

**Behavioral studies on the role of opioid system in
palatability and acquisition of reinforcement for
dietary fat**

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General introduction

Sweet and fatty foods are highly palatable and voluntary intake of these foods can cause addictive behavior through a reinforcing effect in rodents. These addictive behaviors might be induced by the high palatability and energy density of these foods (1, 2). Previous studies have reported that the palatability of sweet and fatty foods can be attributed to several neural circuits in the brain (3). Specifically, there are many reports regarding the role of the endogenous opioid system in the preference for sucrose and dietary fat (4). An endogenous opioid peptide, beta-endorphin, is known to exhibit effects similar to that of morphine in the nervous system (e.g., analgesia and euphoria). Indeed, it has been reported that intake of sucrose and dietary fat raise beta-endorphin levels in the cerebrospinal fluid of rodents (5, 6). Additionally, it has been reported that administration of opioid receptor antagonists reduced the intake volume of sucrose and dietary fat in rodents (7, 8). These reports suggest that the opioid system contributes to ingestive behavior of sweet and fatty foods. However, the role of the opioid system in ingestive and reward-related behavior is still not clear. In this thesis, we report the results of our studies on the contribution of the opioid system to ingestive and reward-related behavior for sucrose and fat in mice using behavioral and pharmacological methods.

Recently, it has been reported that pro-opiomelanocortin neurons in the hypothalamus, which release beta-endorphin, are more strongly activated after fat ingestion than after to sucrose ingestion in rodents (9). Moreover, the orexigenic or anorexigenic effects of opioid receptor agonists or antagonists are stronger in mice that are fed a high-fat diet compared with those fed a high-carbohydrate diet (10). These reports suggest that the contribution of the opioid system to the palatability of fat is different from its contribution to the palatability of sucrose. In the first chapter, to compare the contribution of the opioid system to the palatability of sucrose and fat, we examined the effects of opioid receptor antagonists on short-term ingestive behavior towards sucrose solutions or fat emulsions in a two-bottle choice test and a licking test. In order to exclude preferences for neutral stimuli, such as touch and smell, acquired by learning, the current study was performed with mice that had no experience ingesting sucrose or fat.

Spontaneous ingestion of sucrose and dietary fat also induces reinforcement for ingestion of these foods, which can be determined by using the conditioned place preference (CPP) test and operant lever-pressing test (11-13). It has been also reported that the opioid system is involved in the regulation of reinforcement for ingestion of these foods (8, 14). However, it is not clear whether the opioid system contributes to the learning and development of palatability and reinforcement or simply contributes to the stimulation itself. In the second chapter, to investigate the physiological role of the opioid system in the palatability of fat and the reinforcement properties of fat in the learning process, we examined the effects of an opioid receptor antagonist on these behaviors in mice that were either habituated or naïve to dietary fat ingestion.

Chapter 1

The opioid system majorly contributes to preference for fat emulsions but not sucrose solutions in mice

Introduction

Sucrose and dietary fat are important energy sources for mammals, including humans, and are strongly preferred by mammals in comparison with other food sources. The recognition of sucrose and dietary fat in the oral cavity is important for their palatability, and it has been reported that the preference for sucrose and dietary fat can be observed within a few tenths of a second in mice (15, 16).

Recent studies have shown that, similar to the receptors for primary tastes, candidate fat taste receptors that bind to fatty acids are expressed on the tongue (17-20). Because mice lacking these fatty acid-binding proteins do not show a strong preference for fat (21-23), it is thought that, like the sweetness of sucrose, chemoreception in the oral cavity is important for dietary fat preference. However, we are not able to recognize the taste of dietary fat while we can recognize the strong sweet taste of sucrose. In rodents, only a slight gustatory nerve response to fat has been observed compared to the relatively large response to sucrose (22, 24). Nonetheless, animals preferably ingest fat over sucrose (25), and they show a stronger craving for fat than for sucrose in an operant task (14). These findings suggest the possibility that the recognition mechanisms in the central nervous system for sucrose and fat are distinct, although information from the oral cavity is still important.

It has been suggested that the endogenous opioid system contributes to the preference and reinforcing effects of fat and sucrose (4). Indeed, the intake of fat and sucrose increases the concentration of beta-endorphin, an endogenous opioid peptide, in the brain (5, 6). In addition, administration of an opioid receptor agonist increases the intake of sucrose and fat, while an opioid receptor antagonist decreases the consumption of both (7, 8, 26, 27). However, pro-opiomelanocortin neurons, which release beta-endorphin, are more strongly activated after fat ingestion compared to sucrose ingestion (9). Similarly, the orexigenic or anorexigenic effects of opioid receptor agonists or antagonists are stronger in mice fed a high-fat diet than in those fed a high-carbohydrate diet (10, 28). These reports suggest that the opioid system is highly

involved in the ingestion of dietary fat compared to sucrose. Because it is thought that the opioid system is involved in food palatability, it is possible that the contribution of the opioid system to the palatability of fat and sucrose is different. Although previous studies have examined the contribution of the opioid system to the amount of fat and sucrose intake over several hours, few studies have examined the contribution of the opioid system to their palatability.

In order to compare the contribution of the opioid system to the palatability of sucrose and fat, we examined the effects of opioid receptor antagonists on short-term ingestive behavior towards sucrose solutions or fat emulsions (Intralipid) in a two-bottle choice test and a licking test. Animals acquire preferences for neutral stimuli, such as touch and smell, indirectly with repeated sucrose and fat ingestion. In order to exclude these factors, the current study was performed with mice that had no experience ingesting sucrose or fat.

Materials and Methods

Animals

Eight-week-old male BALB/c mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan) for each experiment. The mice were housed individually in a vivarium that was maintained at $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ under a 12:12-h light/dark cycle (lights off: 06:00–18:00). Commercial standard laboratory chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water were available *ad libitum*. The mice were allowed to acclimate to their surroundings for at least 1 week after their arrival before they were tested. Newly purchased animals were used for all experiments, which were conducted during the dark phase (08:00–16:00). This study was conducted in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee, and in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Kyoto University Animal Care and Use Committee. Efforts were made to minimize the number of animals that were used and to limit experimentation to that necessary to produce reliable scientific information.

Reagents

Intralipid, which is a commercially available intravenous fat emulsion that contains 10% or 20% soybean oil, 1.2% lecithin, and 2% glycerol to adjust the osmotic pressure, was purchased from Terumo Corporation (Tokyo, Japan). The sucrose was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Naltrexone, naloxonazine, naltrindole, and norbinaltorphimine (nor-BNI) were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA).

Two-bottle choice test procedure

In order to impart training for the selection of the preferred fluid, the mice were simultaneously offered two bottles containing saline and water for 10 min daily over 3 days. In the test session, mice were deprived of water and food for 30 min and then offered the pair of test fluids for 10 min. Intake weights were measured daily. The test fluids were presented randomly in an attempt to avoid any effect of concentration order. Additionally, the left-right positions of the test fluids were alternated daily to avoid a side preference.

Effects of several opioid receptor antagonists on the relative preference between sucrose and Intralipid in naïve mice

We examined the effects of several opioid receptor antagonists in naïve mice. After the training sessions, the two-bottle choice test was conducted with 20% sucrose and 10% Intralipid. Mice were subcutaneously administered the non-selective opioid receptor antagonist naltrexone (0.5 and 2 mg/kg), mu-selective opioid receptor antagonist naloxonazine (2 mg/kg), delta-selective opioid receptor antagonist naltrindole (2 mg/kg), kappa-selective opioid receptor antagonist nor-BNI (2 mg/kg), or saline 30 min before the test.

Effects of naltrexone on daily changes in the relative preference between sucrose and Intralipid

To investigate the influence of fat and sucrose intake experience on the contribution of the opioid system to food preference, we next examined the effects of intermittent naltrexone administration on the daily changes in relative preference between sucrose and Intralipid in naïve mice. After the training sessions, the two-bottle choice test was conducted with 20% sucrose and 10% Intralipid for 10 days. On days 1–3 and 9, the mice were administered saline or naltrexone (2 mg/kg) 30 min before the test. On days 4–8 and 10, the two-bottle choice test was conducted with 20% sucrose and 10% Intralipid without any drug/saline administration.

Effects of food deprivation on relative preference between sucrose and Intralipid in naïve mice

We examined the effects of food deprivation on the relative preference between sucrose and Intralipid in naïve mice. After the training sessions, the mice were deprived of laboratory chow for 0, 12, and 24 h, and the two-bottle choice test was conducted with 20% sucrose and 10% Intralipid.

Effects of naltrexone on the preference for sucrose and Intralipid compared to water in naïve mice

We next examined the effects of naltrexone on the preference of sucrose and Intralipid compared to water in naïve mice. After the training sessions, the two-bottle choice test was conducted with 5%, 10%, and 20% Intralipid and 10%, 20%, and 40%

sucrose paired with water. Mice were subcutaneously administered naltrexone (2 mg/kg) or saline 30 min before the test. The 5% Intralipid was prepared by diluting 10% Intralipid with water.

Apparatus for the licking test

The licking test was conducted in a handmade chamber. The principle and apparatus were similar to those described by Hayar's method (29). Briefly, the mice were placed in a stainless-steel cage (32 cm × 22 cm × 10.5 cm) connected to a standard analog-to-digital converter (Mac Lab/8S; ADInstruments Pty Ltd., Castle Hill, NSW, Australia) through two cables. One cable was attached to a stainless-steel drinking spout (3.5 cm long with an 8 mm orifice; the spout contained an internal stainless-steel ball), and the other was attached to the cage. The spout was protected by a silicone sleeve, into which it was recessed ~5 mm to restrict contact with the paws, nose, jaw, and other body parts. Licking rates were converted from the positive voltage peak every 60 s.

