Elucidation of the central role of long-chain fatty acids in the palatability of dietary fat by neuroscientific and animal behavioral studies

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CONTENTS

GENERAL INTRODUCTION

Most animals, including rodents and humans prefer fat-rich foods (1, 2). The ingesting of corn oil has been reported to produce a reward effect in mice, and the dopaminergic pathway in the nervous system has been implicated in the manifestation of this effect (3, 4). Additionally, sham feeding of corn oil were reported to induce the activation of the midbrain dopamine (DA) system, which is involved in reward behavior (5). The mesolimbic system is thought to play a critical role in the reward effect, and the release of DA has been demonstrated when a natural and drug reward is acquired or when its acquisition is anticipated (6, 7) The midbrain dopaminergic circuits originate from the ventral tegmental area (VTA) and project to different sites, such as the nucleus accumbens (NAc), amygdala, and the prefrontal area, which are related to motivation, palatability, and addiction (8–10). Beta-endorphin is known as an endogenous opioid peptide and μ-opioid receptor ligand. Tanda *et al*. demonstrated that intra-VTA infusion of naloxonazine, μ_1 opioid receptor antagonist, prevented the NAc DA increase by ingestion of palatable food and suggested that μ_1 receptors in the VTA depend on the activation of the midbrain dopamine system by the palatability food intake (11). Matsumura *et al.* found fat emulsion ingestion increased c-fos expression in beta-endorphin neurons of the mouse hypothalamus (12). Mizushige *et al.* demonstrated corn oil ingestion induced beta-endorphin levels in the serum and cerebrospinal fluid in the rats (13). Iwakura *et al.* showed that the secretion of ghrelin known as a hormone to stimulate eating was induced by DA in a ghrelin-producing cell line MGN3-1 (14). Since the extracellular concentration of DA in the NAc of the rat has been increased dose-dependently by self-administration of cocaine or consumption of sucrose, release of DA in the NAc could be considered as a kind of index for the palatability or motivational drive (15, 16). However, which property of fat caused the increase in DA release has not been elucidated. Fat palatability could be explained by such factors as the unique texture of fat, flavor, high caloric density per unit mass, and chemical reception of fatty acids on the tongue (17–19), all of which might result in increased DA release during fat ingestion.

Recent studies have revealed that chemoreceptions of long-chain fatty acids (LCFAs) are involved in the recognition of fatty foods. Kawai *et al.* reported that, when fat was introduced into the oral cavity of rats, a certain percentage of triacylglycerides

was hydrolyzed to LCFAs by lingual lipase (20). Gilbertson *et al.* demonstrated the regulation of K^+ channels in type II taste cells by unsaturated LCFAs and suggested the presence of fatty acid chemoreceptors in taste cells (21). Fukuwatari *et al.* found that CD36 fatty acid transporter was expressed on the apical side of taste cells in the circumvallate papillae (22). In addition, *CD36*-deficient mice were reported to show a low taste preference for fat (23). Moreover, Matsumura *et al.* reported that G protein-coupled receptor GPR120 was also expressed on the apical side of taste cells in the circumvallate papillae (24). The unsaturated LCFAs, such as oleic acid and linolenic acid, induced a rise in concentration of intracellular calcium ion ($[Ca^{2+}]_i$) in Human Embryonic Kidney 293 (HEK293) cells stably expressing GPR120. On the other hand, LCFA esters and capric acid did not induce its rise (25).In a licking behavior test, it was found the mice exhibited equally strong preference for a low concentration of linoleic acid as that for 100% corn oil (26). Additionally, the mice and rats displayed a similar strong preference for LCFAs, such as oleic, linolenic, and linoleic acid, whereas they did not show any preference for LCFA esters nor long-chain fatty alcohols (27, 28). Compared with wild-type mice, GPR120 knock-out mice displayed a lower preference for LCFAs (29). These findings lead us to postulate that, in the oral cavity, fat is hydrolyzed to LCFA by lingual lipase and that chemoreception of this LCFA by receptor proteins, such as CD36 and GPR120 expressed on the taste cells, could be involved in the oral recognition and palatability of fats. The purpose of the present study were to elucidate the involvement of LCFA, which is a component of fat, in the increased release of DA in NAc during corn oil ingestion and to examine which structural characteristics of LCFA are responsible for the palatability of fat.

In chapter 1, we studied the change in the extracellular concentration of DA in NAc by *in vivo* microdialysis when rats ingested a low concentration of LCFA. Similar measurements were made in the basolateral amygdala (BLA), which is projected by the dopaminergic nerve system from VTA, as well as to NAc. Since the DA level in BLA increased with the stimulation of reward expectation (30) and cue-responsive neurons in BLA encode the motivating or reinforcing properties of a cue previously associated with a reward (31), the DA response in BLA is thought to be related to reward acquisition.

In chapter 2, we examined the relationship between the GPR120-agonistic properties of ligands and the palatability of LCFAs. First, using HEK 293 cells stably

expressing human GPR120, we examined the effect of various LCFAs on the concentration of $[Ca^{2+}]_i$ by using a fluorescence spectrophotometer. We then assessed the palatability for a variety of LCFAs at a low concentration by testing the licking behavior of the mice. Next, we studied the change in the extracellular concentration of DA in the NAc of the mice by *in vivo* microdialysis after ingestion of various low-concentration LCFAs.

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CHAPTER 1

Effect of long chain fatty acid on dopamine level increase in the nucleus accumbens by ingesting dietary fat

Animals, including rodents and human beings, prefer fat-rich foods (1, 2). The ingestion of corn oil has a strong reward effect on mice, and the involvement of dopaminergic pathways in the nervous system has been implicated in the manifestation of this effect (3, 4). The mesolimbic system in the brain is thought to play a critical role in the reward effect, and the release of dopamine (DA) has been demonstrated when a natural or drug reward is acquired or its acquisition is anticipated (5, 6). The midbrain dopaminergic circuits originate from the ventral tegmental area (VTA), and project to such sites as the nucleus accumbens (NAc), amygdala, and prefrontal area which are respectively related to motivation, palatability, and addiction (7–9). Since the extracellular concentration of DA in NAc has been increased dose-dependently by self-administering cocaine and ingesting sucrose in rats, the DA release in NAc could be considered as a kind of index of palatability or motivational drive (10, 11). In fact, sham feeding corn oil or sucrose has been reported to increase the DA level in NAc of rats (12), and a similar increase in DA released by cocaine administration has also been reported to occur in the amygdala (13). However, which property of fat caused the increase in DA release has not been elucidated. Fat palatability could be explained by such factors as the unique texture of fat, odor, high caloric density per unit mass, and chemical reception of fatty acids on the tongue (14–16), all of which might result in increased DA release during fat ingestion.

We focused the present study on the chemoreception of fatty acids on the tongue. Kawai *et al.* have reported that when fat was introduced into the oral cavity of rats, a certain percentage of triacylglycerides was hydrolyzed to long-chain fatty acids (LCFAs) by lingual lipase (17). Gilbertson *et al*. have shown the regulation of K^+ ion channels in type II taste cells by long-chain unsaturated fatty acids and suggested the presence of fatty acid chemoreceptors in taste cells (18). The protein candidates related to the recognition of LCFAs in the oral cavity are CD36 and G-protein-coupled receptor 120 (GPR120). We have found the expression of CD36 and GPR120 on the apical side of taste cells in the circumvallate papillae (19, 20). In addition, CD36- or GPR120 gene-

deficient mice have been reported to show a low taste preference for fat (21, 22). These findings lead us to postulate that fat introduced into the oral cavity is hydrolyzed into LCFA by lingual lipase and that chemoreception of this LCFA by proteins such as CD36 and GPR120 expressed on the taste cells would function as energy sensors which transduce the presence of fat in the oral cavity. The purpose of the present study was to elucidate the involvement of LCFA, which is a component of fat, in the increased release of DA in NAc during corn oil ingestion. We studied the change in the extracellular concentration of DA in NAc by *in vivo* microdialysis when rats ingested a low concentration of LCFA. We used 1% linoleic acid diluted with mineral oil in the experiments. This concentration of fatty acid was in the range that could be released from fat by lingual lipase and had only a 1/100 calorie content when compared with the same weight of fat. Similar measurements were made in the basolateral amygdala (BLA), which is projected by the dopaminergic nerve system from VTA, as well as to NAc. Since the DA level in BLA increased with the stimulation of reward expectation (23) and cue-responsive neurons in BLA encode the motivating or reinforcing properties of a cue previously associated with a reward (24), the DA response in BLA is thought to be related to reward acquisition.

Materials and Methods

Animals. 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, and was approved by the above-mentioned committee. Male Wistar rats (Japan SLC, Hamamatsu, Japan) at 8 weeks old were housed in stainless wire mesh cages in a room controlled by a 12-h light–dark cycle (dark phase of 18:00–6:00) and constant temperature (24±1 ºC). They were separately housed for a week for acclimatization to the environment. The animals were fed tap water and regular MF rat food (Oriental Yeast, Tokyo, Japan) ad libitum.

Materials. Corn oil was purchased from Ajinomoto (Tokyo, Japan), and mineral oil was purchased from Kaneda Company (Tokyo, Japan). Linoleic acid from Sigma (St. Louis, MO, USA) was 99% pure, stored at –20 ºC until needed and then diluted 1%

with mineral oil for use in this experiment. The other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

Training protocols for oil ingestion. To allow the rats to get accustomed to ingesting corn oil, mineral oil, and 1% linoleic acid, the rats were fed these liquids in their home cages before surgery and then in the microdialysis cage after recovery from surgery. The rats were deprived of water and food for 4 h and the liquids presented for 30 min. Before surgery, the rats were presented with corn oil and mineral oil at the same time on days 1 and 4, with mineral oil and 1% linoleic acid on days 2 and 5, and with 1% linoleic acid and corn oil on days 3 and 6. To confirm the rats' preference for corn oil *versus* mineral oil, the rats were subjected on day 7 to a 2-bottle preference test for corn oil *vs*. mineral oil. The bottle of each liquid was positioned randomly. After recovering from the surgery, the rats were presented one kind of liquid in the microdialysis cage as follows: corn oil on days 1 and 4, mineral oil on days 2 and 5, and 1% linoleic acid on days 3 and 6. The rats were then subjected to a microdialysis test on day 7.

Microdialysis. Surgery: The animals were anesthetized with pentobarbital sodium (Nembutal; Dainippon Pharmaceutical Co., Tokyo, Japan) and placed in a stereotaxic frame adapted for rat surgery. The skull was subsequently exposed, and holes for microdialysis were drilled. The respective coordinates for the NAc shell and amygdala guide cannula (AG-10; Eicom, Kyoto, Japan) were AP, 1.7; ML, 0.2; and DV, 8.0; and AP, –2.8; ML, 5.0; and DV, 7.5 from the bregma. All coordinates were determined according to the stereotaxic atlas of Paxinos and Watson (25). The cannulas were secured to the skull with a LOCTITE 454 adhesive bond (Henkel Japan, Yokohama, Japan). A dummy AD-10 cannula (Eicom) was inserted into the guide cannula and secured with an AC-1 cap nut (Eicom). The rats were allowed 5–7 d to recover from the surgery. Each rat implanted with a probe in NAc or BLA was provided for one-time microdialysis with one kind of test liquid.

