ns, F[1,9]=1.591, ns, and F[1,9]=0.585, ns, respectively).

4.1. Discussion

The purpose of Experiment 2 was to examine whether the destruction of the mNST altered the amount of food intake in rats with ABA. To determine whether the rats in the ABA group had ABA, the body weights of the ABA and control groups in Experiment 2 were compered at first. The results showed that although the body weights of rats in ABA and control groups gradually decreased per day, there was no significant difference between the two groups. Next, the running distance was compared, which is an important factor in the development of ABA. Although the running distance in the ABA group was increased, the difference in the distance between Day1 and Day8 was not statistically significant. Furthermore, the amount of food intake had no statistically significant change in the ABA rats and control rats. Therefore, it can be considered that the rats in the ABA group did not have ABA because their running distance did not increase from day to day, and the NST lesions prevented the progression of ABA by suppressing the running distance.

As with the ABA group in Experiment 1, data of rats in the ABA group in Experiment 2 were obtained from those with a running volume of at least 1000 turns. All rats in the ABA group in Experiment 2 had no aversion to exercise and had at least 1000 turns, as was the case of rats in the ABA group in Experiment 1. Nevertheless, the rats in the ABA group in Experiment 2 did not develop ABA.

Therefore, it can be considered that the decrease in running volume in this study was not due to individual differences.

The NST, destroyed in Experiment 2, is not only a regulator of feeding but also the regulator of heart rate and blood pressure. For example, the NST regulates exercise-induced tachycardia via oxytocin [40]. It has been reported that when rats were exposed to a certain amount of running, the exercise increased oxytocin levels in their bodies. Compared with rats that did not receive exercise training, running stimulated oxytocinergic projections in the NST-dorsal motor nucleus of the vagus complex and suppressed tachycardia among the exercise-trained rats.

In addition to oxytocin, various other substances are involved in regulating the heart rate and blood pressure in the NST. For example, vasopressinergic synapses in the NST are important modulators of baroreceptor reflex function, and elevated concentrations of arginine vasopressin in the rat's NST attenuate baroreceptor reflex-mediated bradycardia [41].

Moreover, leptin, neuropeptide Y, α-melanocyte-stimulating hormone, agouti-related protein, cocaine and amphetamine-regulated transcript peptide (CART), ghrelin, orexins, and corticotrophin-releasing factor are known as the neural regulators of blood pressure. In particular, neuropeptide Y, CART, and ghrelin have been found to be directly related to the NST [42].

Therefore, it is possible that the NST lesion caused the suppression of the increase in running distance rather than the dysregulation of the amount of food intake. However, it is not clear from the present experiment whether abnormal heart rate or blood pressure caused by the NST lesion affected running in these studies. In addition, previous studies have shown that rats with running addiction have decreased blood pressure at rest [43]. Thus, the NST lesion in rats with ABA may not only have caused abnormal heart rate and blood pressure during exercise but also at rest, which may have inhibited the increase in running distance. Future experiment should focus on the heart rate and blood pressure in rats with ABA with destroyed the NST.

Chapter 4. General discussion

The purpose of Experiment 1 was to investigate whether the hypothesis that the excessive release of opioids during running was responsible for the failure of rats with ABA to increase their food intake to a level appropriate for exercise was peripheral or central in origin and if it is central, which brain regions were the primary sites of action. In previous experiments, the opioid antagonist, naloxone, was injected intraperitoneally; therefore, in the current Experiment 1, naloxone was microinjected into the NST to examine whether the amount of food intake was altered. The results showed that the amount of food intake of ABA rats did not change and was not significantly increased compared with those in the control group. Thus, Experiment 1 concluded that the effect of naloxone on the NST did not block the ABA in rats. The NST may not be associated with the occurrence (suppression of the increase in the amount of food intake) of ABA in rats.

Based on the results of Experiment 1, the purpose of Experiment 2 was to examine whether the NST is involved in the suppression of the increased food intake in ABA rats. If the NST itself or the pathway through the NST, one of the two pathways through which the brain receives satiety signals, the NST should be involved in enhancing satiety information, the NST lesions might have been eliminate the inhibition of and increase food intake. However, if there was no change in the amount

of food intake, it is unlikely that the NST or the pathway through the NST was involved in the progression of ABA, even if it was a pathway through the satiety signal. Therefore, the body weights of rats in the ABA group were not significantly different from those in the control group, and the rats did not have ABA. To determine why ABA was not observed, other factors were examined and found that the running distance in the ABA group did not show a significant increase from day to day. In contrast, the amount of food intake in the ABA group was not significantly different from that in the control group, indicating that there was no change due to the creation of the NST lesion. Surprisingly, the NST was not related to the suppression of the increase in food intake of the ABA rats but to the increase in running distance.