Effects of naltrexone on licking behavior for sucrose and Intralipid in naïve mice

To rule out postingestive effects of the fluids, we examined the effects of naltrexone on the licking behavior for the test samples in naïve mice. The training sessions lasted 5 days. On days 1 to 3 the mice were given saline, and on days 4 to 5 the mice were given water in the licking chamber for 10 min. In the test session, the mice were administered naltrexone (2 mg/kg) or saline 30 min before the test and then offered water, 20% sucrose, or 10% Intralipid for 10 min. The initial licking rate was calculated for 60 s from the first lick. We also measured the latency time, which was defined as the time from the presentation of the solution to ingestion.

Statistical analyses

All values are presented as mean ± SEM. The intake volumes of each fluid in the two-bottle choice test were statistically evaluated by paired *t*-tests. The preference ratios between the groups in the two-bottle choice test were analyzed by unpaired *t*-tests. The initial licking rates and latency times in the licking test were analyzed with one-way analyses of variance followed by Dunnett's post-hoc test (Prism 5.0; GraphPad Software, Inc., San Diego, CA, USA).

Results

Effects of several opioid receptor antagonists on the relative preference between sucrose and Intralipid in naïve mice

Saline-administered mice ingested nearly equivalent amounts of 20% sucrose and 10% Intralipid. However, administration of the non-selective opioid receptor antagonist naltrexone (0.5 and 2 mg/kg) reduced 10% Intralipid intake, and the mice ingested a significantly larger amount of 20% sucrose compared to 10% Intralipid (Fig. 1A). Similarly, administration of the mu-selective opioid receptor antagonist naloxonazine (2 mg/kg) reduced 10% Intralipid intake, and the mice ingested a significantly larger amount of 20% sucrose compared to 10% Intralipid (Fig. 1B). These effects were not seen after administration of the delta-selective opioid receptor antagonist naltrindole (2 mg/kg) or the kappa-selective opioid receptor antagonist nor-BNI (2 mg/kg).

Effects of naltrexone on the daily change in the relative preference between sucrose and Intralipid

On days 1 and 2, saline-administered mice ingested nearly the same amount of 20% sucrose and 10% Intralipid. However, on days 3–10, these mice ingested a significantly larger amount of 10% Intralipid compared to 20% sucrose (Fig. 2A). In contrast, naltrexone administration reduced 10% Intralipid intake, and the mice ingested a significantly larger amount of 20% sucrose than 10% Intralipid on days 1–3. On days 4–8 (no drug administration), the mice gradually ingested larger amounts of 10% Intralipid compared to 20% sucrose. In the mice well habituated to ingesting sucrose and Intralipid, naltrexone-administered mice ingested a still larger amount of 10% Intralipid compared to 20% sucrose (day 9 in Fig. 2B). Compared to saline-administered mice on days 2, 3, 4, and 6, naltrexone-administered mice exhibited a significant reduction in preference for Intralipid compared to sucrose (Fig. 2C). The preferences between the groups were similar on days 7–10 (Fig. 2C).

Effects of food deprivation on the relative preference between sucrose and Intralipid in naïve mice

There were no differences in the relative preferences for 20% sucrose and 10% Intralipid between the freely fed group and the 12- and 24-h fasting groups (Fig. 3).

Effects of naltrexone on the preference for sucrose and Intralipid compared to water in naïve mice

Saline-administered mice ingested a significantly larger amount of 10%, 20%, and 40% sucrose and 5%, 10%, and 20% Intralipid than water (Fig. 4A). However, naltrexone-administered (2 mg/kg) mice ingested nearly the same amount of 5%, 10%, and 20% Intralipid compared to water while ingesting still larger amounts of 10%, 20%, and 40% sucrose compared to water (Fig. 4B). In addition, these mice exhibited a significantly lower preference for Intralipid than saline-administered mice (Fig. 4D), while the two groups showed similar preference for sucrose (Fig. 4C).

Effects of naltrexone on licking behavior for sucrose and Intralipid in naïve mice

In the saline-administered group, the mice showed a significantly higher lick count for the initial 1 min. In the naltrexone-administered mice, the initial lick count for Intralipid was similar to that for water, while the lick count for sucrose was still higher than for water in naïve mice (Fig. 5A). There were no differences in the latency times in all cases (Fig. 5B).

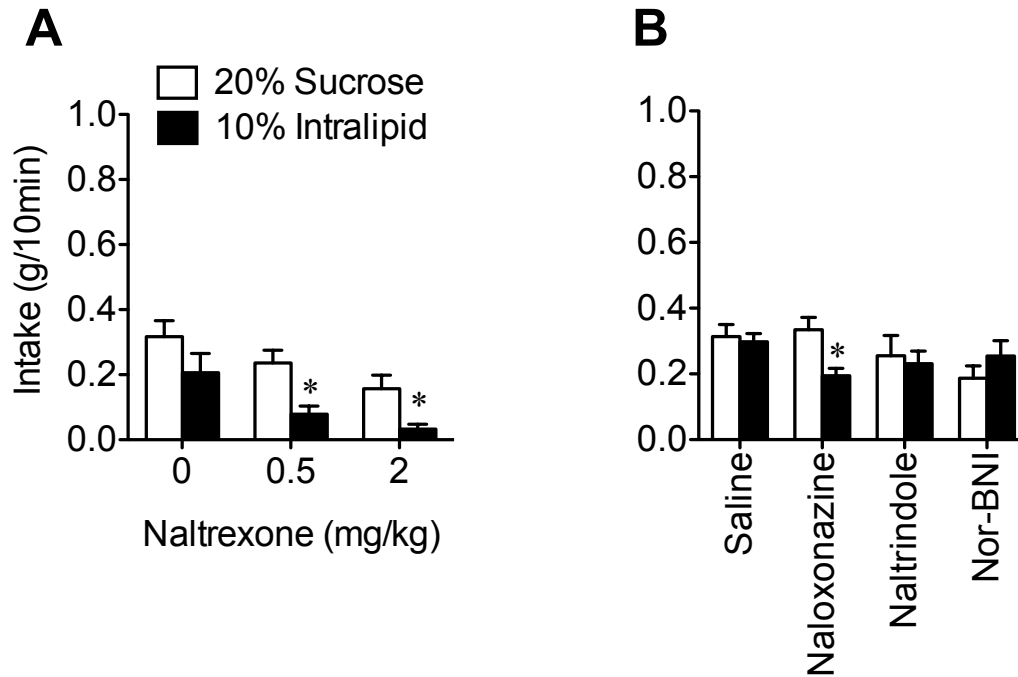


Fig. 1. Effects of various opioid receptor antagonists on the relative preference of 20% sucrose and 10% Intralipid in the 10-min two-bottle choice test.

Fluid intake in mice that were subcutaneously administered saline and 0.5 and 2 mg/kg of naltrexone (A) and saline, 2 mg/kg naloxonazine, 2 mg/kg naltrindole, and 2 mg/kg norbinaltorphimine (nor-BNI) (B) 30 min before the test. The values indicate the mean $\pm$ SEM ($n = 8$). Statistical significance key: * $P < 0.05$ for 20% sucrose vs. 10% Intralipid.

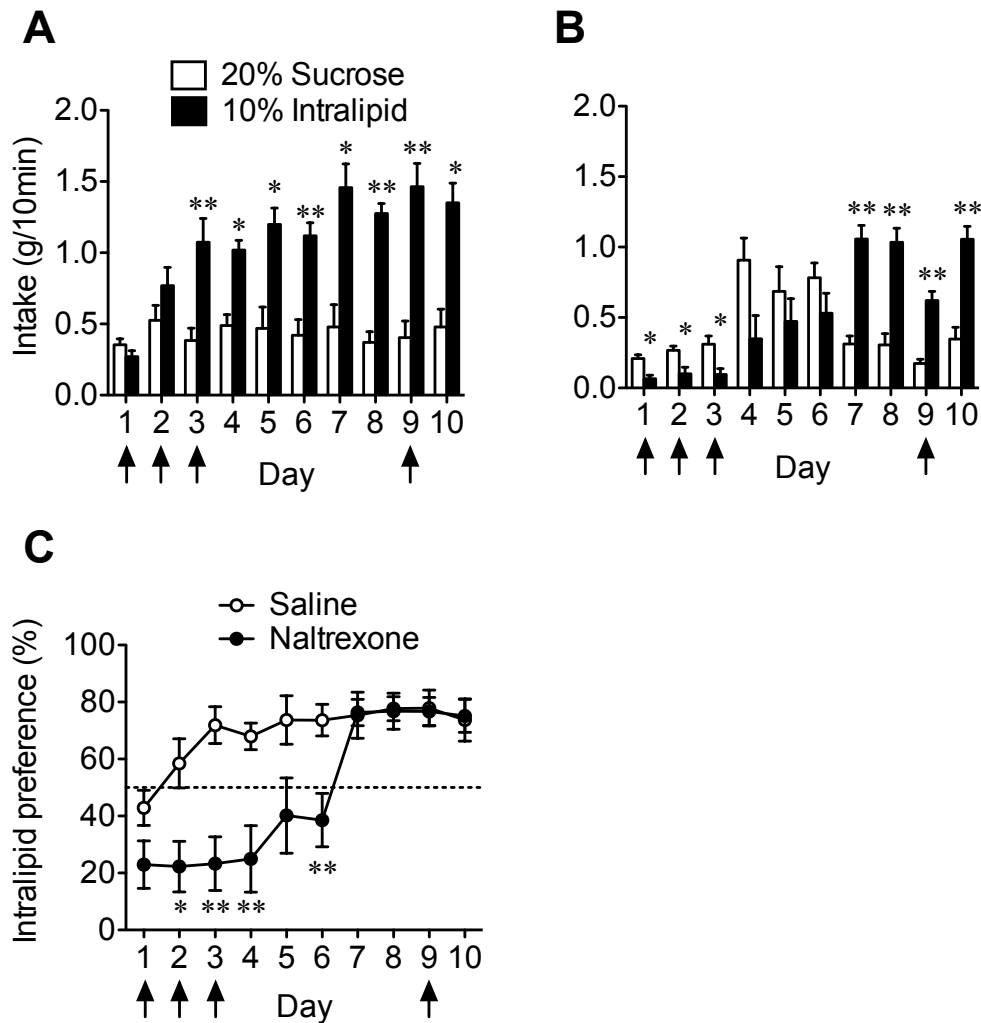


Fig. 2. Effects of naltrexone on the daily change of the relative preference for 20% sucrose and 10% Intralipid in the 10-min two-bottle choice test.