Procedure: The experiments were conducted during the light period. The dummy cannula was removed on the day of the experiment, and the AI-10-2 microdialysis probe (Eicom, 2.0mm membrane length) was inserted into NAc or the amygdala via the guide cannula. The rats were placed in the microdialysis cage at 10:00 a.m. for 4 h without food and water, and then presented with the test liquid at 02:00 p.m. for 10 min. The

amount of liquid ingested was also recorded. The rats remained in the microdialysis cage for another 110 min after presenting the test liquid. Ringer's solution, containing 147mM Na^+, 4mM K^+, 2.3mM Ca^{2+}, and 155.6mM Cl^- was perfused at 2 mL/min by using an ESP-64 micro-syringe pump (Eicom), dialysate collection being started 20 min before the liquids were presented and conducted every 5 min for a total of 140 min thereafter. To quantify the DA levels in the dialysate, samples were analyzed by reversed-phase HPLC with an electrochemical detector, using an Eicompak PP-ODS column (4.6 i.d. × 30 mm long; Eicom). The applied voltage was set at 450mV (relative to an Ag/AgCl reference electrode). The mobile phase at a flow rate of 500 mL/min consisted of a 99% (v/v) 0.1M phosphate buffer at pH 6.0, 1% (v/v) methanol, 500 mg/L of sodium decane sulfate, and 50 mg/L of 2Na-EDTA. The mean value obtained from 3 samples from –20 to –5 min was set as the 100% baseline level, and all subsequent sample values were expressed as a percentage of the baseline value.

Histological analysis. After completing the experiment, the rats were deeply anesthetized with sodium pentobarbital. The brain was removed from the skull, frozen and cut into 30-μm sections. The placement of the microdialysis probe was verified by thionine blue staining. Data obtained from the rats with inappropriate probe placement were excluded from the analysis.

Statistics. Data are expressed as the mean ± SEM. Data from the 2-bottle preference test were analyzed by a paired *t*-test. Changes in DA levels were compared with the corresponding baseline value by one-way ANOVA and Tukey's multiple-comparison test as a post-hoc test. Mean differences among 3 groups at each time point were analyzed by two-way repeated-measures ANOVA and Bonferroni's multiple-comparison test as a post-hoc test. The amounts of each fluid ingested during microdialysis were analyzed with one-way ANOVA and the Tukey–Kramer test as a post-hoc test. *p* values of 5% or less were considered statistically significant. Statistical analyses were conducted by using the Prism 4 software package (GraphPad, San Diego, CA, USA).

Results

Validation of the preference for oil after ingestion training

The 2-bottle preference test on day 7 before surgery demonstrated that the rats significantly preferred corn oil to mineral oil (Fig. 1, $p < 0.05$ by the paired *t*-test).

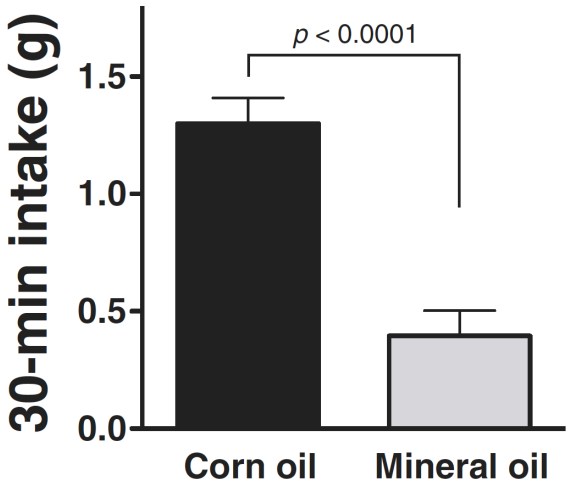

Fig. 1. Preference for Corn Oil before Surgery. The rats (n = 35) were subjected to a 2-bottle choice test with the presentation of 100% corn oil and 100% mineral oil at the same time for 30 min, the amount of each liquid ingested being recorded. Data are presented as the mean intake ± SEM summed for 30 min per rat ($p < 0.0001$, corn oil *vs*. mineral oil intake by a paired *t*-test).

Effect of oil intake on the extracellular DA level in the NAc shell

The intake of 1% linoleic acid was greater than that of mineral oil during the microdialysis test (Fig. 2B; $p < 0.05$ by the Tukey–Kramer test). No significant difference was apparent between other combinations of liquids. Figure 2A shows the time-course changes to the DA level in the NAc shell of rats that had ingested each liquid. There was no difference in the baseline extracellular DA concentrations in NAc among those rats respectively ingesting corn oil, 1% linoleic acid, and mineral oil (0.49 ± 0.16 pg/mL, 0.76 ± 0.22 pg/mL, and 0.52 ± 0.11 pg/mL). There was no significant change to DA level in the rats ingesting mineral oil; however, the DA level in the rats ingesting corn oil was significantly higher than the baseline value at times of 0–15 min (*vs*. baseline by Tukey's multiple-comparison test: 123.4 ± 3.8% at 0 min, $p < 0.01$; 128.1 ± 4.6% at 5 min, $p < 0.001$; 129.8 ± 6.2% at 10 min, $p < 0.001$; and 120.4 ± 5.0% at 15 min, $p < 0.05$). The DA level in the rats ingesting 1% linoleic acid was significantly higher than the baseline value at times of 0–20, 40, and 45 min (*vs.* baseline: 121.9 ± 4.3% at 0 min, 124.8 ± 5.5% at 5 min, 125.9 ± 9:0% at 10 min, 120.1 ± 3.6% at 15 min, 117.1 ± 5.8% at 20 min, 112.5 ± 3.1% at 40 min, and 113.8 ± 5.0% at

45 min). The *p* values at these times were as follows: $p < 0.001$ at 0, 5, 10, 15 min, $p < 0.01$ at 20 min, $p < 0.05$ at 40 and 45 min. The DA levels in the rats ingesting corn oil and 1% linoleic acid were significantly higher than those in the rats ingesting mineral oil at 5 and 10 min by Bonferroni's multiple-comparison test (*vs.* mineral oil: $p < 0.01$ at 5 min and $p < 0.001$ at 10 min for corn oil; $p < 0.05$ at 5 min and 10 min for 1% linoleic acid). Figure 2C shows the typical position of the microdialysis probe inserted into the NAc shell.

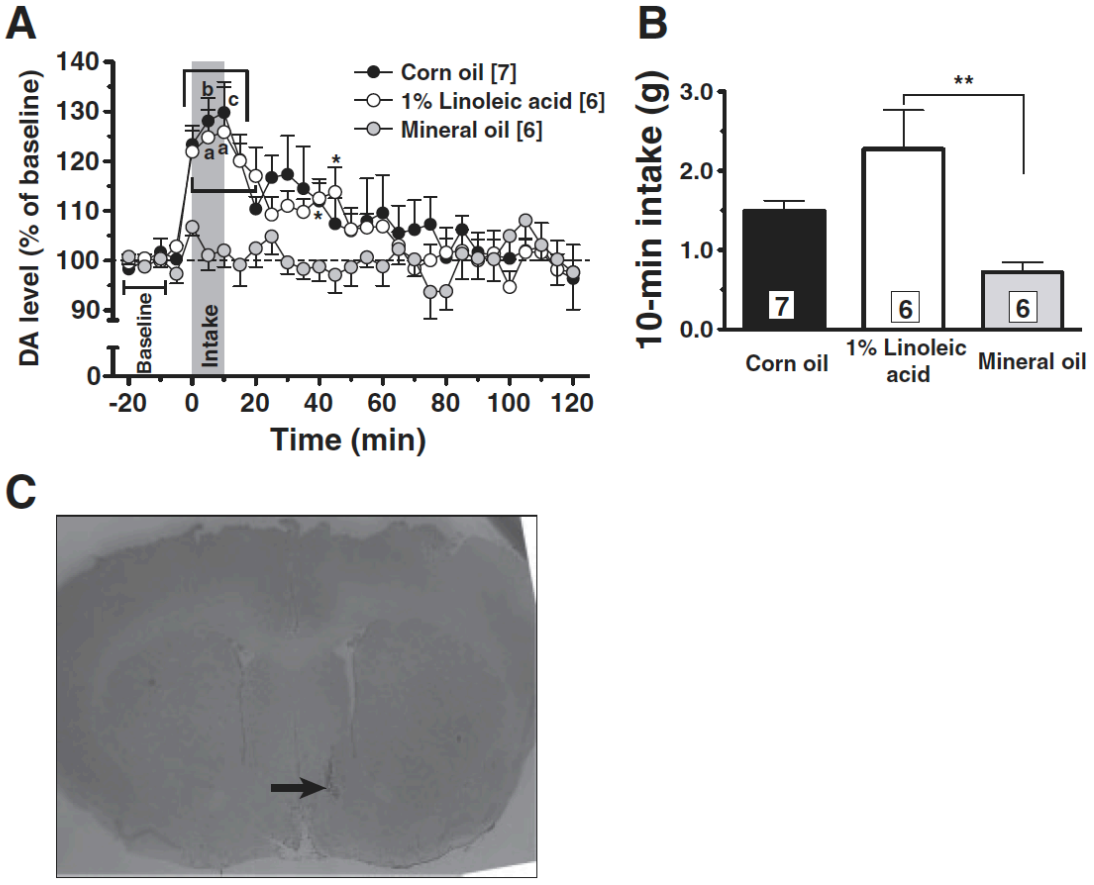

Fig. 2. Time-Course Change in the Extracellular Dopamine (DA) Levels in the Rat Nucleus Accumbens (NAc) Shell during Ingestion of the Test Liquids by Rats. A, Each rat was presented with 100% corn oil, 1% linoleic acid, or 100% mineral oil at 0 min for 10 min. The mean value obtained from 3 samples before the liquid had been presented was set at 100% (baseline), all subsequent sample values being expressed as a percentage of this baseline value, and as the mean ± SEM at each time point (n = 7 for corn oil; n = 6 for 1% linoleic acid; n = 6 for mineral oil). Interval lines of 0–15 min for corn oil (upper), 0–20min (lower), and * for 1% linoleic acid represent significant increases from the baseline value ($p < 0.05$). Lowercase letters show the statistical significance of the difference from the value for the mineral oil group at corresponding time points (a, for 1% linoleic acid, $p < 0.05$; b, for corn oil, $p < 0.01$; c, for corn oil, $p < 0.001$). B, Mean intake of each liquid per rat with the 10-min presentation during the microdialysis session (mean ± SEM; $p < 0.01$). C, The photograph shows typical placement of the microdialysis probe inserted into the NAc shell. The tip of the probe is indicated with an arrow.