As increased running is also an important factor in the progression of ABA, it can be considered that the suppression of food intake was due to substances that increase with running. However, the NST was related to an increase in running.

In addition, a specific comparison of the running distances of rats in the ABA group in Experiments 1 and 2 showed that rats in the ABA group in Experiment 1 showed a statistically significant increase in running distance after Day 5 compared with Day 1 on the first day of the experiment (except Day 7), whereas those in the ABA group in Experiment 2 did not show a significant difference from Day 1 on any day of the experiment.

However, the NST lesion did not inhibit running completely, as the running distance of rats in the ABA group in Experiment 2 increased to approximately twice the number of kilometers on Day 7 and 8 compared with Day 1. This suggests that the NST lesions may have inhibited a response to exercise above a certain amount, thus avoiding running dependence. For example, in a previous study [40][44], rats experienced physical discomfort while running because the NST was lesioned, and exercise-induced tachycardia suppression by oxytocin and vasopressin did not work. In addition, each withdrawal symptom of addiction has a specific response, depending on the addictive substance. Occasionally, exact opposite withdrawal symptoms occur; however, it is also considered that all changes in systolic blood pressure occur at rest [45]. Therefore, it is possible that the changes in the resting body that would have occurred if ABA rats had become dependent on running no longer required running as a result of the NST lesions.

In future experiments, it is necessary to first examine the heart rate, blood pressure, and respiratory status of rats with ABA with and without lesioned the NST during exercise, as well as the blood pressure at rest. After examining these responses, the role of the NST in ABA rats will become clearer if substances are prevented from functioning by the creation of the NST lesion are examined. Moreover, previous studies have reported that feeding inhibitors suppress the amount of ABA-induced

running [46] and that they also act on the NST [42]. In addition, especially from the present Experiment 2, it was found that the NST of ABA rats was involved in running regulation and not in satiety. Therefore, previous studies and the present studies suggest that the brain regions that modify the regulation of feeding, such as the NST, might also be related to regulation of running in ABA rats. Future studies should not focus only on feeding or running, but on both of them in order to uncover the mechanism of ABA.

Chapter 5. References

[1] Routtenberg A, Kuznesof AW. Self-starvation of rats living in activity wheels on a restricted feeding schedule. J Comp Physiol Psychol 1967;64:414–21. https://doi.org/10.1037/h0025205.

[2] W. Frank Epling, W. David Pierce LS. A theory of activity - based anorexia. Int J Eat Disord 1983;3:27–46. https://doi.org/10.1002/1098-108X(198323)3:1<27::AID-EAT2260030104>3.0.CO;2-T.

[3] Carrera O, Fraga Á, Pellón R, Gutiérrez E. Rodent model of activity-based anorexia. Curr Protoc Neurosci 2014:1–11. https://doi.org/10.1002/0471142301.ns0947s67.

[4] Gutierrez E. A rat in the labyrinth of anorexia nervosa: Contributions of the activity-based anorexia rodent model to the understanding of anorexia nervosa. Int J Eat Disord 2013;46:289–301. https://doi.org/10.1002/eat.22095.

[5] Pardo M, Roca-Rivada A, Al-Massadi O, Seoane LM, Camiña JP, Casanueva FF. Peripheral leptin and ghrelin receptors are regulated in a tissue-specific manner in activity-based anorexia. Peptides 2010;31:1912–9. https://doi.org/10.1016/J.PEPTIDES.2010.06.022.

[6] Boersma GJ, Liang NC, Lee RS, Albertz JD, Kastelein A, Moody LA, et al. Failure to upregulate Agrp and Orexin in response to activity based anorexia in

weight loss vulnerable rats characterized by passive stress coping and prenatal stress experience. Psychoneuroendocrinology 2016;67:171–81. https://doi.org/10.1016/J.PSYNEUEN.2016.02.002.

[7] Ishihara E, Aoyama K. Naloxone increased food intake of rats under the activity-based anorexia procedure. Japanese J Anim Psychol 2018;68:188.

[8] Wildmann J, Krüger A, Schmole M, Niemann J, Matthaei H. Increase of circulating beta-endorphin-like immunoreactivity correlates with the change in feeling of pleasantness after running. Life Sci 1986;38:997–1003. https://doi.org/10.1016/0024-3205(86)90233-X.

[9] Kelley AE, Bakshi VP, Haber SN, Steininger TL, Will MJ, Zhang M. Opioid modulation of taste hedonics within the ventral striatum. Physiol Behav 2002;76:365–77. https://doi.org/10.1016/S0031-9384(02)00751-5.

[10] Cohen MR, Cohen RM, Pickar D, Murphy DL. Naloxone Reduces Food Intake in Humans. Psychosom Med 1985;47:132–8.