Fluid intake in the mice that were subcutaneously administered saline (A) and 2 mg/kg of naltrexone (B) 30 min before the test on days 1–3 and 9 and the preference ratio of these groups (C). The arrows indicate the day of administration. The values indicate the mean $\pm$ SEM ($n = 8$). Statistical significance key: * $P < 0.05$, ** $P < 0.01$ for 20% sucrose vs. 10% Intralipid, saline vs. naltrexone. The arrows indicate the day of administration.

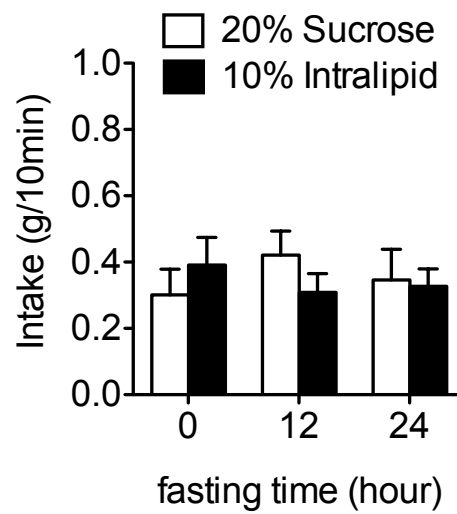


Fig. 3. Effects of food deprivation on relative preference between 20% sucrose and 10% Intralipid in the 10-min two-bottle choice test.

The values indicate the mean $\pm$ SEM ($n = 7$).

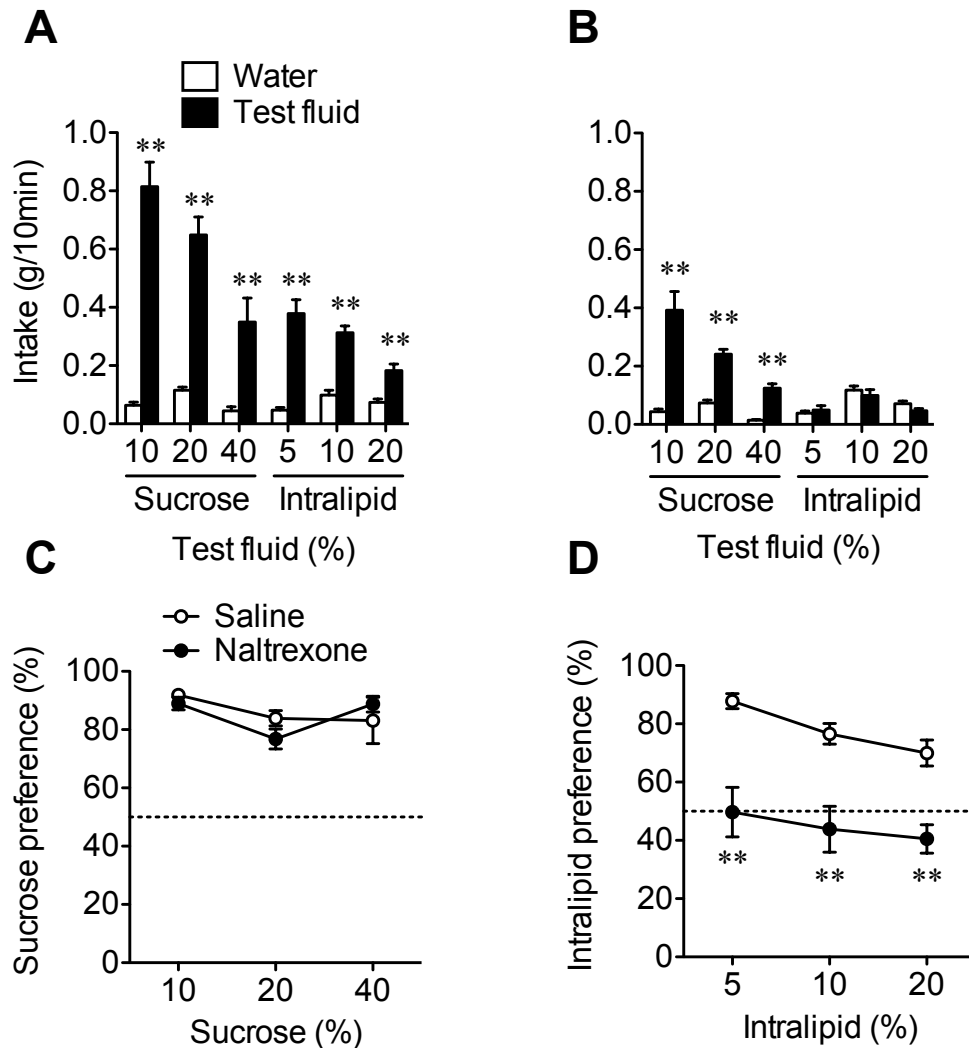


Fig. 4. Effects of naltrexone on the preference for 10%, 20%, and 40% sucrose and 5%, 10%, and 20% Intralipid compared to water in the 10-min two-bottle choice test.

Fluid intake in the mice that were subcutaneously administered saline (A) and 2 mg/kg of naltrexone (B) 30 min before the test and the preference ratio for sucrose (C) and Intralipid (D). The values indicate the mean $\pm$ SEM (n = 8). ** P < 0.01 for water vs. sucrose and Intralipid, saline vs. naltrexone.

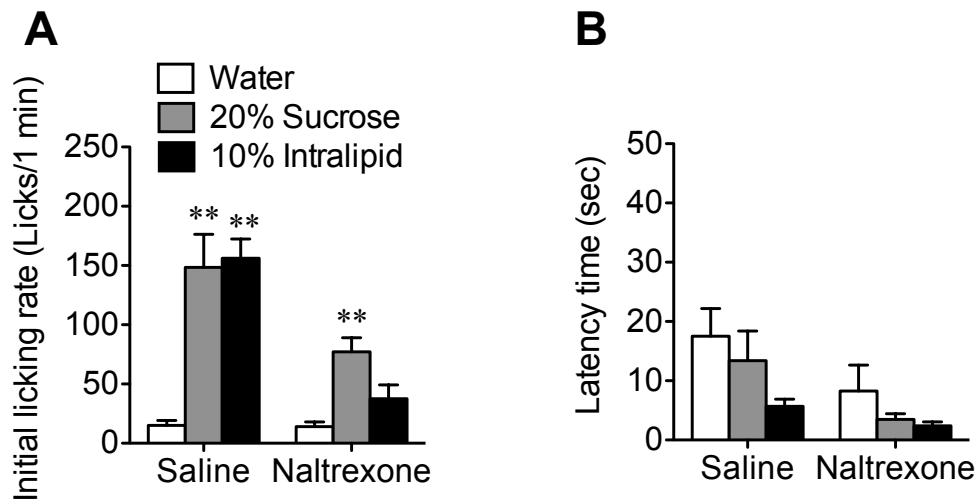


Fig. 5. Effects of naltrexone on short-term licking behavior for water, 20% sucrose, and 10% Intralipid.

The initial licking rate (A) and latency time (B) in mice subcutaneously administered saline and 2 mg/kg of naltrexone 30 min before the test. The values indicate the mean $\pm$ SEM (n = 10). Statistical significance key: ** $P < 0.01$ for water vs. 20% sucrose and 10% Intralipid.

Discussion

Sweet and umami (savory) taste signaling is mediated by the TRPM5 cation channel and the expression of the G-protein gustducin in taste cells (30-32). Mice lacking these proteins show a lower preference for sucrose compared to wild-type mice (31, 33, 34). However, even in mice lacking these proteins, the preference for fat does not differ much from wild type animals (33). In addition, we have reported that fat metabolism inhibitors suppress the palatability of fat, while a fat or carbohydrate metabolism inhibitor does not affect the palatability of sucrose (35, 36). These observations suggest that the mechanisms for the palatability of sucrose and fat differ. In this study, administration of opioid receptor antagonists strongly suppressed the preference for Intralipid compared to sucrose in the two-bottle choice test (Fig. 1). These results suggest that the opioid system is more involved in the palatability of Intralipid than of sucrose. Furthermore, opioid receptor antagonists abolished the preference for Intralipid but not sucrose in the two-bottle choice test and licking test, suggesting that the palatability (even only in the oral cavity) of Intralipid relies heavily on the opioid system, whereas the palatability of sucrose is regulated by other mechanisms in addition to the opioid system (Fig. 4, 5). Additionally, the outcomes of our experiments examining the effect of intake experience and food deprivation on relative preferences suggest that the fluid preferences acquired by intake experience or induced by food deprivation are not significantly regulated by the opioid system (Fig. 2, 3).

It has been reported that the opioid system contributes to the ingestion of foods with high palatability, such as fats and sugars, but is not involved in the ingestion of a standard diet or water (7, 37). In addition, it has been reported that the contribution of the opioid system to food intake is dependent on the intensity of the palatability of the food (38). However, in this study, opioid receptor antagonist administration preferentially suppressed Intralipid ingestion compared to that of sucrose, even though there was no difference between the preference of sucrose and Intralipid in the two-bottle choice test and the licking test (Fig. 1, 5). These results suggest that the opioid system contributes significantly to the palatability of Intralipid rather than sucrose, regardless of the intensity of the palatability. In addition, such effects were observed when the mice were administered a μ -selective opioid receptor antagonist, but not delta- and kappa-selective opioid receptor antagonists. Therefore, these findings

suggest that, unlike preference for sucrose, preference for Intralipid is strongly governed by the opioid system through the mu-opioid receptor (Fig. 1B).