Effect of oil intake on the extracellular DA level in the amygdala

The intake of 1% linoleic acid was greater than that of mineral oil during the microdialysis test (Fig. 3B; $p < 0.05$ by the Tukey–Kramer test). There was no significant difference in intake between the other combinations of liquids. Figure 3A shows the time-course changes in the extracellular level of DA in the amygdala of rats ingesting each liquid. There was no difference among the baseline extracellular DA concentrations of the rats respectively ingesting corn oil, 1% linoleic acid, and mineral oil (0.58 ± 0.18 pg/mL, 0.67 ± 0.57 pg/mL, and 0.75 ± 0.18 pg/mL). There were small but significant increases in the DA level of the rats ingesting mineral oil at 0–10 min (*vs.* the baseline by Tukey's multiple-comparison test: 109.8 ± 0.6% at 0 min, $p < 0.05$; 110.5 ± 0.7% at 5 min, $p < 0.01$; 110.5 ± 0.9% at 10 min, $p < 0.01$). The DA levels in the rats ingesting corn oil were significantly higher than the baseline values at time points 0–20 min (*vs.* baseline by Tukey's multiple-comparison test: 129.9 ± 4.9% at 0 min, 129.0 ± 3.0% at 5 min, 128.3 ± 4.5% at 10 min, 125.5 ± 2.5% at 15 min, 119.0 ± 3.4% at 20 min, and 122.4 ± 3.6% at 25 min; $p < 0.001$ at 0, 5, 10, 15, and 25 min, $p < 0.01$ at 20 min). Significantly higher levels of extracellular DA were also observed in the rats ingesting 1% linoleic acid at time points 0–25 min (*vs*. baseline: 127.9 ± 3.4% at 0 min, 125.2 ± 3.1% at 5 min, 125.1 ± 6.2% at 10 min, 125.2 ± 5.7% at 15 min, 124.0 ± 4.4% at 20 min, and 121.5 ± 5.3% at 25 min; $p < 0.001$ at 0, 5, 10, 15, and 20 min, $p < 0.01$ at 25 min). There was no significant difference among the DA levels of the 3 groups at corresponding time points. Figure 3C shows the typical position of the microdialysis probe inserted into the amygdala.

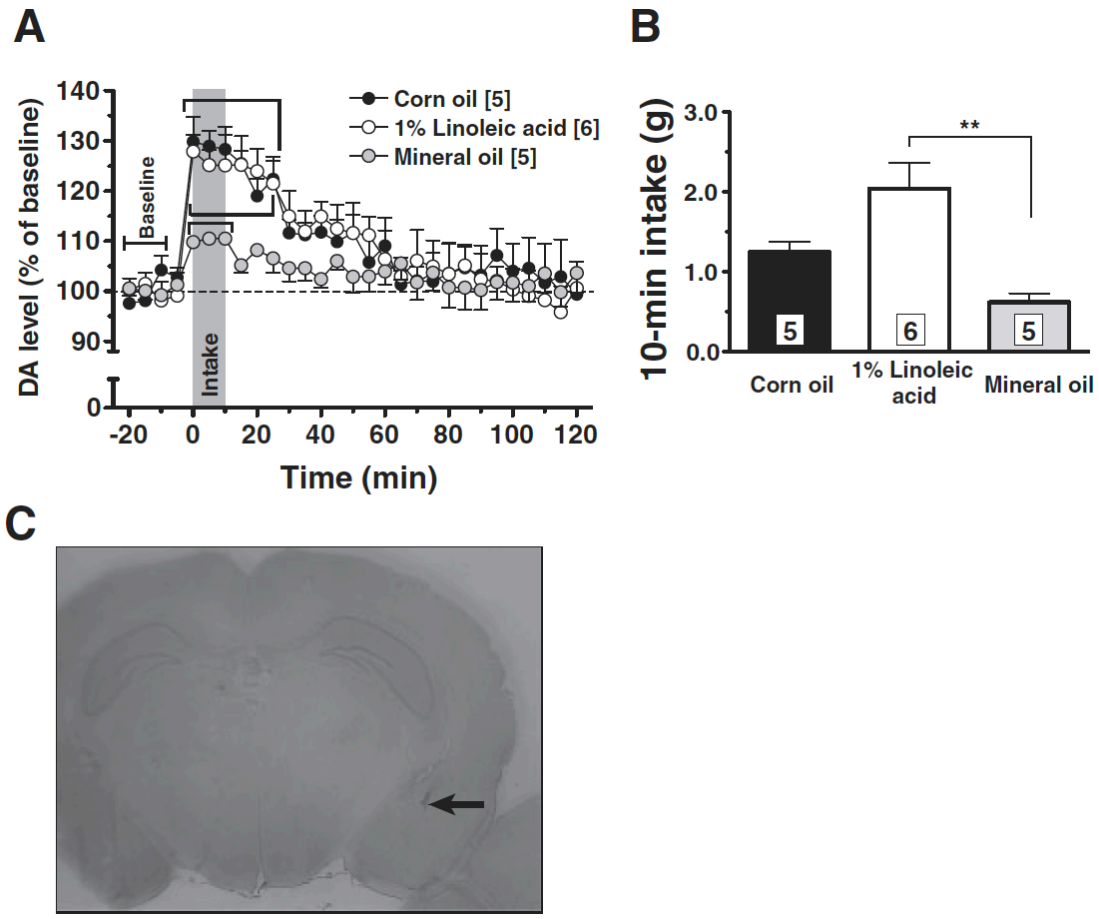

Fig. 3. Time-Course Change in the Extracellular Dopamine (DA) Levels in the Rat Basolateral Amygdala (BLA) during Ingestion of the Test Liquids by Rats. The test liquids and presentation procedures were the same as those described in Fig. 2. A, Time-course change in the extracellular levels of DA in BLA are shown after ingesting the liquids (n = 5 for corn oil; n = 6 for 1% linoleic acid; n = 5 for mineral oil). Interval lines of 0–25min for corn oil (upper) and 1% linoleic acid (middle), and 0–10 min for mineral oil (lower) represent significant increases from the baseline value ($p < 0.05$). B, Mean intake of each liquid per rat with the 10-min presentation during the microdialysis session (mean ± SEM; $^{**}p < 0.01$). C, The photograph shows typical placement of the microdialysis probe inserted into the amygdala. The tip of the probe is indicated with an arrow.

Discussion

Previous reports have shown that the higher the concentration of sucrose (10) or cocaine (11) ingested or self-administered by rats, the greater the increase in DA level in their NAc shell. These reports indicate the possibility that the increased DA level in NAc might be correlated with the degree of pleasantness and palatability, and could be considered an index of the reward value or motivational state of the animal. In respect of oil ingestion, sham feeding of corn oil (the rats were operated on to drain out the ingested solution before it had reached the stomach) has been reported to increase the DA level in NAc of rats (12), this finding suggesting that information from the oral cavity was sufficient for increasing the DA level and that feedback from the digestive tract was not necessary. Yoneda *et al*. reported in a previous study that the licking ratio for a low concentration of LCFA was similar to that for 100% corn oil and higher than that for mineral oil in mice within a very short measurement time (60 s); hence, the postprandial effect of the ingested liquid could be eliminated (4). We have also demonstrated in this present study that the rats preferred corn oil and a low concentration of linoleic acid to mineral oil during training sessions and that the amount of 1% linoleic acid ingested was higher than that of mineral oil during the test session. Therefore, despite the low caloric content, a low concentration of LCFA seemed to have similar properties to corn oil with respect to both the reward value and palatability. Mineral oil has a similar texture to corn oil but does not contain LCFA and is not digestible. Consequently, it cannot be utilized by animals and has very little reward value, based on the increase in DA level in the NAc shell observed in this study.

Dopaminergic neurons in the VTA project to the amygdala, as well as to the NAc shell, and the amygdala is thought to be involved in the manifestation of negative emotions like fear and in the evaluation of a negative reward value. Inglis and Moghaddam have reported that there was an increase in extracellular DA level in the amygdala of rats exposed to handling stress (26). Furthermore, Yokoyama *et al*. have reported that a conditioned stimulus, in addition to an unconditioned stimulus (foot shock), caused the DA level to rise in the amygdala of rats in a conditioned-fear experiment involving foot shock (27). However, some recent studies have reported the involvement of the amygdala in both negative emotions and in positive ones. For example, Polston et al. have reported an increased DA level in the amygdala in response

to a reward stimulus such as methamphetamine (28), and See *et al.* have shown that the administration of a D1-receptor antagonist to the amygdala inhibited the reinstatement of cocaine self-administration (29). Food addiction scores have been reported to be correlated with activation of the amygdala in fMRI measurements on young women (30), and the activity of the amygdala observed during food intake (also with fMRI) increased more in response to an increase in taste intensity than to taste affective valence (31). Moreover, the amygdala has been more strongly activated by high-fat food ingestion than by low-fat food ingestion (32). In our experiment, more palatable food clearly resulted in an increased DA level in the amygdala, the extracellular DA level there increasing after the ingestion of corn oil and 1% linoleic acid, these liquids being consumed by rats in preference to mineral oil.

Mineral oil was clearly preferred to water by rat pups aged 12–15 d, and had similar acceptability to corn oil by adult rats (33). Taka and Fushiki also observed that mineral oil was preferentially consumed and that the ingested volume of mineral oil was more than that of water by mice (unpublished data). Although the method for administration was different via an oral cannula, mineral oil ingestion resulted in a low but significant increase in the extracellular concentration of DA in the NAc shell of rats (Adachi *et al.*, manuscript in preparation). However, the reward value of mineral oil would be lower than that of corn oil, and the level of the DA increase in NAc might not have been captured by unknown factor in this experiment. The different response to mineral oil ingestion between the two brain nuclei should be further studied.

A main ingredient of cooking oil is triacylglyceride that consists of esterified glycerol with LCFA. Kawai and Fushiki have demonstrated that a few percent of triacylglycerol administered into the oral cavity was hydrolyzed into LCFA by lingual lipase secreted by von Ebner's glands present in the circumvallate papillae of rats (17). Gilbertson et al. have elucidated the regulation of K^+ channels in type II taste cells by long-chain unsaturated fatty acids and suggested a chemical receptor for fatty acids in taste cells (18). Fukuwatari *et al.* have demonstrated the expression of CD36, which is a translocator of LCFA, on the apical surface of taste cells of the circumvallate papillae in rats (19), and Laugerette *et al*. (21) and Martin *et al.* (34) have found no preference for fatty acids in CD36-deficient mice. These findings suggest the chemoreception of dietary fat via CD36 in the oral cavity. In addition, Matsumura *et al.* have studied the expression of GPR120 on the tongue (20, 35), and reported a decrease in preference for

fat by GPR120-deficient mice (22). Moreover, Yoneda *et al.* have demonstrated that mice preferred about a 1% concentration of LCFA and that a low concentration of LCFA manifested similar palatability to that of corn oil in the oral cavity (4). These reports indicate that chemo- reception of LCFA via fatty acid receptors like CD36 and GPR120, which are expressed on the tongue, was involved in fat palatability.

Liu *et al.* (36) have reported that information on fatty acid reception was intracellularly transduced via transient receptor potential channel type M5 (TRPM5). Taste cells from TRPM5-deficient mice showed no intracellular increase in the calcium ion concentration by stimulation with linoleic acid. They have indicated that certain GPCRs were present upstream of this transduction mechanism. Additionally, Shah *et al*. (37) have demonstrated that, although not present in taste cells but in STC-1, an enteroendocrine cell line, TRPM5 played a critical role in cholecystokinin (CCK) release by linoleic acid stimulation. The stimulation of GPR120 was necessary in the release of glucagon-like peptide-1 (GLP-1) (38) and CCK (39) with LCFA from the same cell line. Taken together, the information of LCFA reception in a taste cell was partly transduced *via* GPR120, and TRPM5 would function downstream. The result that TRPM5-deficient mice showed no preference for and had reduced sensitivity to linoleic acid (36) could support this notion. Although the sweet, bitter and umami sensations in taste cells were received by distinct receptors, each signal converged on TRPM5 (40). LCFA is now known to share this signaling pathway. However, the relationship between GPR120 and CD36 has not been elucidated. CD36 will affect the energy status of the whole cell as it translocates LCFA into the cell. The recognition mechanism for LCFA *via* CD36 would therefore be independent of the pathway utilizing GPR120 which functions as a receptor and transduces signals by activating associated G-protein.