[11] Moore R, Mills IH, Forster A. Naloxone in the treatment of anorexia nervosa: Effect on weight gain and lipolysis. J R Soc Med 1981;74:129–31. https://doi.org/10.1177/014107688107400208.

[12] Brown AJ, Avena NM, Hoebel BG. A high-fat diet prevents and reverses the development of activity-based anorexia in rats. Int J Eat Disord 2008;41:383–9. https://doi.org/10.1002/EAT.20510.

[13] Verhagen LAW, Luijendijk MCM, Hillebrand JJG, Adan RAH. Dopamine antagonism inhibits anorectic behavior in an animal model for anorexia nervosa. Eur Neuropsychopharmacol 2009;19:153–60. https://doi.org/10.1016/J.EURONEURO.2008.09.005.

[14] Verty ANA, Evetts MJ, Crouch GJ, McGregor IS, Stefanidis A, Oldfield BJ. The Cannabinoid Receptor Agonist THC Attenuates Weight Loss in a Rodent Model of Activity-Based Anorexia. Neuropsychopharmacol 2011 367 2011;36:1349–58. https://doi.org/10.1038/npp.2011.19.

[15] Bass CE, Martin BR. Time course for the induction and maintenance of tolerance to Δ9-tetrahydrocannabinol in mice. Drug Alcohol Depend 2000;60:113–9. https://doi.org/10.1016/S0376-8716(99)00150-7.

[16] Asahina S, Asano K, Sunaga M, Hisamitu T, Sato M, Horikawa H. The influence of exercise intensity, frequency, and duration, on beta-endorphin production in rat hypothalamus. J Showa Med Assoc 2007;67:414–21. https://doi.org/10.14930/jsma1939.67.414.

[17] Sanger DJ, McCarthy PS. Differential effects of morphine on food and water intake in food deprived and freely-feeding rats. Psychopharmacology (Berl) 1980;72:103–6. https://doi.org/10.1007/BF00433813.

[18] Kunihara M, Lee S. Opposite effects of morphine on feeding behavior in rats, associated with administration time. Kitakanto Med J 1985;35:451–62. https://doi.org/10.2974/kmj1951.35.451.

[19] Brown DR, Holtzman SG. Suppression of deprivation-induced food and water intake in rats and mice by naloxone. Pharmacol Biochem Behav 1979;11:567–73. https://doi.org/10.1016/0091-3057(79)90043-1.

[20] Morton GJ, Meek TH, Schwartz MW. Neurobiology of food intake in health and disease. Nat Rev Neurosci 2014;15:367–78. https://doi.org/10.1038/nrn3745.

[21] Reidelberger RD, Hernandez J, Fritzsch B, Hulce M. Abdominal vagal mediation of the satiety effects of CCK in rats. Am J Physiol Integr Comp Physiol 2004;286:R1005–12. https://doi.org/10.1152/ajpregu.00646.2003.

[22] Koda S, Date Y, Murakami N, Shimbara T, Hanada T, Toshinai K, et al. The role of the vagal nerve in peripheral PYY3–36-induced feeding reduction in rats. Endocrinology 2005;146:2369–75. https://doi.org/10.1210/EN.2004-1266.

[23] Le Merrer J, Becker JAJ, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. Physiol Rev 2009;89:1379–412. https://doi.org/10.1152/physrev.00005.2009.

[24] Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates, 6th Edn. New York: Elsevier; 2009.

[25] Hurtado MD, Sergeyev VG, Acosta A, Spegele M, La Sala M, Waler NJ, et al. Salivary peptide tyrosine–tyrosine 3–36 modulates ingestive behavior without inducing taste aversion. J Neurosci 2013;33:18368–80. https://doi.org/10.1523/JNEUROSCI.1064-13.2013.

[26] Bronzino JD, Oley N, Kelly ML, Cordova C, Morgane PJ. EEG effects of microinjection of naloxone in the region of the nucleus tractus solitarius of the rat. Brain Res 1983;271:33–40. https://doi.org/10.1016/0006-8993(83)91362-8.

[27] Aoyama K. Effects of an activity-based anorexia procedure on within-session changes in nose-poke responding. Learn Motiv 2012;43:48–54. https://doi.org/10.1016/j.lmot.2012.02.001.

[28] Kanarek RB, D'Anci KE, Jurdak N, Mathes WF. Running and addiction: precipitated withdrawal in a rat model of activity-based anorexia. Behav Neurosci 2009;123:905–12. https://doi.org/10.1037/a0015896.

[29] Field KJ, White WJ, Lang CM. Anaesthetic effects of chloral hydrate, pentobarbitone and urethane in adult male rats. Lab Anim 1993;27:258–69. https://doi.org/10.1258/002367793780745471.