We also examined the effects of the continuous administration of opioid receptor antagonists on the daily changes in the ingestion of sucrose and Intralipid in the two-bottle choice test. In the first 3 days, the opioid receptor antagonist-administered mice ingested a significantly larger amount of sucrose compared to Intralipid (days 1–3 in Fig. 2). In the mice that repeatedly ingested the sucrose and Intralipid, the same effect of the opioid receptor antagonist was not observed (day 9 in Fig. 2). Several studies have demonstrated that opioid receptor antagonist administration does not affect the preference for a palatable food that is acquired by experience with food ingestion (39-41). Therefore, it is possible that in this study, repeated exposure led mice to acquire an opioid system-independent preference based on an association with a feature, such as texture or smell, of the sucrose or Intralipid.

We examined the effects of food deprivation on the relative preference between sucrose and Intralipid. In the current study, food deprivation did not affect preference between sucrose and Intralipid in the 10-min two-bottle choice test, even though previous studies have reported that food deprivation influences intake selection for high-fat diet and high-carbohydrate diet within a few hours. Therefore, deprivation-induced physiological need for nutrients seems to not play an important role in the short-term selection of food, as in the present experimental condition. The current experimental results appear to be the result of the effects of naltrexone on palatability in the oral cavity, but not on the physiological need for nutrients (42, 43).

In the two-bottle choice test and the licking test with mice that had never ingested fat and sucrose, the opioid receptor antagonist-administered mice did not show a preference for Intralipid, although the mice still showed a preference for sucrose (Figs. 4, 5). Although dietary fat is a good energy source, lack of fat ingestion does not affect our biological activity on a short time scale. In addition, animals can accumulate large quantities of fat in their bodies as triglycerides when they excessively ingest fat. These findings suggest that fat intake is regulated mainly by hedonic palatability, which is dependent on the opioid system. Previous studies implied that the opioid system also contributes to sweet taste preference (44, 45). In agreement with these studies, opioid receptor antagonist administration reduced intake volume and licking behavior for sucrose solutions in this study (Figs. 1, 2, 4, and 5). These results indicate that

preference for sucrose is partially regulated by the opioid system. On the other hand, in human volunteer experiments, it has also been reported that administration of opioid receptor antagonists suppresses palatability, but not the sweetness intensity, of sucrose (46, 47). In accordance with these reports, opioid receptor antagonist administration did not entirely abolish the sucrose preference in our study (Figs. 4, 5). Therefore, the preference for sucrose might not only be dependent on the opioid system, but also complexly regulated by other feeding regulatory mechanisms, especially since carbohydrates are required to maintain blood glucose levels. Unfortunately, in the present study, we could not reveal the mechanism underlying the sucrose preference that was not dependent on the opioid system. Further studies are needed to clarify this point.

Opioid receptors are highly expressed in the ventral tegmental area and nucleus accumbens, which are core components of the brain's reward system (48, 49). Dopaminergic neurons that project to the nucleus accumbens from the ventral tegmental area are involved in the craving and palatability of food (50, 51). Because recent studies have reported that mesolimbic dopamine circuits are also involved in the taste-independent palatability of sucrose (52), the dopaminergic system, as well as the opioid system, might play an important role in the palatability of fat compared to sucrose. Other neuropeptides such as orexin and endocannabinoids are also known to be involved in the ingestive behavior of highly palatable food, such as sucrose and fat (53, 54). Future studies are required to address these issues.

In conclusion, the results of the current study suggest that the opioid system is more involved in the palatability of fat than of sucrose. Our results also suggest that in mice that have never ingested fat and sucrose, the palatability of fat primarily involves the opioid system, and that of sucrose is regulated by not only the opioid system, but also through other mechanisms. Our results lend new insights regarding the differences in the palatability between sucrose and dietary fat.

Grants

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Chapter 2

The opioid system contributes to the acquisition of reinforcement for dietary fat but is not required for its maintenance

Introduction

Dietary fats are attractive food components because they make foods palatable. Recent studies indicate that mice and rats prefer consuming not only fatty foods but also dietary fat itself (55-57). We and several other researchers have revealed that rodents and humans have specific receptors for fat molecules in taste bud cells, which indicates that fat makes foods palatable not only through the olfactory system but also through the gustatory system (17, 19, 22, 58). In addition, we reported that the spontaneous ingestion of corn oil induces conditioned place preference (CPP) in a test that evaluated the reinforcing properties of a test sample (8, 57). In an operant responding paradigm, we demonstrated that the rewarding effect of corn oil is enhanced in proportion to its concentration (12) and that it is significantly stronger than that of xanthan gum solution (imitation of oil texture) or non-caloric oil. Thus, dietary fat is not only palatable but also reinforcing.

The endogenous opioid circuit has long been implicated in the regulation of appetite and, in particular, hedonic processes associated with food choice, consumption, and reinforcement (4). There are mainly three subtypes of opioid receptors (μ -, δ -, and κ -opioid receptor); the μ -opioid receptor is especially involved in these behaviors. For example, the administration of non-selective and μ -selective opioid receptor agonists into the nucleus accumbens increases intake of highly palatable foods, such as sucrose solutions, fatty foods, and dietary fat (26, 28, 59). It has also been reported that non-selective and μ -selective opioid receptor antagonists suppress ingestive behavior related to fatty foods (26, 60-62). The opioid system does not affect ingestive behavior for water or standard laboratory chow, which exhibit hedonically neutral palatability (7, 38, 63), suggesting that the opioid receptor is involved only in ingestive behavior related to palatable foods. Recently, we reported that the ingestion of a sweet solution (glucose or sucrose) and dietary fat, but not water or laboratory chow activates beta-endorphin neurons in the hypothalamus, thus promoting beta-endorphin release (6, 9). Beta-endorphin is a known endogenous μ -opioid receptor ligand; hence,

beta-endorphins released by palatable food ingestion might activate the opioid system and promote further ingestion of these foods.

Animals, including humans, show strong reinforcement (craving) toward eating fatty foods and sometimes over-consume them (64). Repetitive ingestion and positive post-ingestion effects are thought to reinforce these types of behaviors. Previously, we reported that daily repetitive ingestion of dietary fat increases the intake and licking response for fat in mice (65, 66). In addition, as mentioned above, we observed that corn oil ingestion induces a reinforcing effect in a CPP test where mice could learn an association between a distinctive environmental cue and a novel and motivationally significant event or rewarding stimulus (8, 11).

These combined results suggest that experience and learning could enhance fat preference and reinforcement for consumption behaviors. Some reviews have suggested that experience and learning are important components in the process of developing the palatability and reinforcement for ingestion (3, 67). It is possible that the opioid system is involved not only in the palatability of fat but also in developing these behavioral processes. Indeed, beta-endorphin-deficient mice showed lower operant responses for fat as a reinforcer when compared to wild-type mice (14, 68). However, it is not clear whether the opioid system contributes to the learning and development of palatability and reinforcement or simply contributes to stimulation itself, because the experimental design in the previous study always inhibited the opioid system during the experimental period.

The present study was designed to investigate the physiological role of the opioid system that underlies the palatability of fat and fat's reinforcement properties in the learning process; we examined the effects of an opioid receptor antagonist (naltrexone) on these behaviors in mice that were either habituated or naïve to dietary fat ingestion.

Materials and Methods

Animals

Eight-week-old male BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan) for each experiment. Newly purchased mice were used for all experiments and 286 mice were used in total. The mice were housed together in groups of 6 in one cage in an animal housing facility maintained at $23 \pm 2^{\circ}\text{C}$ under a 12:12-h (reverse) light/dark cycle (lights off 06:00–18:00; lights on 18:00–06:00). Commercial standard laboratory chow (MF; Oriental Yeast, Tokyo, Japan) and water were available ad libitum. The mice were allowed to acclimate to their surroundings for at least one week after arrival before testing began. All experiments were carried out during the dark phase. This study was conducted in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee and was in complete compliance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals.” All procedures used were approved by the Kyoto University Animal Care and Use Committee. Efforts were made to minimize the number of animals that were used and to limit experimentation only to that which was necessary to produce reliable scientific information.

Effect of naltrexone and naltrindole on Intralipid intake

Mice were used to investigate the effect of naltrexone (n=12) and naltrindole (n=10). All these mice were housed individually, and all training and tests were performed individually. During the training sessions, mice were given access to a bottle containing 20% Intralipid in their home cages for 10 min every alternate day. Mice that ingested 20% Intralipid at least 3 times were considered habituated and as having achieved a stable consumption level. To eliminate the effect of stress, before each training session, the mice were handled to simulate the conditions for injection. And then the mice were access to 20% Intralipid was allowed 30 min after food and water were removed from the cages. During the test session, mice received a subcutaneous (s.c.) injection of the non-selective opioid receptor antagonist naltrexone (0.5 or 2.0 mg/kg), the delta-opioid receptor antagonist naltrindole (2 or 4 mg/kg), or saline concurrent with the removal of food and water. The doses of each drug were determined according to previous reports (8, 13). After 30 min, the mice were offered 20% Intralipid, which was weighed at 0-, 10-, 30-, and 60-min time points, and the

cumulative intake was calculated. Each drug administration was separated by at least 3 days, and mice were offered 20% Intralipid in the absence of either drug between each treatment to confirm that their intake had returned to the baseline amount. The order of testing naltrexone and naltrindole treatments was randomized across mice such that on each test day an approximately equal number of mice received each treatment.