Fat palatability seems to have been established by associative learning of energy acquisition information from the gastrointestinal tract, and from taste, smell and texture (41–43). However, the increase in extracellular DA level in the rat NAc shell occurred immediately after oil ingestion, so this response is thought to have been caused by the properties of fat sensed in the oral cavity had already been associatively learned, and not by information from the gastrointestinal tract. Although 1% linoleic acid diluted in mineral oil was very low in energy density, ingesting this liquid resulted in a similar increase in extracellular DA level to that of corn oil. 1% of linoleic acid was in the range of concentration of LCFA released from corn oil by lingual lipase digestion, and

this would be matched by the properties of oil which had been associatively learned by the rats. This would be the reason for a similar response being caused with 1% linoleic acid ingestion, as was observed in the case of corn oil, and suggests that the reward obtained by 1% linoleic acid stimulation in the oral cavity was from its palatability, and not from its calorie content.

To summarize, the results of the present study corroborate the increase in extracellular DA levels in the NAc shell and BLA of rats after ingesting 1% linoleic acid, which has a very low calorie level, these levels being comparable to those of full caloric 100% corn oil. These data support the notion that the chemoreception of LCFA released from fat by lingual lipase plays a critical part in the detection of fat in the oral cavity and the manifestation of a reward effect.

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CHAPTER 2

Involvement of GPR120-agonistic activity of long chain fatty acid in the palatability of dietary fat

Most animals, including rodents, as well as humans prefer fat-rich foods (1, 2). The consumption of corn oil has been reported to produce a reward effect in mice, and the dopaminergic pathway in the nervous system has been implicated in the manifestation of this effect (3, 4). Additionally, sham and real feeding of corn oil were reported to induce the activation of the midbrain dopamine (DA) system, which is involved in reward behavior (5,6, and chap.1). The mesolimbic system is thought to play a critical role in the reward effect, and the release of DA has been demonstrated when a natural and drug reward is acquired or when its acquisition is anticipated (7, 8). The midbrain dopaminergic circuits originate from the ventral tegmental area (VTA) and project to different sites, such as the nucleus accumbens (NAc), amygdala, and the prefrontal area, which are related to motivation, palatability, and addiction (9–11). Iwakura *et al.* showed that the secretion of ghrelin, a hormone that stimulates food intake, is induced by DA in a ghrelin-producing cell line MGN3-1 (12). Since the extracellular concentration of DA in the NAc of the rat increases in a dose-dependent manner after self-administration of cocaine or consumption of sucrose, release of DA in the NAc could be considered as a form of index for the palatability or motivational drive (13, 14).

Recent studies have revealed that chemoreceptors of long-chain fatty acids (LCFAs) are involved in the recognition of fatty foods. Kawai *et al.* reported that, when fat was introduced into the oral cavity of rats, a certain percentage of triacylglycerides was hydrolyzed to LCFAs by lingual lipase (15). Gilbertson *et al.* demonstrated the regulation of K^+ channels in type II taste cells by unsaturated LCFAs and suggested that fatty acid chemoreceptors were present within taste cells (16). Fukuwatari *et al.* found that CD36 fatty acid transporter was expressed on the apical side of taste cells in the circumvallate papillae (17). Additionally, *CD36*-deficient mice were reported to show a low taste preference for fat (18). Moreover, Matsumura *et al.* reported that GPR120 was also expressed on the apical side of taste cells in the circumvallate papillae (19). The

unsaturated LCFAs, such as oleic acid and linolenic acid, induced a rise in concentration of intracellular calcium ion ($[Ca^{2+}]_i$) in Human Embryonic Kidney 293 (HEK293) cells stably expressing GPR120. On the other hand, LCFA esters and capric acid did not induce this increase in concentration (20). When the licking behavior of the mice was tested, it was found that the mice exhibited equally strong preference for a low concentration of linoleic acid as that for 100% corn oil (21). In addition, the mice exhibited a similar strong preference for LCFAs, such as oleic, linolenic, and linoleic acid, whereas they did not display any preference for LCFA esters nor long-chain fatty alcohols (22). Moreover, ingesting linoleic acid at a low concentration increased extracellular DA release in the NAc of rats (6 and chap.1). Compared with wild-type mice, GPR120 knock-out mice showed a lower preference for LCFAs and lower response of the chorda tympani and glossopharyngeal nerve to LCFAs (23). These findings lead us to postulate that, in the oral cavity, fat is hydrolyzed to LCFA by lingual lipase and that chemoreception of this LCFA by receptor proteins, such as CD36 and GPR120 expressed on the taste cells, could be involved in the oral recognition and palatability of fats. However, it remains unclear as to which structural characteristics of LCFA are responsible for the GPR120-agonistic activity and if these activities could be implicated in the palatability of LCFA. Therefore, in this study, we examined the relationship between the GPR120-agonistic properties of ligands and the palatability of LCFAs. First, using HEK 293 cells stably expressing human GPR120, we examined the effect of various LCFAs on the concentration of $[Ca^{2+}]_i$ by using a fluorescence spectrophotometer. We then assessed the palatability for a variety of LCFAs at a low concentration by testing the licking behavior of the mice. Next, we studied the change in the extracellular concentration of DA in the NAc of the mice by *in vivo* microdialysis after ingestion of various low-concentration LCFAs. In both the tests, we used 0.0322 mol/L fatty acid in mineral oil. The molar concentration was equal to a volume/volume% concentration of 1% linoleic acid. This concentration of fatty acid was in the range that could be released from fat by lingual lipase and had only $1/100^{th}$ of the calorie content of fats of the same weight.

Materials and Methods

[Ca2+]$_i$ analysis

Cell Culture. HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified, 5% CO_2 atmosphere at 37°C. Human GPR120 cDNA from the lung was provided by Pharmafoods International Co., Ltd. (Kyoto, Japan).

Transfection into cells. Human GPR120 was transfected into HEK293 cells using the lipofection method as per the manufacturer's instructions. For the control, an empty vector was transfected into the HEK293 cells (Empty). Forty-eight hours after transfection, the medium was replaced with fresh medium containing 400 μg/mL G418 (Wako, Osaka, Japan) for the selection of the transfected cells, following which a single clone was selected by the standard limiting dilution method. GPR120 expression in cells was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). Transfected cells were maintained in DMEM containing 10% FBS, 1% penicillin-streptomycin, and G-418 (400 μg/mL).

Reagents for [Ca2+]i analysis. All fatty acids—caprylic acid, C8:0; capric acid, C10:0; lauric acid, C12:0; myristic acid, C14:0; myristoleic acid, C14:1; palmitic acid, C16:0; palmitoleic acid, C16:1; stearic acid, C18:0; oleic acid, C18:1; linoleic acid, C18:2; linolenic acid, C18:3; stearidonic acid, C18:4; arachidic acid, C20:0; arachidonic acid, C20:4; eicosapentaenoic acid (EPA), C20:5; behenic acid, C22:0; docosahexaenoic acid (DHA), C22:6; methyl oleate, methyl linoleate, and methyl linolenate—were purchased from Sigma (St. Louis, MO, USA) and stored at -20°C until use. All cell culture reagents (HEPES, Hanks buffer, DMEM, FBS, penicillin-streptomycin, and Lipofectamine) were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals, unless stated otherwise, were purchased from Sigma.

Ca^{2+} mobilization assay. Ca^{2+} loading buffer comprised of 5 μL Fluo-3AM (1 μM; Dojindo, Kumamoto, Japan) and 10 μL pluronic F-127 (Wako), diluted to yield 10 mL Ca^{2+} assay buffer (20 mM HEPES pH 7.6, 0.01% BSA, 1 mM Probenecid [Wako] in Hanks solution) (24). On the day before the assay, 5×10^4 cells were seeded in 96-well,

poly-D-lysine-coated plates (BD BioCoat, Franklin Lakes, NJ, USA). The cells were washed once with phosphate buffered saline (PBS) and incubated in a final volume of 100 µL/well in Ca^{2+} loading buffer for 60 min at 37°C. Then, the cells were then washed twice with Ca^{2+} assay buffer and the assay was carried out in 100 µL of Ca^{2+} assay buffer. Changes in Ca^{2+} levels were monitored by a fluorescence spectrophotometer (Powerscan HT, DS Pharma Biomedical Co., Ltd., Osaka, Japan) at an excitation wavelength of 485 nm and emission wavelength of 528 nm. The maximum intracellular Ca^{2+} ($[Ca^{2+}]_i$) fluorescence intensity was obtained as the mean of triplicate assays. Test samples for the Ca^{2+} mobilization assay were prepared by sonication in Ca^{2+} buffer just prior to the assay.

Behavioral Test

Animals. This study was conducted in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee, in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and it was approved by the above-mentioned committee. Male BALB/c mice (Japan SLC, Hamamatsu, Japan) at 8 weeks of age were housed in plastic cages in a room with a 12-h light–dark cycle (dark phase of 18:00–6:00) and constant temperature (24 ± 1°C). They were separately housed for > 5 days for acclimatization to the environment. The animals were provided with tap water and regular MF mouse food (Oriental Yeast, Tokyo, Japan) ad libitum.

Materials. Corn oil was purchased from Ajinomoto (Tokyo, Japan) and mineral oil was purchased from Kaneda Company (Tokyo, Japan). All fatty acids—caproic acid, C6:0; caprylic acid, C8:0; capric acid, C10:0; lauric acid, C12:0; myristic acid, C14:0; myristoleic acid, C14:1; palmitic acid, C16:0; palmitoleic acid, C16:1; stearic acid, C18:0; oleic acid, C18:1 *cis*-9; elaidic acid, C18:1 *trans*-9; *cis*-vaccenic acid, C18:1 *cis*-11; *trans*-vaccenic acid, C18:1 *trans*-11; linoleic acid, C18:2; linolenic acid, C18:3; stearidonic acid, C18:4; arachidic acid, C20:0; arachidonic acid, C20:4; behenic acid, C22:0; docosahexaenoic acid (DHA), C22:6; lignoceric acid, C24:0; methyl oleate, methyl linoleate, and methyl linolenate—were purchased from Sigma (St. Louis, MO, USA). They were 99% pure, stored at -20°C until use and then diluted in mineral oil to 0.0322 mol/L, which is equivalent to a v/v% concentration of 1% linoleic acid. Capric

acid, lauric acid, myristic acid, palmitic acid, stearic acid, elaidic acid, *trans*-vaccenic acid, arachidic acid, behenic acid, and lignoceric acid do not dissolve at room temperature. Therefore, we heated each fatty acid solution to 75°C to eliminate the effects of difference in temperature. For testing the licking behavior, we used all the above fatty acids. In the microdialysis test, we used capric acid, lauric acid, stearic acid, oleic acid, linoleic acid, docosahexaenoic acid, and methyl linoleate. The other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

Evaluation of the licking behavior

Apparatus for the test. Licking behavior was evaluated in a custom-made licking test chamber (Muromachi Kikai, Tokyo, Japan) previously described (25). In brief, the test chamber (150 × 120 × 130 mm) was made of Plexiglas with an automatic shutter placed on the front wall, 1.5 cm above the metal-grid floor. When the shutter opened, mice gained access to a stainless steel drinking spout. The licking response was recorded by a computer. The licking rate was calculated for 60 s starting from the first lick. Given this very short period, we can rule out any contribution of post-ingestive feedback to the licking behavior.