[30] Schalla MA, Stengel A. Activity based anorexia as an animal model for anorexia nervosa–a systematic review. Front Nutr 2019;6. https://doi.org/10.3389/fnut.2019.00069.

[31] Van Bockstaele EJ, Rudoy C, Mannelli P, Oropeza V, Qian Y. Elevated μ-opioid receptor expression in the nucleus of the solitary tract accompanies attenuated withdrawal signs after chronic low dose naltrexone in opiate-dependent rats. J Neurosci Res 2006;83:508–14. https://doi.org/10.1002/jnr.20738.

[32] Ngai SH, Berkowitz BA, Yang JC, Hempstead J, Spector S. Pharmacokinetics of naloxone in rats and in man: basis for its potency and short duration of action. Anesthesiology 1976;44:398–401. https://doi.org/10.1097/00000542-197605000-00008.

[33] Wittert G, Hope P, Pyle D. Tissue distribution of opioid receptor gene expression in the rat. Biochem Biophys Res Commun 1996;218:877–81. https://doi.org/10.1006/BBRC.1996.0156.

[34] Le Merrer J, Becker JAJ, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. Physiol Rev 2009;89:1379–412. https://doi.org/10.1152/physrev.00005.2009.

[35] Holt MK, Cook DR, Brierley DI, Richards JE, Reimann F, Gourine A V., et al. PPG neurons in the nucleus of the solitary tract modulate heart rate but do not mediate GLP-1 receptor agonist-induced tachycardia in mice. Mol Metab 2020;39:101024. https://doi.org/10.1016/J.MOLMET.2020.101024.

[36] Higa KT, Mori E, Viana FF, Morris M, Michelini LC, Morris M, et al. Baroreflex control of heart rate by oxytocin in the solitary-vagal complex. Am J Physiol Regul Integr Comp Physiol 2002;282:537–45. https://doi.org/10.1152/ajpregu.00806.2000.-Previous.

[37] Thomas JJ, Keel PK, Heatherton TF. Disordered eating attitudes and behaviors in ballet students: Examination of environmental and individual risk factors. Int J Eat Disord 2005;38:263–8. https://doi.org/10.1002/eat.20185.

[38] Sundgot-Borgen J, Torstveit MK. Prevalence of eating disorders in elite athletes is higher than in the general population. Clin J Sport Med 2004;14:25–32. https://doi.org/10.1097/00042752-200401000-00005.

[39] Lutz TA, Senn M, Althaus J, Del Prete E, Ehrensperger F, Scharrer E. Lesion of the Area Postrema/Nucleus of the Solitary Tract (AP/NTS) Attenuates the

Anorectic Effects of Amylin and Calcitonin Gene-Related Peptide (CGRP) in Rats. Peptides 1998;19:309–17. https://doi.org/10.1016/S0196-9781(97)00292-1.

[40] Braga DC, Mori E, Higa KT, Morris M, Michelini LC. Central oxytocin modulates exercise-induced tachycardia. Am J Physiol Regul Integr Comp Physiol 2000;278. https://doi.org/10.1152/AJPREGU.2000.278.6.R1474.

[41] Michelini LC, Bonagamba LGH. Baroreceptor reflex modulation by vasopressin microinjected into the nucleus tractus solitarii of conscious rats. Hypertension 1988;11:I-75-I–79. https://doi.org/10.1161/01.HYP.11.2_PT_2.I75.

[42] Matsumura K, Tsuchihashi T, Fujii K, Iida M. Neural regulation of blood pressure by leptin and the related peptides. Regul Pept 2003;114:79–86. https://doi.org/10.1016/S0167-0115(03)00116-2.

[43] Kolb EM, Kelly SA, Garland T. Mice from lines selectively bred for high voluntary wheel running exhibit lower blood pressure during withdrawal from wheel access. Physiol Behav 2013;112–113:49–55. https://doi.org/10.1016/j.physbeh.2013.02.010.

[44] Dufloth DL, Morris M, Michelini LC. Modulation of exercise tachycardia by vasopressin in the nucleus tractus solitarii. Am J Physiol - Regul Integr Comp Physiol 1997;273.

https://doi.org/10.1152/AJPREGU.1997.273.4.R1271/ASSET/IMAGES/LARGE/AREG7095609.JPEG.

[45] Kolb EM, Kelly SA, Garland T. Mice from lines selectively bred for high voluntary wheel running exhibit lower blood pressure during withdrawal from wheel access. Physiol Behav 2013;112–113:49–55. https://doi.org/10.1016/J.PHYSBEH.2013.02.010.

[46] Hillebrand JJG, Koeners MP, De Rijke CE, Kas MJH, Adan RAH. Leptin treatment in activity-based anorexia. Biol Psychiatry 2005;58:165–71. https://doi.org/10.1016/j.biopsych.2005.03.011.