After several exposures to fat ingestion, the animals ingested higher volumes of dietary fat and developed a preference and reinforcement to consume dietary fat (11, 12, 66, 69). To test whether the opioid system might also be involved in this behavior change, we administered naltrexone to mice that had never ingested 20% Intralipid and measured their daily 20% Intralipid intake. Newly purchased mice were used for each experimental group (n=8). Training for fluid ingestion was carried out by using the same protocol described above but using a 20% sucrose solution instead of 20% Intralipid. During the test session, the mice received naltrexone (0.5 or 2.0 mg/kg) or saline via a single administration or 3 times by subchronic administration. In the single-administration group, on Day 1, 30 min after administration, the mice were offered 20% Intralipid for 10 min. From Day 2 to Day 5, in order to exclude the possibility of non-specific effects of naltrexone (e.g., neophobia, aversion learning), intake of 20% Intralipid was recorded for 10 min using the same procedure as used on Day 1 but without any drug/saline administration. For the subchronic-administration group, from Day 1 to Day 3 the mice were offered 20% Intralipid for 10 min every alternate day at 30 min after naltrexone administration. From Day 4 to Day 5, their intake of 20% Intralipid was recorded for 10 min by using the same procedure that was used on Day 1 to Day 3 but without any drug/saline administration.

Measurement of food intake

Newly purchased mice were used to investigate the effect of naltrexone (n=10) and naltrindole (n=10). Food intake was measured 8 h after the lights went off (10:00). The mice were housed individually and were allowed food and water ad libitum during the experiment. Naltrexone (0.5 or 2.0 mg/kg), naltrindole (2 or 4 mg/kg), and saline were administered subcutaneously. Pre-weighed food pellets in each cage were measured for 1-, 2-, 4-, and 8-h periods, and cumulative food intake was calculated. Spilled food was weighed, and food intake was corrected as necessary. Each drug administration was separated by at least 3 days. The order of testing with naltrexone and

naltrindole treatments was randomized across mice such that on each test day an approximately equal number of mice received each treatment.

Apparatus for the operant responding paradigm

The apparatus employed for the operant responding paradigm consisted of outer chambers with a small fan for ventilation, which attenuated the ambient sounds, and an inner chamber that was used as the operant task chamber (Med Associates, St. Albans, VT, USA). The operant task chamber (20 cm × 24 cm × 18 cm) was constructed from Plexiglass and had a metal grid floor. The right wall contained a liquid dipper 1 cm above the floor in the center of the wall, a single retractable lever 6 cm from the dipper on the left side and 2 cm above the floor with stimulus light directly above the lever, and a buzzer on the right side. When the mice accomplished a defined task, the stimulus light and buzzer were activated for 1 s followed by the delivery of 10 µl of the reinforcer into the dipper. A house light was located 10 cm above the floor on the opposite wall, which was illuminated throughout the session.

Procedure for the operant responding paradigm

In order to quantify the reinforced behavior, we measured the break points of operant responses under a progressive ratio-3 (PR3) schedule, which was originally developed as a procedure to measure the magnitude of a reward (70). The procedure was based on previous reports (14, 68) and our previous study (12), with small modifications. To facilitate conducting the training sessions, mice were food-restricted for 5 days to maintain their body weight at 80–90% of their original weight. Five sessions were conducted under a fixed ratio-1 (FR1) schedule using sweetened condensed milk (Morinaga, Tokyo, Japan) as a reinforcer, which was consequently delivered by a single lever press. The session was conducted once a day for 30 min. Mice that did not press the lever more than 50 times within 30 min at last session of the FR1 schedule training were excluded. The mice were then tested in two FR5 sessions. Following this, they were returned to ad libitum feeding for 3 days. After two FR5 sessions under ad libitum feeding, the mice were presented with a progressive ratio (PR) session.

The PR3 schedule involved increasing the number of lever presses by 3 for each subsequent reinforcer presentation (i.e., the first reinforcer required 3 lever presses, the

second reinforcer required 6, the third reinforcer required 9, etc.; $= 3n + 3$). Each PR session was stopped when the mice did not receive the reinforcer within a 15-min time limit on one response schedule. The break point in the PR session was defined as the last ratio level that was completed before 15 min had elapsed without the mouse receiving a reinforcer. After 5 PR3 sessions conducted using condensed milk as a reinforcer, another 5 PR3 sessions were carried out using 20% Intralipid as a reinforcer to stabilize their behavior.

During the experimental session, the PR3 test session was conducted using various concentrations of Intralipid (5 and 20%) and water as the reinforcer. Newly purchased 18 mice were divided into 3 groups (no mice were excluded), and each group received one test sample. The PR3 test session for each test sample was repeated 5 times (5 consecutive days) to stabilize their behavior. All of the mice were tested for all concentrations of Intralipid, and the order of the test solution was randomized across each group (a total of 15 sessions for each subject).

Effects of naltrexone and naltrindole on the break point tested under the PR3 schedule

We next investigated whether naltrexone and naltrindole administration could affect the reinforced behavior for various concentrations of Intralipid. In each experiment, 18 newly purchased mice were used to investigate the effect of naltrexone ($n=16$; 2 mice were excluded), naltrindole ($n=15$; 3 mice were excluded), and (–)-sulpiride ($n=16$; 2 mice were excluded). Mice were trained during the FR and PR sessions described above and used to investigate the effects of the opioid receptor antagonists under a PR3 schedule. The PR schedule was applied as described above by using various concentrations of Intralipid (2, 5, and 20%) as a reinforcer once a day for 5 days (basal setting). During the experimental session, naltrexone (0.5 or 2.0 mg/kg), naltrindole (2 or 4 mg/kg), or a vehicle was administered s.c. 30 min before the test. We also used a dopamine D2 receptor antagonist, (–)-sulpiride (50 and 100 mg/kg), as a positive control described in previous studies (11, 12, 71). Each treatment was separated by at least 3 days, and the mice were trained for the PR3 session by using 2, 5, and 20% Intralipid in the absence of drugs between each treatment to confirm that their break point had returned to baseline. The order of testing with each drug was randomized across mice such that on each test day an approximately equal number of mice received each treatment.

Effects of naltrexone on operant response under the fixed ratio schedule

Mice (n=18) were purchased and divided into 3 groups (no mice were excluded). The mice trained in the FR1 and FR5 sessions through the same procedure described above and were used to investigate the effect of naltrexone on lever pressing behavior under the FR5 schedule. The FR5 schedule was applied as described above using 20% Intralipid as a reinforcer once a day for 5 days (basal setting). During the experimental session, naltrexone (0.5 or 2.0 mg/kg) or saline was administered s.c. 30 min before the test. The number of reinforcers acquired in 30 min was measured under the FR5 schedule. The order of testing with each drug was randomized across the mice such that on each test day an approximately equal number of mice received each treatment.

Apparatus for the conditioned place preference test

We used an automated measurement system modified for the light/dark test. The test chamber (Toyo Sangyo, Toyama, Japan) consisted of two compartments of equal size (200 × 150 × 150 mm) and a small connecting zone (50 × 100 × 150 mm). The walls of the dark box were black, the walls of the light box were transparent, and the lids of both boxes were transparent. Both boxes were equipped with a grid floor, and a hatched transparent sheet was placed on the grid of the dark box. These two compartments were joined at a connecting zone, which had two light-brown walls, a transparent lid, and a rough-textured, dark-brown plate on the grid floor. Two guillotine doors—one black (on the dark-box side) and one transparent (on the light-box side)—separated this zone from each box. The test chamber was set in a Scanet MV-10LD (Toyo Sangyo) equipped with infrared beam sensors to detect animal motion and track the time spent by the mice in each box, not including the connecting zone. The photosensors were set at 6-mm intervals in two rows; the position of the animal was detected by disrupting the path of light transmitted to the photosensors. The sensors measured motion and time spent in each compartment on the basis of changes in the animal's position. The data thus obtained were simultaneously transferred from three detectors to a computer and subsequently stored. The detector and the test chamber were placed in a soundproof box, which contained a small fan and a fluorescent 8 W light above the test chamber.

Procedure for the conditioned place preference test

Each complete cycle of the CPP test was 10 days in duration. On days 1–3, baseline preferences for the light and dark boxes were measured; on days 4–9, mice were conditioned in each box; on day 10, changes in preference as a result of the conditioning were measured. On days 1–3 and 10, the mice were not treated and the time spent in each box was measured for 20 min. On days 4–9, the mice were provided with Intralipid or water and confined to each box for 30 min. On days 1–3 and 10, mice were placed in the connecting zone and both guillotine doors were opened. On days 4–9, one door was opened and closed immediately after the mice entered the open box. With the exception of the mice in the test chamber, all other animals were given ad libitum access to food and water.

The conditioning sessions were carried out as follows. Mice (n=30) were purchased and divided into 3 groups (n=10) and were given access to Intralipid (5 and 20%) or water ad libitum from a bottle inserted through the lid in the light box. On the next day, they were provided with water in the dark box. The mice were thus alternately exposed to the two boxes, where they were offered a specific fluid or treatment on days 4–9. Therefore, during the conditioning session on these days, they were exposed to both conditioning boxes and formed an association between the environment of each box and the intake of a particular fluid.

On the test day (Day 10), each mouse was placed in the box and allowed to move freely between the light and dark zones. The time spent in each zone was measured, and the reinforcing effects were evaluated on the basis of these data.

Effect of naltrexone during conditioning and test sessions on CPP induction

Next, in order to clarify the opioid system contribution to the learning processes of reinforcement for dietary fat, we examined the effect of naltrexone during conditioning and test processes on CPP induced by 20% Intralipid ingestion. In each experiments, 30 mice were purchased and divided into 3 groups (n=10). Measurement of baseline place preference (days 1–3) was conducted normally. When we examined the effect of naltrexone during the conditioning session, mice were treated with naltrexone (0.5 and 2 mg/kg) or saline 30 min before every conditioning session (days 4–9) but not before the test session (day 10). On the other hand, when we examined the effect of naltrexone during the test session on CPP induction, mice were treated with

naltrexone (0.5 and 2 mg/kg) or saline 30 min before the test session (day 10) but not before conditioning sessions (days 4–9).