Evaluation of the licking behavior. To allow the mice to be habituated to the test environment and to get accustomed to ingesting corn oil and mineral oil, they were kept in the test chamber for 30 min and offered corn oil and mineral oil for 30 min. This training lasted until the mice could discriminate corn oil from mineral oil to the same degree as the previous report (21). After training, the mice were offered linoleic acid and stearic acid. We confirmed that the preference for linoleic acid was high and that for stearic acid was low, similar to the previous report (22). The licking behavior of the mice was then tested. In the test, the mice were offered the test fluids for 30 min once a day in the test chamber. We recorded the licking rate for 1 min from the first lick and the intake for 30 min from the start of presentation of the test fluid. To avoid order effects, each mouse was offered the test fluids in a different order.

Microdialysis test

Training protocols for oil ingestion in the microdialysis test. To allow the mice to get accustomed to ingesting corn oil, mineral oil, and fatty acid fluid, the mice were fed

these liquids in their cages before surgery and then in the microdialysis cage after recovery from surgery. The mice were deprived of water and food for 30 min, and then the liquids were kept in front of the mice for 10 min. Before surgery, the mice were presented with corn oil and mineral oil at the same time on days 1 and 2. To confirm their preference for corn oil over mineral oil, the mice were subjected to a 2-bottle preference test for corn oil vs. mineral oil on day 3. The liquid bottles were positioned randomly. The mice were randomly presented with mineral oil, lauric acid, and linoleic acid on days 4 to 6 and capric acid, stearic acid, oleic acid, methyl linoleate, and docosahexaenoic acid on days 7 to 11. After recovering from the surgery, the mice were presented with corn oil on day 1 and all other liquids in random order on days 2 to 9 in the microdialysis cage. The mice were then subjected to a microdialysis test on day 10.

Microdialysis surgery. The animals were anesthetized with pentobarbital sodium (Nembutal; Dainippon Pharmaceutical Co., Tokyo, Japan) and placed in a stereotaxic frame modified for surgery in mice. The skulls of the mice were subsequently exposed and holes were drilled for microdialysis. The coordinates for the NAc guide cannula (AG-5; Eicom, Kyoto, Japan) were AP, 1.2; ML, 0.6; and DV, 3.2 from the bregma. The coordinates were determined according to the stereotaxic atlas of Paxinos and Franklin (26). The cannulas were secured to the skull with a LOCTITE 454 adhesive bond (Henkel Japan, Yokohama, Japan). A dummy AD-5 cannula (Eicom) was inserted into the guide cannula and secured with an AC-1 cap nut (Eicom). The mice were allowed 3 to 5 days to recover from the surgery. Each mouse implanted with a probe in the NAc was used for a single microdialysis procedure with a single test liquid.

Procedure. The experiments were conducted during the light period of the light-dark cycle. The dummy cannula was removed on the day of the experiment, and the AI-5-1.5 microdialysis probe (Eicom, 1.5 mm membrane length) was inserted into the NAc via the guide cannula. The mice were placed in the microdialysis cage at 8:00 a.m. for 3.5 h without food and water, and then presented with the test liquid at 11:30 p.m. for 10 min. The amount of liquid ingested was also recorded. The rats remained in the microdialysis cage for another 80 min after presenting the test liquid. Ringer's solution containing 147 mM Na^+, 4 mM K^+, 2.3 mM Ca^{2+}, and 155.6 mM Cl^- was perfused at 3 μL/min by an ESP-64 micro-syringe pump (Eicom). Dialysate collection was started 30 min before

the liquids were presented, and the collection conducted every 10 min for a total of 120 min thereafter. To quantify DA and 5-HT levels in the dialysate, samples were analyzed by reversed-phase high performance liquid chromatography (HPLC) with an electrochemical detector, using an Eicompak PP-ODS II column (4.6 i.d. × 30 mm long; Eicom). The voltage applied was set at 400 mV (relative to an Ag/AgCl reference electrode). The mobile phase at a flow rate of 500 μL/min consisted of a 98% (v/v) 0.1 M phosphate buffer at pH 6.0, 2% (v/v) methanol, 500 mg/L sodium decane sulfate, and 50 mg/L EDTA-2Na. The mean value obtained from 3 samples from -30 to -10 min was set as the 100% baseline level, and all subsequent sample values were expressed as a percentage of the baseline value.

Histological analysis. Upon completion of the experiment, the mice were deeply anesthetized with sodium pentobarbital. The brain was removed from the skull, frozen, and cut into 20-μm sections. The placement of the microdialysis probe was verified by thionine blue staining. Data obtained from the mice with inappropriate probe placement were excluded from the analysis.

Statistics. Data are expressed as the mean ± SEM. Data from $[Ca^{2+}]_i$ assay were analyzed using a one-way ANOVA and Dunnett's post hoc test. Data obtained by testing the licking behavior were analyzed using one-way repeated ANOVA and Dunnett's post hoc test. Data from the 2-bottle preference test were analyzed by a paired *t*-test. Changes in DA and 5-HT levels were compared with the corresponding baseline value by one-way repeated ANOVA and Tukey's multiple-comparison test as a post-hoc test. Mean differences among the 3 groups at each time point were analyzed by two-way repeated-measures ANOVA and Bonferroni's multiple-comparison as a post-hoc test. The amount of each fluid ingested during microdialysis was analyzed by a one-way ANOVA and Dunnett's test as a post-hoc test. Correlation coefficient was obtained by Pearson correlation test. *p* values of 5% or less were considered statistically significant. Statistical analyses were conducted by using the Prism 6 software package (GraphPad, San Diego, CA, USA).

RESULTS

Intracellular Ca^{2+} Assay in HEK293 cells

Fig. 1 shows the maximum intracellular Ca^{2+} fluorescence intensity in the response induced by 10 μM of different fatty acids. Intracellular calcium levels in the response induced by myristic acid (C14:0), myristoleic acid (C14:1), palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), stearidonic acid (C18:4), arachidonic acid (C20:4), eicosapentaenoic acid (C20:5), and docosahexaenoic acid (C22:6) were higher than those induced by the buffer (*versus* buffer by Dunnett's test: $p < 0.01$ for palmitic acid; $p < 0.001$ for other fatty acids). On the other hand, the level of response induced by caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), stearic acid (C18:0), methyl oleate (C18:1-CH_3), methyl linoleate (C18:2-CH_3), methyl linolenate (C18:3-CH_3), arachidic acid (C20:0), and behenic acid (C22:0) were not significant.

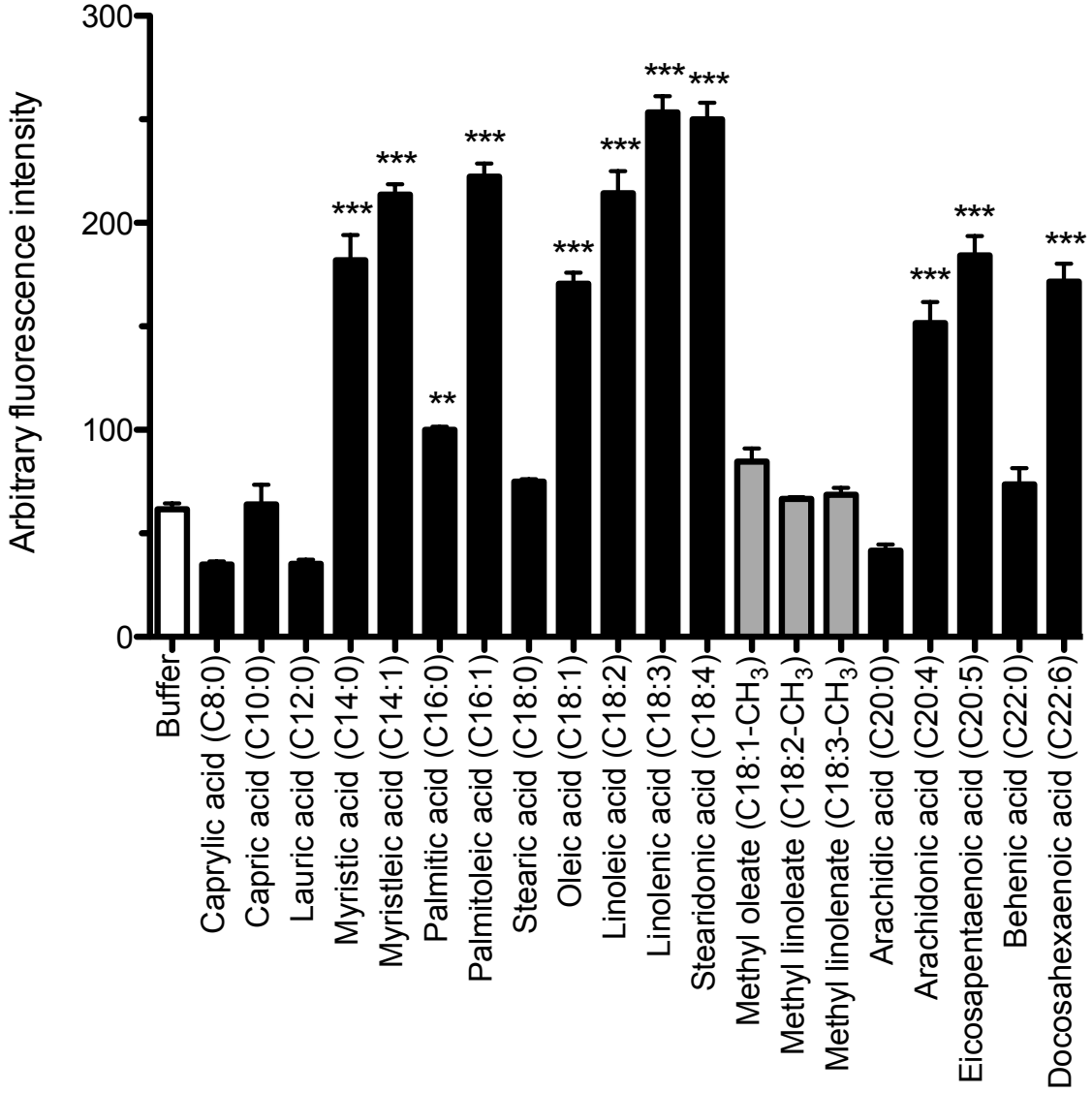

Fig. 1 Intracellular Ca^{2+} ($[Ca^{2+}]_i$) level increases induced by 10 µM of various fatty acids in HEK293 cells stably expressing GPR120. $[Ca^{2+}]_i$ response induced by 10 µM test fatty acid in HEK 293 cells GPR120 was monitored by a fluorescence spectrophotometer for 1 min at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. $[Ca^{2+}]_i$ level was expressed as the maximum fluorescence intensity observed in 1 min. Data are presented as the mean ± SEM (n = 3, $^{**}p < 0.01$, $^{***}p < 0.001$, *versus* control buffer).

Evaluation of the Licking behavior

Fig. 2 shows the initial licking rate with various fatty acids. The mice exhibited a significantly higher licking rate with palmitoleic acid (C16:1), oleic acid (C18:1 *cis*-9), linoleic acid (C18:2), linolenic acid (C18:3), stearidonic acid (C18:4), arachidonic acid (C20:4), and docosahexaenoic acid (C22:6) than with mineral oil (*versus* mineral oil by Dunnett's test: $p < 0.01$ for arachidonic acid; $p < 0.001$ for other fatty acids). On the other hand, the mice did not respond significantly to caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1), palmitic acid (C16:0), stearic acid (C18:0), elaidic acid (C18:1 *trans*-9), *cis*-vaccenic acid (C18:1 *cis*-11), *trans*-vaccenic acid (C18:1 *trans*-11), methyl oleate (C18:1-CH_3), methyl linoleate (C18:2-CH_3), methyl linolenate, (C18:3-CH_3), arachidic acid (C20:0), behenic acid (C22:0), and lignoceric acid (C24:0).