Reagents

Intralipid, a commercially available intravenous fat emulsion that contains 20% soybean oil, 1.2% lecithin, and 2% glycerol to adjust osmolarity, was purchased from Terumo (Tokyo, Japan). Naltrexone, naltrindole, and sulpiride were purchased from Sigma (St. Louis, MO, USA). All other chemicals used in this study were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Statistical analyses

All values are presented as the mean $\pm$ SEM. The effects of naltrexone or naltrindole administration on intake of Intralipid, chow, and daily fluid were examined by two-way repeated-measures analysis of variance (ANOVA) with the Bonferroni post-hoc test (Prism 5.0; GraphPad Software, Inc.; San Diego, CA, USA). The effects of naltrexone, naltrindole, and sulpiride administration on the break point in the operant responding paradigm test were examined by one-way repeated measures ANOVA with a post-hoc Tukey's multiple comparison test to compare the values to those of the control treatment. The statistical significance of the CPP index was analyzed by Student's paired t-tests, comparing the data from days 3 and 10.

Results

Effect of naltrexone and naltrindole on Intralipid intake

Compared to saline administration (Fig. 1A), administration of naltrexone (0.5 or 2.0 mg/kg) decreased 20% Intralipid intake over a 10-min period from the start of ingestion. Cumulative intake continued to increase in all groups for at least 60 min from the start of ingestion. However, cumulative intake was lower in the naltrexone-treated group than in the saline-treated group during the entire test period. In contrast, naltrindole administration did not affect Intralipid intake at any dose (Fig. 1B).

Effects of naltrexone and naltrindole on food intake

There was no significant effect of naltrexone treatment (0.5 and 2 mg/kg) on food intake over 1-, 2-, 4-, and 8-h periods (Fig. 2A). Similarly, naltrindole treatment (2 and 4 mg/kg) had no significant effect on food intake over 1-, 2-, 4-, and 8-h periods (Fig. 2B).

Effects of naltrexone and naltrindole on the break point tested under the PR3 schedule

The break point for Intralipid reward increased in a concentration-dependent manner (Fig. 3A). At a dose of 0.5 and 2 mg/kg, naltrexone administration did not change the break point for any concentration of Intralipid (Fig. 3B). Subcutaneous administration of naltrindole did not change the break point at any dose (Fig. 4A). Similar to a previous report (12), sulpiride administration significantly reduced the break point in a dose-dependent manner (Fig. 4B).

Effects of naltrexone on operant responses under the fixed ratio schedule

Unlike the result for the PR3 schedule, naltrexone administration significantly reduced the number of reward acquisitions (Fig. 5).

Effects of naltrexone on daily changes in Intralipid intake

In the saline-administered group, Intralipid intake increased daily beginning on the second ingestion (Day 2) and continued to increase until at least Day 5. Naltrexone administration on Day 1 delayed the daily increase of Intralipid intake but mice showed a similar increase in Intralipid intake from Day 2 to Day 5 (Fig. 6A, C). Naltrexone administration 3 times during testing (Day 1 to Day 3) more severely delayed the daily

increase of Intralipid intake (Fig. 6B, D). Especially in the mice administered 2 mg/kg dose of naltrexone, the Intralipid intake did not change during the administration of the drug (Fig. 6D). After the cessation of naltrexone administration, Intralipid intake rapidly increased in the naltrexone-administered group (Fig. 6D). The effects of naltrexone were transient.

Effects of naltrexone during conditioning and test sessions on CPP induction

Mice spent more time in the light box on day 10 conditioned with Intralipid (5 and 20%) intake but not with water intake (Fig. 7). Acquisition of CPP by 20% Intralipid intake was not observed in mice pretreated with naltrexone (2 mg/kg) in any conditioning session (Fig. 8A). In addition, administration of naltrexone in the test session (i.e., after conditioning) did not affect CPP induction by 20% Intralipid ingestion (Fig. 8B).

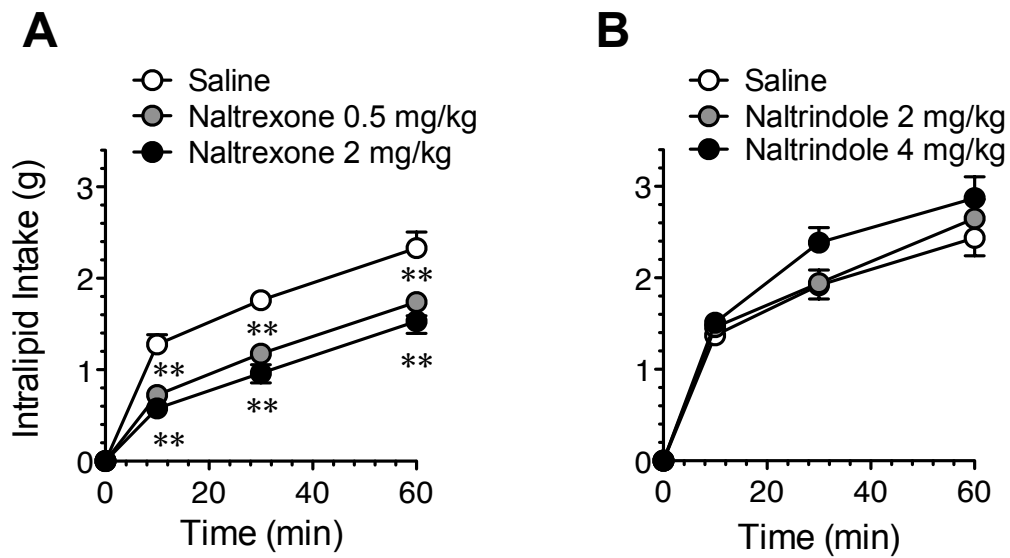


Fig. 1. Effect of naltrexone (A) and naltrindole (B) administration on cumulative 20% iIntralipid intake.

The values are presented as the mean $\pm$ SEM ($n = 10-12$). Statistical significance key:

** $p < 0.01$ for saline vs. 0.5 and 2 mg/kg naltrexone.

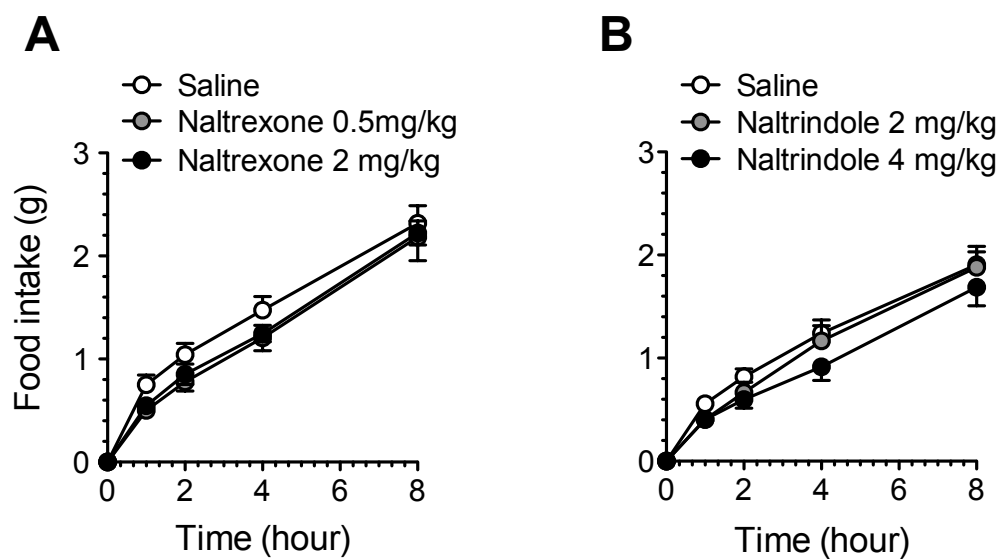


Fig. 2. Effect of naltrexone (A) and naltrindole (B) administration on cumulative food intake.

The values are presented as the mean $\pm$ SEM (n = 10).

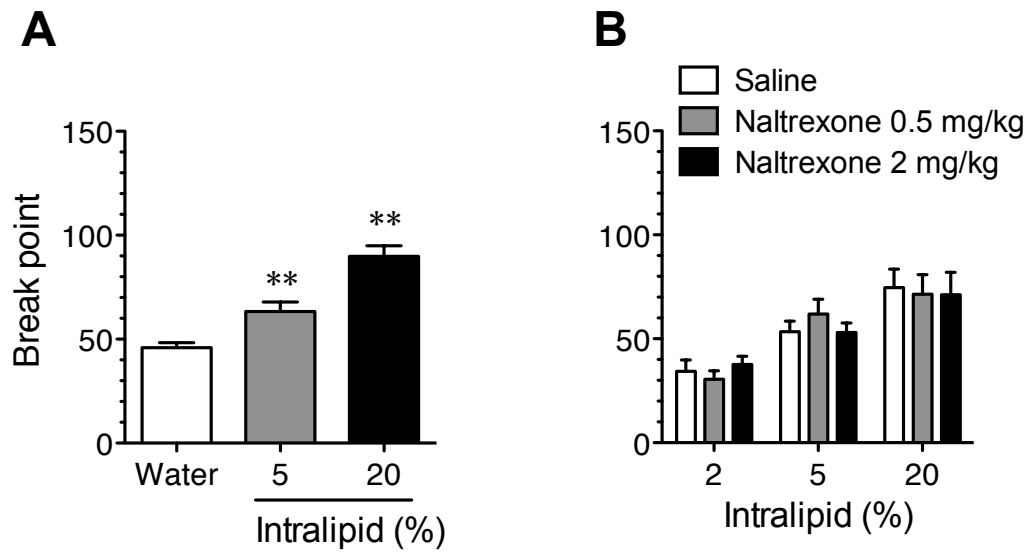


Fig. 3. Effect of various concentrations of Intralipid (5%, and 20%) and water on the break point (A) and effect of naltrexone administration on the break point for various concentrations of Intralipid (B) under PR3 schedule.

The values are presented as the mean $\pm$ SEM ($n = 16-18$). Statistical significance key:

** $p < 0.01$ for water vs. 5% and 20% Intralipid.

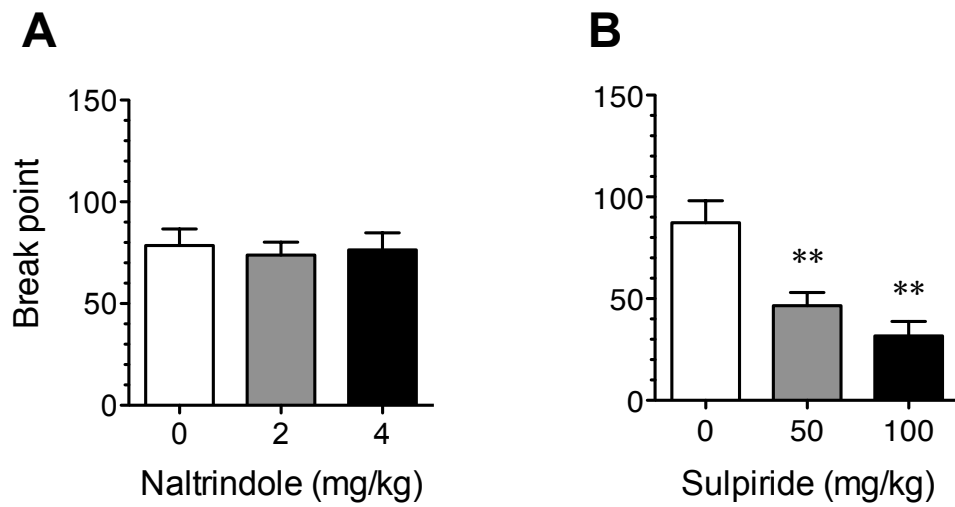


Fig. 4. Effects of naltrindole (A) and sulpiride (B) administration on the break point of 20% Intralipid under the PR3 schedule.

The values are presented as the mean $\pm$ SEM ($n = 15-16$). Statistical significance key:

** $p < 0.01$ for the vehicle vs. 50 and 100 mg/kg sulpiride.

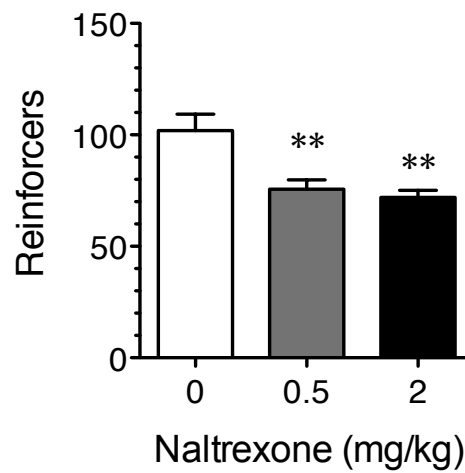


Fig. 5. Effects of naltrexone administration on the number of reward acquisitions (20% Intralipid) under the FR5 schedule for 30 min.

The values are presented as the mean $\pm$ SEM ($n = 18$). Statistical significance key: ** $p < 0.01$ for saline vs. 0.5 and 2 mg/kg naltrexone.

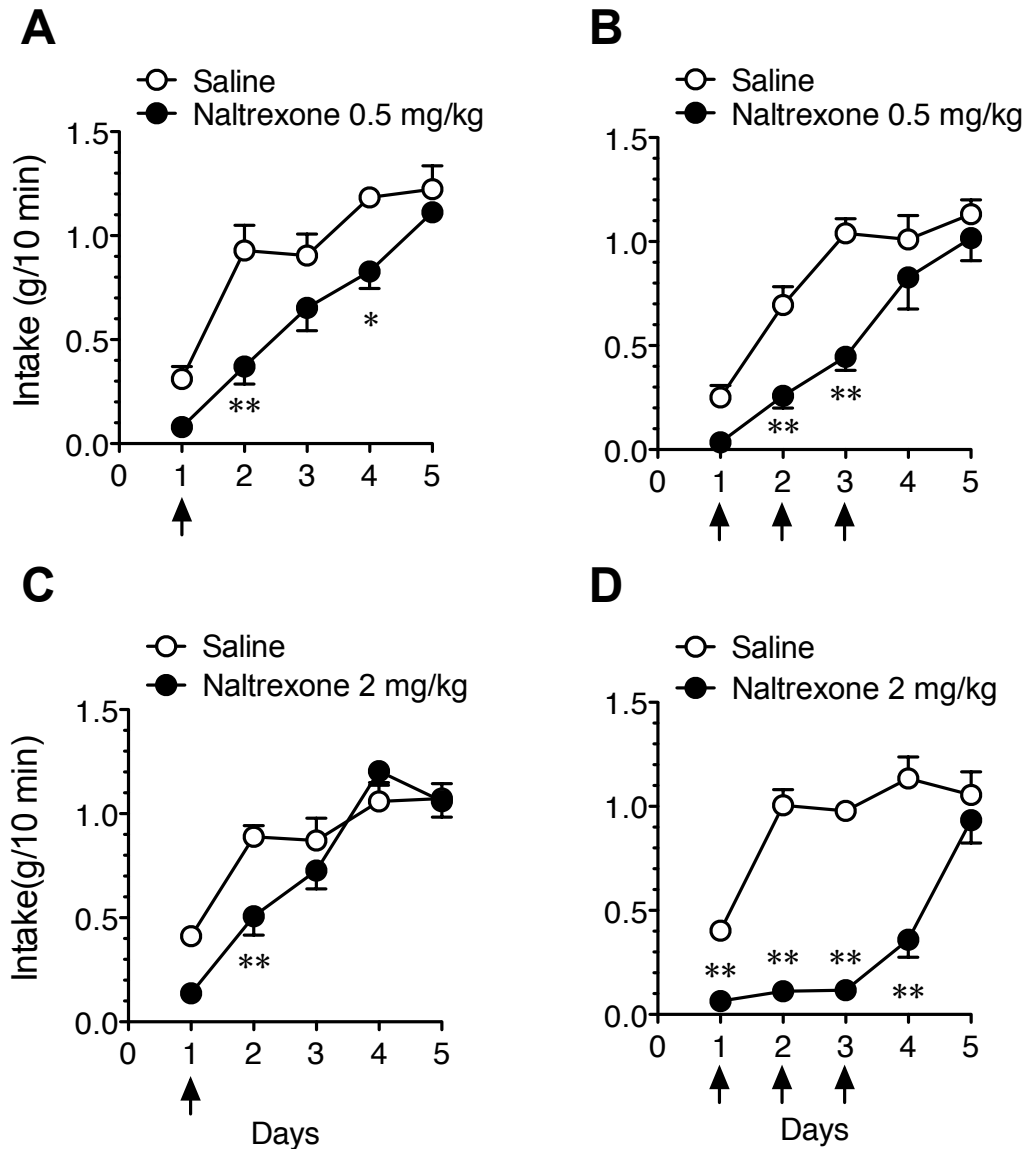


Fig. 6. Effects of naltrexone on daily changes in 20% Intralipid intake.

(A)(C) Mice were administered saline or naltrexone only on Day 1. (B)(D) Mice were administered saline or naltrexone on Day 1 to Day 3. Thirty min after administration, the intake of 20% Intralipid for 10 min was measured daily. The values are presented as the mean $\pm$ SEM (n = 8). Statistical significance key: * $p < 0.05$, ** $p < 0.01$ for saline vs. naltrexone. The arrows indicate the day of administration.

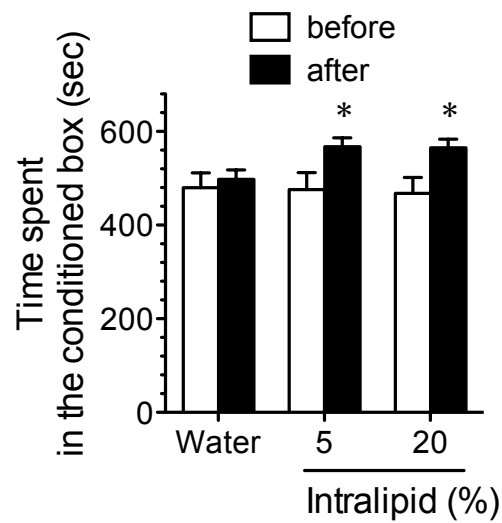


Fig. 7. The reinforcing effects of voluntary ingestion of Intralipid (5%, and 20%) and water in the conditioned place preference test (CPP).

The values are presented as the mean $\pm$ SEM (n = 10). Statistical significance key: * $p < 0.05$ for the before vs. after conditions.