Similar to the result for the licking rate, the intake amount for palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1 *cis*-9), elaidic acid (C18:1 *trans*-9), linoleic acid (C18:2), *cis*-vaccenic acid (C18:1 *cis*-11), linolenic acid (C18:3), and stearidonic acid (C18:4) were significantly greater than that of mineral oil (*versus* mineral oil by Dunnett's test: $p < 0.01$ for palmitic acid and elaidic acid; $p < 0.001$ for other fatty acids) (Table 1). However, the intake amount for all other fatty acids was not significant.

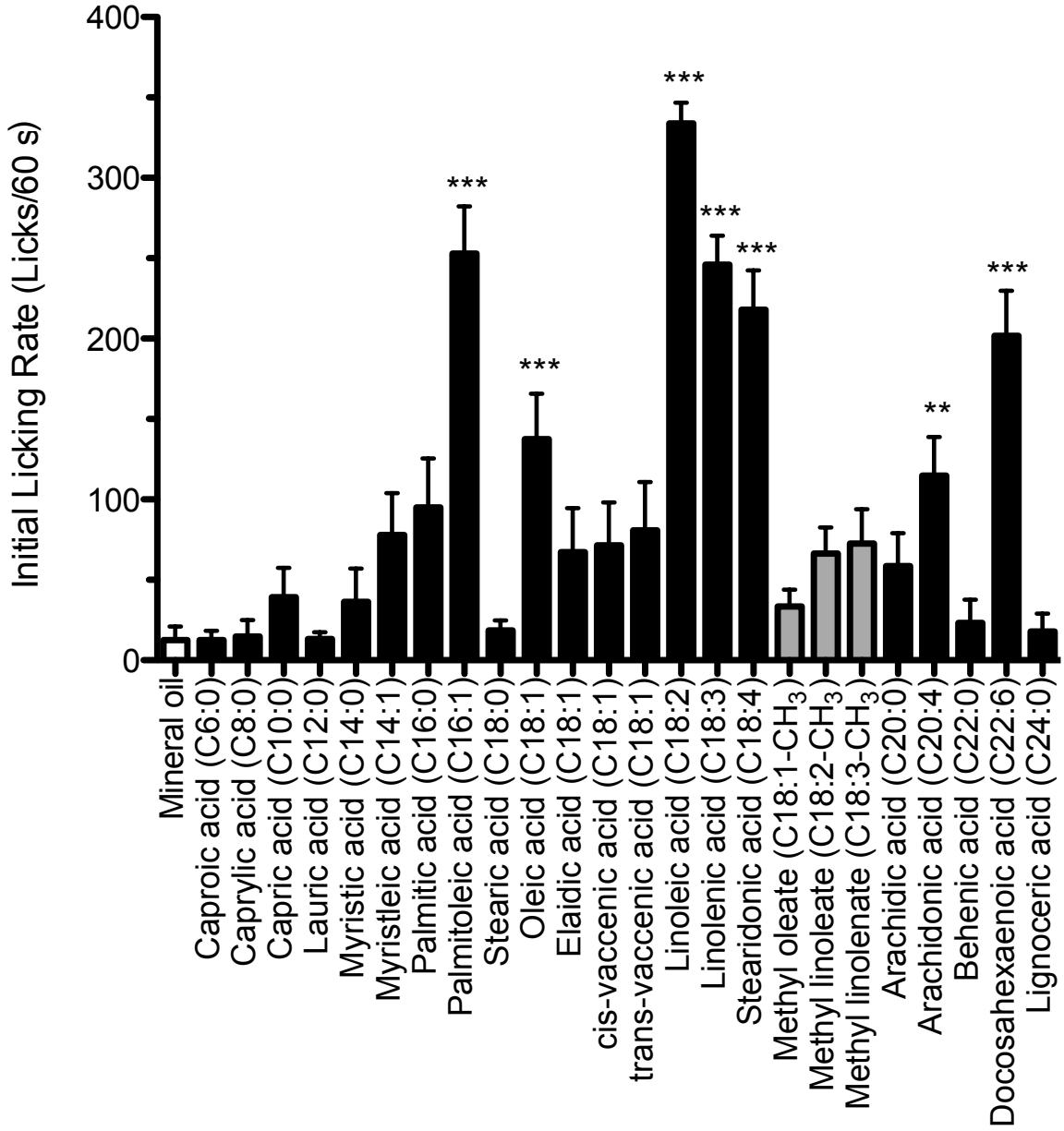

Fig. 2 Initial licking rate with various fatty acids at a concentration of 0.0322 mol/L in the test performed to evaluate licking behavior. Mice (n = 15) were offered a bottle of the test fluid and the licking rate over the first 60 s was recorded as the initial licking rate. Data are presented as the mean ± SEM (**$p < 0.01$, ***$p < 0.001$, *versus* initial licking rate with mineral oil).

Table 1

Intake amounts for various types of fatty acid in the licking test for 30 min

Fatty acid	Carbon chain length	Intake (g/30 min)
Mineral oil		0.02±0.01
Caproic acid	C6:0	0.03±0.01
Caprylic acid	C8:0	0.07±0.05
Capric acid	C10:0	0.30±0.18
Lauric acid	C12:0	0.10±0.06
Myristic acid	C14:0	0.42±0.16
Myristleic acid	C14:1	0.31±0.14
Palmitic acid	C16:0	0.74±0.22**
Palmitoleic acid	C16:1	1.84±0.19***
Stearic acid	C18:0	0.06±0.04
Oleic acid	C18:1	1.51±0.27***
Elaidic acid	C18:1	0.80±0.24**
cis-Vaccenic acid	C18:1	0.74±0.23**
trans-Vaccanic acid	C18:1	0.56±0.18
Linoleic acid	C18:2	2.28±0.10***
Linolenic acid	C18:3	1.53±0.13***
Stearidonic acid	C18:4	1.07±0.17***
Methyl oleate	C18:1-CH_3	0.27±0.11
Methyl linoleate	C18:2-CH_3	0.19±0.10
Methyl linolenate	C18:3-CH_3	0.20±0.07
Arachidic acid	C20:0	0.07±0.02
Arachidonic acid	C20:4	0.19±0.04
Behenic acid	C22:0	0.09±0.05
Docosahexaenoic acid	C22:6	0.33±0.07
Lignoceric acid	C24:0	0.05±0.01

Values are means±SEM (n = 15). **P<0.01, ***P<0.001, vs. mineral oil.

Microdialysis Test

1. Validation of the preference for oil after ingestion training

The 2-bottle preference test on day 3 before surgery demonstrated that the mice significantly preferred corn oil to mineral oil (Fig. 3, $p < 0.05$ by the paired *t*-test).

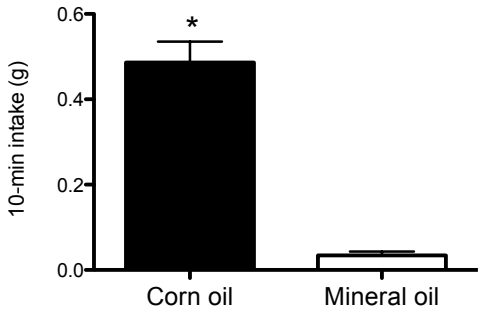

Fig. 3 Preference for corn oil before surgery. Mice (n = 48) were subjected to a 2-bottle choice test with the presentation of 100% corn oil and 100% mineral oil at the same time for 10 min, and the amount of each liquid ingested was recorded. Data are presented as the mean intake ± SEM summed for 10 min per mouse ($p < 0.0001$, corn oil vs. mineral oil intake by a paired *t*-test).

2. Effect of intake on the extracellular DA and 5-HT level in the NAc

(1) Fatty Acid Intake in the microdialysis test

The intake of oleic acid and linoleic acid was greater than that of mineral oil during the microdialysis test (Fig. 4; $p < 0.05$ vs. mineral oil by Dunnett's test). The intake of corn oil, caprylic acid, lauric acid, stearic acid, methyl linoleate, and docosahexaenoic acid was not significant.

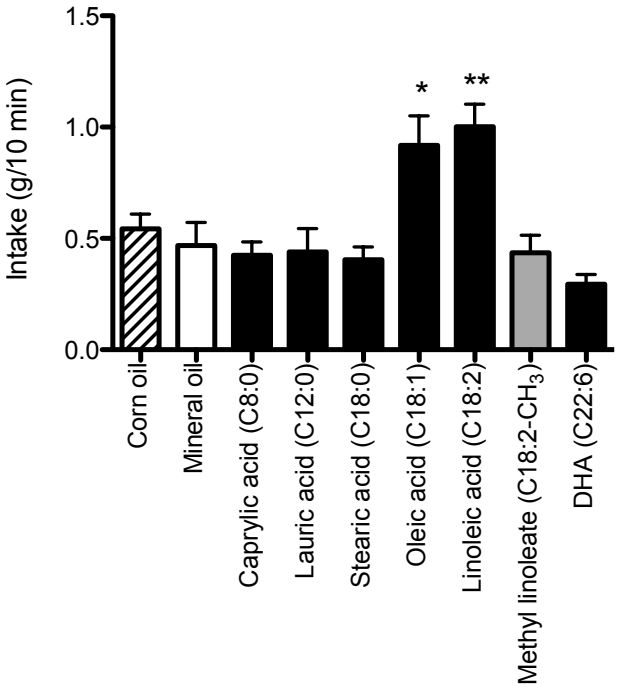

Fig. 4 Mean intake of each liquid per mouse with the 10-min presentation during the microdialysis session. Each mouse was presented with the test liquids (n = 6 for corn oil; n = 5 for mineral oil; n = 5 for capric acid; n = 5 for lauric acid; n = 5 for stearic acid; n = 6 for oleic acid; n = 6 for linoleic acid; n = 5 for methyl linoleate; n = 5 for DHA) at 0 min for 10 min. Data are presented as the mean intake ± SEM (*$p < 0.05$; **$p < 0.01$, *versus* mineral oil intake).