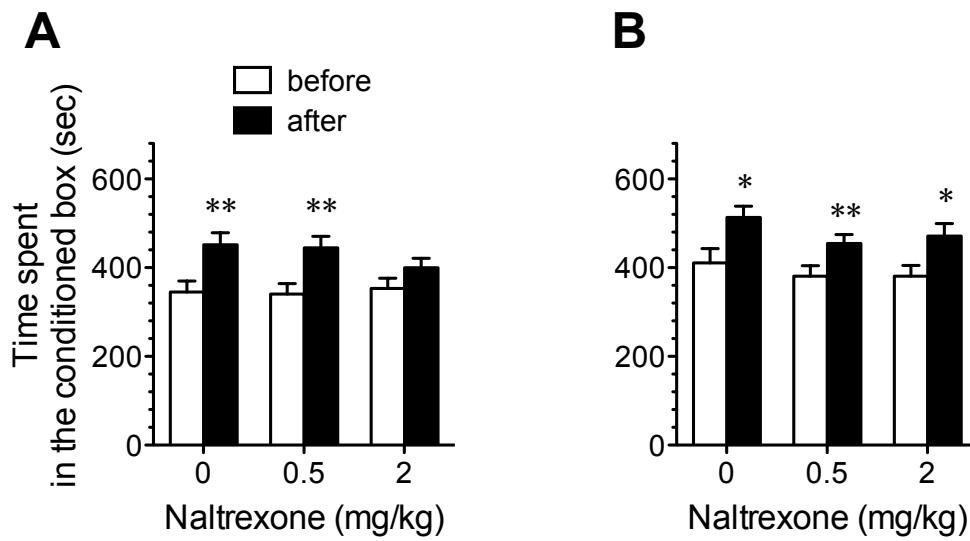


Fig. 8. Effects of naltrexone administration during conditioning session (A) and test session (B) on CPP induced by 20% Intralipid ingestion.

The values are presented as the mean $\pm$ SEM ($n = 10$). Statistical significance key: * $p < 0.05$, ** $p < 0.01$ for the before vs. after conditions.

Discussion

In this study, we observed that opioid signaling plays an important role in the regulation of dietary fat intake and learning processes underlying reinforcement. However, once mice acquired reinforcement for ingesting fat, the effect of naltrexone became weakened against the reinforced behavior in an operant task under PR scheduling and the CPP test. These results suggest that the reinforcement for ingesting fat may be divided into two phases: acquisition and maintenance. It also suggests that the opioid system is mainly involved in the acquisition phase of reinforcement.

Several studies demonstrated that the non-selective opioid receptor antagonist naltrexone decreases the intake of sweet-tasting solutions and dietary fat (13, 26, 65, 72, 73). Similar to these previous studies, we observed that naltrexone decreases Intralipid intake. It has been reported that naltrexone shows high affinity for the mu-opioid receptor and little affinity for the delta-opioid receptor (74, 75). In this study, naltrindole, which is a delta-opioid receptor antagonist, did not change Intralipid ingestion. In addition, naltrexone administration did not change food intake of the mice.

Taken together, our current findings and previous reports suggest that the opioid system regulates the amount ingested of dietary fats or sweet-tasting solutions but not of laboratory chow. In particular, the mu-opioid receptor seems to be involved in this regulation. Food intake is regulated not only by food preference but also by various humoral or neuronal factors and information about ingested food availability and nutrients that are stored in the body. Considering that the preference for laboratory chow is neutral (similar to water), our results suggest that naltrexone is unlikely to change the factors that regulate food intake, except for food preference. Alternatively, the naltrexone-treated mice might have acquired a sense of satiation earlier than the saline-treated mice did. Further studies are needed to test this.

To investigate the role of opioid signaling on daily changes in Intralipid intake, we administered naltrexone to mice that had never ingested Intralipid. Because mice are neophobic to unfamiliar foods, both groups showed a neophobic response and consumed less Intralipid on Day 1. For mice that were administered naltrexone, the average Intralipid intake was below 0.1 g and did not increase further during the naltrexone treatment (Day 1 to Day 3; Fig. 6D), whereas the saline-administered group showed a daily increase in Intralipid intake. These results suggest that opioid signaling is involved in the daily increase in Intralipid intake by naïve animals. After the cessation

of naltrexone administration, Intralipid intake rapidly increased in the naltrexone-administered group (Day 4 and Day 5; Fig. 6D), which indicates that naltrexone did not likely induce taste aversion learning associated with nausea.

Several studies have demonstrated that the opioid system is involved in the expression of hedonic behavior and reinforced behavior with respect to palatable food intake (7, 38, 76). Injection of the selective mu-opioid receptor agonist not only elicits the ingestion of a high-fat diet and sucrose solution but also increases the break point under a PR schedule of an operant responding paradigm (27, 28, 62). However, in the current study using Intralipid as a reinforcer, naltrexone could not affect the break point under a PR schedule even for a dose that significantly reduced Intralipid intake (0.5 and 2 mg/kg), as shown in Fig. 1. Similar to naltrexone, the delta-opioid receptor antagonist naltrindole had no effect on the break point. On the contrary, for the FR5 session, naltrexone significantly decreased the operant response (Fig. 5). Considering that the reward volume in our experimental condition was 10 μ l, the total volume of Intralipid intake that was acquired during the FR5 session in Fig. 5 by the saline group (1.02 ± 0.07 g) was comparable to that of spontaneous ingestion, as shown in Fig. 1A in the saline group (1.76 ± 0.1 g). Therefore, the decrease in operant response by naltrexone during the FR5 session may have been due to reduced ingestion rather than a reduction in reinforced behavior or the reward value of Intralipid. The number of reward acquisitions during the PR3 session was lower (total intake volume of 20% Intralipid in Fig. 3A was 0.27 ± 0.02 g), and the test period was longer than that of the FR5 session. Therefore, unlike the FR5 session, the intake volume was not a critical factor for determining the break point and reward value during the PR3 session. Thus, it might be possible that the opioid system regulates mainly the intake volume of dietary fat but not the reinforcement of ingestion.

In order to investigate the contribution of the opioid system to the acquisition process of reinforcement, we examined the effects of naltrexone during the conditioning and test sessions on Intralipid intake-induced CPP in naïve mice. High-dose naltrexone (2 mg/kg) administration suppressed CPP induction, and significant CPP induction was not observed when the drug was administered during conditioning sessions. This result is consistent with past findings (8). Meanwhile, there was no effect of naltrexone when the drug was administered during the test session. These results suggest that the opioid system may play an important role in developing reinforced behaviors, but not in

causing the behaviors themselves. After several experiences of Intralipid ingestion, other factors such as dopamine might overwhelm the opioid system to regulate the reward-related behavior.

Previous studies have reported that naltrexone administration suppressed the consumption of sucrose even in esophagostomized rats (77). It has also been reported that the opioid system was not activated by sucrose ingestion when mice underwent conditioned taste aversion to sucrose or were administered sucrose directly into the stomach (5, 9). These reports suggest that the opioid system contributes to the induction of food palatability in the oral cavity. Furthermore, our previous studies raise the possibility that beta-endorphin in the brain is released immediately after ingestion of fat, although the anticipation of fat intake increases expression of the hypothalamic pro-opiomelanocortin gene (6, 65). Taking into account the current results, the opioid system appears to contribute to enhancing palatability and reinforcement via regulation of palatability in the oral cavity when animals ingest fat. However, contrary our results it has been reported that administration of an opioid receptor antagonist did not suppress CPP induced by sucrose ingestion (78). Considering reports that some opioid receptor antagonists and agonists greatly affect the ingestion of a high-fat diet compared with a high-carbohydrate diet (10, 28), the opioid system may not play an important part in the enhancement of palatability and reinforcement for carbohydrate ingestion.

In conclusion, our study suggests that the opioid system plays two important roles in the regulation of fat ingestive behavior. First, it is involved in the regulation of the amount of ingestion, which may have an influence independent of previous experience with fat ingestion. Additionally, activation of the opioid system may be involved in dietary fat palatability. Second, the opioid system is also involved in the enhancement of ingestive and reinforcing aspects of fat in naïve mice. On the other hand, our results indicate that the opioid system does not play an important role in the reinforcing aspects of dietary fat ingestion in mice habituated to fat ingestion. The opioid system may be indirectly involved in these types of behaviors.

Grants

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Summary

Chapter 1

Rodents show a stronger preference for fat than sucrose, even if their diet is isocaloric. This implies that the preference mechanisms for fat and sucrose differ. To compare the contribution of the opioid system to the preference of fat and sucrose, we examined the effects of mu-, delta-, kappa-, and non-selective opioid receptor antagonists on the preference of sucrose and fat, assessed by a two-bottle choice test and a licking test, in mice naïve to sucrose and fat ingestion. Administration of non-selective and mu-selective opioid receptor antagonists more strongly inhibited the preference of fat than sucrose. While the preference of fat was reduced to the same level as water by the antagonist administration that of sucrose was still greater than water. Our results suggest that the preference of fat relies strongly on the opioid system, while that of sucrose is regulated by other mechanisms in addition to the opioid system.

Chapter 2

The opioid system plays an important role in ingestive behavior, especially with regard to palatable high-fat or sweetened foods. In the present study, we investigated the role of the opioid system in the regulation of ingestive behavior in mice with regard to dietary fat intake, reinforcement, and particularly the processes involved in development of these behavior types. Subcutaneous administration of the non-selective opioid receptor antagonist naltrexone reduced the spontaneous intake of fat. We investigated the effect of naltrexone on reinforcement by using an operant behavioral paradigm under a progressive ratio schedule. Mice showed stronger reinforcement by fat as a function of concentration. However, naltrexone did not affect reinforcement at any concentration of fat in mice that had repeatedly ingested fat before testing was carried out. Fat ingestion also induced conditioned place preference (CPP), which is another evaluation index of reinforcement. High-dose naltrexone administration during CPP conditioning suppressed the reinforcement induced by fat ingestion, although the drug administration during CPP testing did not affect reinforced behavior. Our results indicate that the opioid system plays an important role in acquiring reinforcement for fat but is not required for maintenance of learned reinforcement.

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2. Sakamoto, K. *, Matsumura, S. *, Okafuji, Y., Eguchi, A., Yoneda, T., Mizushige, T., Tsuzuki, S., Inoue, K., and Fushiki, T. (2015) The opioid system contributes to the acquisition of reinforcement for dietary fat but is not required for its maintenance. *Physiology & behavior*, in press

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