(2) Time-course change in the DA level in the mouse NAc

Fig. 5A shows the time-course changes in DA level in the NAc of mice that had ingested each liquid. There was no difference in the baseline extracellular DA concentrations in the NAc (Table 2). There were no significant changes to DA levels in the mice ingesting mineral oil, capric acid (C10:0), lauric acid (C12:0), stearic acid (C18:0), methyl linoleate (C18:2-CH_3), or docosahexaenoic acid (C22:6); however, the DA level in the mice ingesting corn oil was higher than baseline value at 0 to 20 min (*versus* baseline by Tukey's multiple-comparison test: 142.3 ± 4.7% at 0 min, $p < 0.05$; 147.3 ± 9.8% at 10 min, $p < 0.05$; 142.7 ± 14.2% at 20 min, $p < 0.05$). The DA level in the mice ingesting oleic acid (C18:1) was significantly higher than baseline value at times of 0 to 10 min (*versus* baseline: 132.3 ± 6.4% at 0 min, $p < 0.05$; 140.8 ± 2.6% at 10 min, $p < 0.01$). The DA level in the mice ingesting linoleic acid was significantly higher than baseline value at time point from 0 to 10 min (*versus* baseline: 148.0 ± 10.7% at 0 min, $p < 0.05$). The DA level in the mice ingesting corn oil was significantly higher than that in the mice ingesting mineral oil at 10 min by Bonferroni's multiple-comparison test (*versus* mineral oil: $p < 0.05$). The DA level in the mice ingesting linoleic acid (C18:2) was significantly higher than that in the mice ingesting mineral oil at 0 min by Bonferroni's multiple-comparison test (*versus* mineral oil: $p < 0.05$). The DA levels in the mice ingesting capric acid, lauric acid, stearic acid, oleic acid, methyl linoleate, and docosahexaenoic acid were not significantly higher than that in the mice ingesting mineral oil. At the time when the mice was ingesting fluid (at 0 min), DA levels in the mice presented with corn oil, oleic acid, and linoleic acid were higher than that in the mice presented with mineral oil (Fig. 5B; *versus* mineral oil by Dunnett's test: $p < 0.01$ for corn oil and linoleic acid; $p < 0.05$ for oleic acid).

Table 2

The basal extracellar dopamine (DA) and serotonin (5-HT) concentraitons in the mouse nucleus accumbens in the microdialysis test

	DA concentration (ng/μl)	5-HT concentration (ng/μl)
Corn oil	0.037±0.005	0.023±0.002
Mineral oil	0.042±0.018	0.022±0.003
Capric acid	0.051±0.019	0.029±0.003
Lauric acid	0.047±0.018	0.024±0.005
Stearic acid	0.031±0.012	0.026±0.003
Oleic acid	0.049±0.020	0.022±0.003
Linoleic acid	0.043±0.018	0.030±0.011
Methyl linoleate	0.047±0.018	0.023±0.003
Docosahexaenoic acid	0.052±0.014	0.023±0.003

Values are means±SEM (n = 5-6 for DA; n = 3-6 for 5-HT).

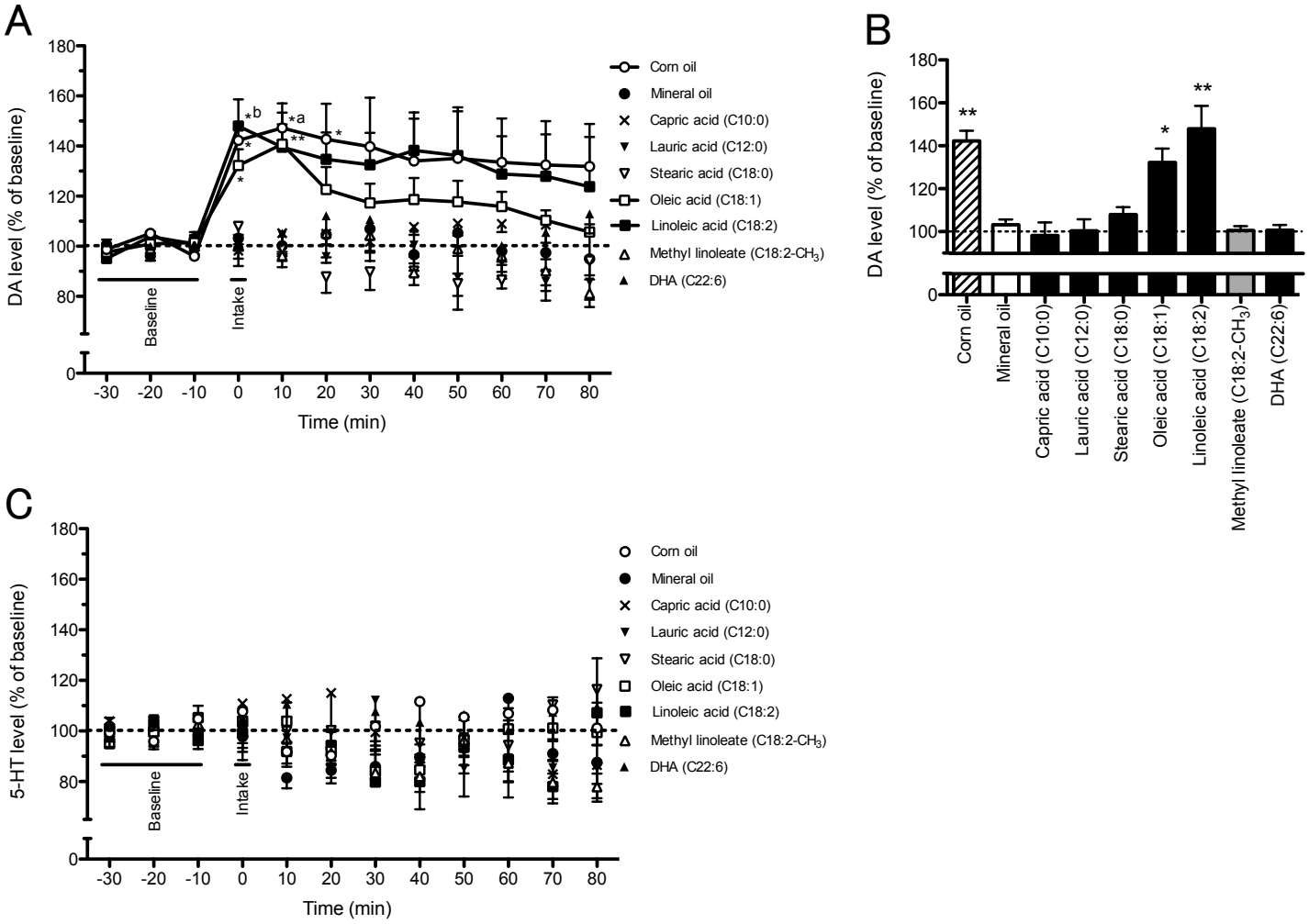

Fig. 5 Time-course change in the extracellular dopamine (DA) and serotonin (5-HT) levels in the mouse nucleus accumbens (NAc) during ingestion of the test liquids (100% corn oil, 100% mineral oil, and 0.0322 mol/L fatty acid) by mice. Each mouse was presented with the test liquids at 0 min for 10 min, all subsequent sample values being expressed as a percentage of this baseline value, and as the mean ± SEM at each time point. A, Time-course change of DA in the NAc are shown after ingesting the liquid (n = 6 for corn oil; n = 5 for mineral oil; n = 5 for capric acid; n = 5 for lauric acid; n = 5 for stearic acid; n = 6 for oleic acid; n = 6 for linoleic acid; n = 5 for methyl linoleate; n = 5 for DHA). Asterisks (*) represents significant increase from the baseline value (*$p < 0.05$; **$p < 0.01$). Lowercase letters shows the statistical significance of the difference from the value for the mineral oil group at corresponding time points (a, for corn oil, $p < 0.05$; b, for linoleic acid, $p < 0.05$). B, DA levels within the duration of 0 to 10 min during presentation of the liquid (*$p < 0.05$; **$p < 0.01$, *versus* the mineral oil group). C, Time-course change of 5-HT in the NAc are shown after ingesting the liquid (n = 5 for corn oil; n = 3 for mineral oil; n = 4 for capric acid; n = 3 for lauric acid; n = 4 for stearic acid; n = 6 for oleic acid; n = 4 for linoleic acid; n = 3 for methyl linoleate; n = 3 for DHA).

(3) Time-course change in the 5-HT level in the mouse NAc

Fig. 5C shows the time-course changes in the 5-HT level in the NAc of mice that had ingested each liquid. There was no difference in the baseline extracellular 5-HT concentrations in the NAc (Table 2). There were no significant changes to 5-HT levels in the mice ingesting each fluid. Fig. 6 shows the placement of all the microdialysis probes into the NAc.

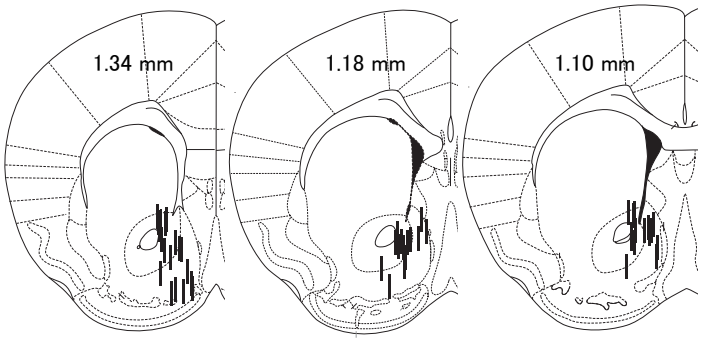

Fig. 6 Placement of all the microdialysis probes into the NAc. Black bar represents the tip of the probe. The number on each section is the distance in millimeters anterior from the bregma according to the stereotaxic atlas of Paxinos and Franklin.

Correlations between Ca^{2+} Fluorescence Changes and Initial Licking Rate between, and Accumbens Dopamine Level

Fig. 7A shows a positive correlation between initial licking rate and the consumption of the different fatty acids in mice and maximum intracellular Ca^{2+} level estimated in cultured cells induced by corresponding fatty acid (Pearson correlation; r = 0.8005, $p < 0.0001$, n = 19). Additionally, there was a positive correlation between the 30 min intake amounts for fatty acids and $[Ca^{2+}]_i$ levels (data not shown; Pearson correlation; r = 0.7308, p = 0.0004, n = 19). Fig. 7B shows a positive correlation between cumulative value of DA levels in the NAc at 0 to 80 min in the mice offered various types of fatty acid and $[Ca^{2+}]_i$ levels (Pearson correlation; r = 0.8688, p = 0.0111, n = 7). Additionally, there was a positive correlation between DA levels at the time (0 to 10 min) when the mice were offered the fluids and $[Ca^{2+}]_i$ levels (data not shown; Pearson correlation; r = 0.7829, p = 0.0374, n = 7).

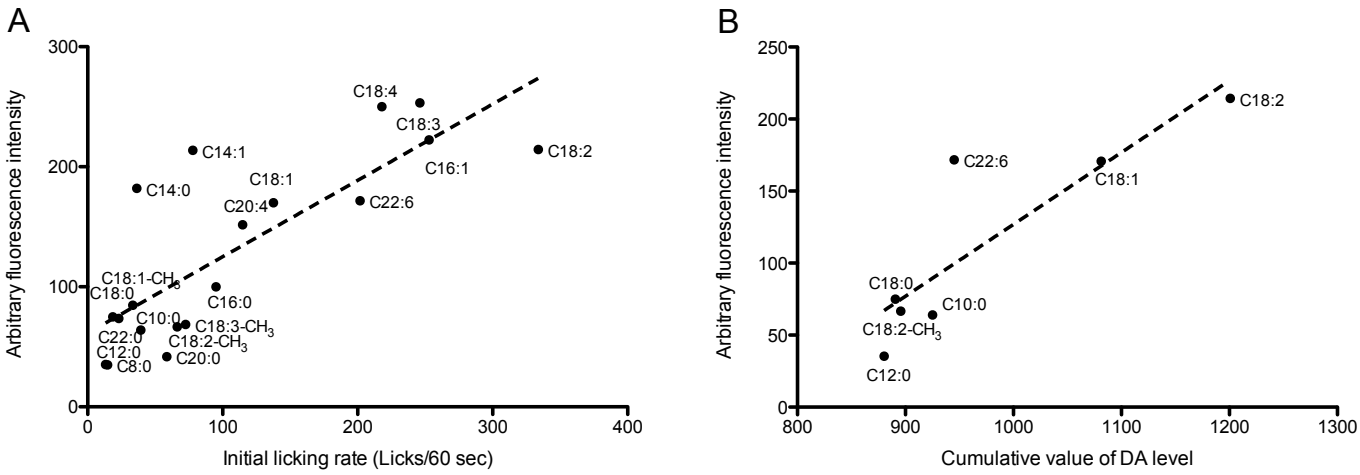

Fig. 7 Correlations of the maximum intracellular Ca^{2+} ($[Ca^{2+}]_i$) fluorescence intensities and the initial licking rates (A), and of the maximum $[Ca^{2+}]_i$ fluorescence intensities and the cumulative values of DA level (B). A, X axis shows the averaged initial licking rate with various fatty acids and Y axis shows the averaged maximum $[Ca^{2+}]_i$ fluorescence intensities in the response induced by corresponding fatty acid (Pearson correlation; r = 0.7421, p = 0.0002, n = 19). B, X axis shows the cumulative value of averaged DA level at 0 to 80 min in the NAc of mice after ingesting various fatty acids and Y axis shows the averaged maximum $[Ca^{2+}]_i$ fluorescence intensities in the response induced by corresponding fatty acid (Pearson correlation; r = 0.8688, p = 0.0111, n = 7).

DISCUSSION

1. Intracellular Ca^{2+} Assay

Since there was no response to fatty acids in the HEK293 cells transfected with an empty vector, it was confirmed that the fluorescence changes stimulated by fatty acids were the GPR120-specific responses (data not shown).

The addition of saturated and unsaturated 14- and 16-carbon fatty acids induced a significantly higher increase in $[Ca^{2+}]_i$ than that of the control buffer in the HEK293 cells stably expressing GPR120. Additionally, in the 18-, 20-, and 22-carbon fatty acids, increases in $[Ca^{2+}]_i$ induced by unsaturated fatty acids were significantly higher than those induced by control buffer, whereas saturated forms of these fatty acids caused no significant response. These results demonstrate that saturated 14- and 16-carbon fatty acids and unsaturated 14-, 16-, 18-, 20-, and 22-carbon fatty acids are strong ligands for GPR120. Moreover, in 18- and 20-carbon fatty acids, $[Ca^{2+}]_i$ levels tended to increase due to poly unsaturated fatty acids like stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), stearidonic acid (C18:4), arachidic acid (C20:0), arachidonic acid (C20:4), and EPA (C20:5). This result shows that the structure of poly unsaturated fatty acids is more suitable for GPR120 ligands in the 18- and 20-carbon acids than in other fatty acids. Further, $[Ca^{2+}]_i$ levels stimulated by methyl esters of unsaturated 18-carbon fatty acid were not significant when compared with that of control. This result implies that the terminal carboxyl group of the fatty acids plays an important role in the recognition of fatty acid structure by GPR120. The results of $[Ca^{2+}]_i$ assay in this study correspond well with previous reports by Hirasawa *et al.* and Galindo *et al.* (20, 27).

2. Evaluation of Licking Behavior

During the evaluation of the licking behavior with middle-chain fatty acids such as 6-carbon caproic acid, 8-C caprylic acid, and 10-C capric acid, as well as shorter-chain LCFAs such as 12-C lauric acid, 14-C myristic acid, and myristoleic acid, the initial licking rates were not significantly different from those observed by using mineral oil. On the other hand, in the test with longer LCFAs such as 16-C palmitoleic acid, 18-C oleic acid, linoleic acid, linolenic acid, stearidonic acid, 20-C arachidonic acid, and 22-C DHA, the initial licking rates were significantly higher than that with mineral oil. This result suggests that carbon chain length is involved in the palatability of fatty acid.

In addition, since the mice displayed high licking response to unsaturated LCFAs (palmitic acid, 16:1; oleic acid, 18:1; linoleic acid, 18:2; linolenic acid, 18:3; stearidonic acid, 18:4; arachidonic acid, 20:4; and DHA, 22:6) and low licking response to saturated LCFAs (palmitic acid, 16:0; stearic acid, 18:0; arachidic acid, 20:0; and behenic acid, 22:0), these results indicate that palatability of fatty acids is affected by the saturation state of the fatty acid. Moreover, the mice showed a significantly higher licking rate with oleic acid, linoleic acid, and linolenic acid. On the other hand, the mice did not respond significantly to corresponding methyl esters, namely, methyl oleate, methyl linoleate, and methyl linolenate. Yoneda *et al.* reported that the mice exhibited low palatability for not only fatty acid ester but also fatty alcohol (22). These results imply that terminal carboxyl group of fatty acids is involved in their palatability. Further, the licking rate with oleic acid (*cis*-9-octadecenoic acid) was significantly higher than that with mineral oil. However, there was no difference in the licking rates with geometric isomer of oleic acid, elaidic acid (*trans*-9-octadecenoic acid) and mineral oil. Additionally, the licking rates with *cis*-vaccenic acid (*cis*-11-octadecenoic acid) and *trans*-vaccenic acid (*trans*-11-octadecenoic acid) which are regioisomers of oleic acid and elaidic acid, respectively, had no such effect. The results show that conformation of the fatty acid is important in the manifestation of palatability, in at least C18:1 fatty acids.

In this study, we observed that the amount of fatty acid intake over 30 min did not necessarily correlate with the initial licking rate. For example, the licking rates of myristoleic acid and elaidic acid were not significantly higher than that of mineral oil; however, their intake was greater than that of mineral oil. A possible explanation for this difference is that the post-ingestive effects of these acids may have promoted their consumption. In contrast, the licking rate of DHA was significantly higher than that of mineral oil, while the intake amount was not. Harden *et al*. reported that DHA stimulated significantly more release of cholecystokinin (CCK) than other LCFAs such as linoleic acid and oleic acid in secretin tumor (STC-1) cells (28). Therefore, release of CCK from the duodenum, which is suppressed after DHA ingestion, may suppress the overall intake of this fatty acid over 30 min.

3. Change in DA Level in Microdialysis Test

Previously, we have reported that extracellular DA level in the NAc of rats was

elevated by ingesting 1% (v/v) linoleic acid, which had a very low calorie level (approximately 0.09 kcal/g), as compared to the similar increase observed with as high as 100% corn oil (9 kcal/g) ingestion (6 and chap.1). This showed that the reward value of fat, at least as estimated from the increase in DA level in the NAc, was determined by the chemoreception of LCFAs in the oral cavity, not by its caloric density. Similarly, in this study using mice, 100% corn oil and 1% linoleic acid significantly increased the NAc DA levels. Oleic acid also resulted in a significant increase in DA level. On the other hand, capric acid, lauric acid, stearic acid, methyl linoleate, and DHA caused no significant change in the DA levels. This result suggests that 18-carbon length, saturated state of fatty acid, and terminal carboxyl group are important for the rise in DA level observed upon the ingestion of these LCFAs. Further, the intake amount of oleic acid and linoleic acid during the 10 min of presentation in the microdialysis test was significantly greater than that of mineral oil, whereas other fatty acids presented in the microdialysis test showed no difference in consumption than that of mineral oil. These results indicated that the characteristics of fatty acids that caused an increase in the DA level corresponded well with the palatability of LCFAs.

4. Correlation between $[Ca^{2+}]i$ level, licking rate, and DA level

In this study, the GPR120-agonistic activity of LCFA as estimated in cell culture correlated with the initial licking rate and the increase in DA level. This suggests that the chemoreception of LCFA via GPR120 is the first step in the manifestation of this effect. $[Ca^{2+}]_i$ levels stimulated by myristic acid and myristoleic acid were higher than those achieved by using the control buffer, and the mice did not show significantly higher licking response to these fatty acids as compared to mineral oil. This difference may be attributed to the influence of the flavors in these fatty acids, which may have resulted in the mice avoiding their consumption. Godinot *et al.* reported that non-fatty acid agonists of GPR120 activated the glossopharyngeal nerve of mice and that the mice did not show any preference for non-fatty acid agonists of GPR120 (29). These findings imply that the palatability of LCFAs at low concentration may not consist of only GPR120 and that composition of the palatability may include other mechanisms such as chemoreception via CD36 and GPR40. Both of CD36 and GPR40 are reported to be expressed in the taste cell, while GPR120 is reported to be involved in the recognition and palatability of fat in the oral cavity (30). Further studies using GPR120 knockout

mice are necessary to determine whether the expression of GPR120 is implicated in the palatability of fat and increase in DA level when ingesting fat. The agonistic activity of receptor proteins expressed in the oral cavity, such as CD36 and GPR40 require investigation and a detailed study is warranted, on the relationship between agonistic activity and palatability using animal models.

To summarize, we found that saturated 14- and 16-carbon fatty acids and unsaturated 14-, 16-, 18-, 20-, and 22-carbon fatty acids are strong ligands for GPR120 that cause an increase in $[Ca^{2+}]_i$ levels in the cultured cells. We also revealed that mice have high palatability for unsaturated 16-, 18-, 20-, and 22-carbon fatty acids. Moreover, DA level in the NAc of mice was elevated after ingestion of unsaturated 18-carbon acid to the same extent as 100% corn oil. Further, the agonistic activity of GPR120 ligands correlated with the palatability of LCFAs in the oral cavity and the reward values based on DA levels in the NAc. These findings suggest that the chemoreception of LCFAs via GPR120 is implicated in the palatability of LCFAs, and they support the notion that the chemoreception of LCFAs released from fat by lingual lipase plays an important role in the detection of fat in the oral cavity as well as fat palatability, including the reward effect.

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SUMMARY

Chapter 1

Changes in the extracellular concentration of dopamine (DA) in the nucleus accumbens (NAc) shell and the basolateral amygdala (BLA) resulting from the voluntary ingestion of either corn oil, mineral oil, or 1% linoleic acid diluted with mineral oil as a vehicle were measured in rats by using in vivo microdialysis after they had been trained to establish a preference for corn oil. Ingesting the mineral oil caused no significant change in DA level in the NAc shell, whereas corn oil ingestion significantly increased the DA level during 0–15 min of the test session, reaching the maximum level of 129.8 ± 6.2% compared with the baseline after 10 min. Ingesting linoleic acid also resulted in a significant increase in DA level during 0–20 min, reaching 125.9 ± 9.0% after 10 min. Similar results were obtained in the BLA. Despite its very low calorie content, a low concentration of non-esterified fatty acid increased the DA levels equivalent to those resulting from corn oil in the brain's reward system.

Chapter 2

Mechanism underlying the involvement of the GPR120-agonistic activity of LCFA in the palatability of dietary fat remains elusive. Therefore, we examined the association between the GPR120-agonistic activity and palatability of LCFA. First, we measured Ca^{2+} signaling in HEK293 cells stably expressing GPR120 under stimulation by various LCFAs. We then assessed the palatability of the various LCFAs by testing the licking behavior in mice and measured the changes in the NAc-DA level by *in vivo* microdialysis. Consequently, 14- to 22-carbon unsaturated LCFAs showed strong GPR120-agonistic activity. Additionally, mice displayed high licking response to unsaturated 16- and 18-carbon LCFAs, and unsaturated 18-carbon LCFA significantly increased the DA level. The licking rate and the LCFA-dependent increase in DA level also correlated well with the GPR120-agonistic activity. These findings demonstrate that chemoreception of LCFA by GPR120 is involved in the recognition and palatability of dietary fat.

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List of publications

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