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POMC and orexin mRNA expressions induced by anticipation of a corn-oil emulsion feeding are maintained at the high levels until oil ingestion

Takafumi MIZUSHIGE[1], Takayuki KAWAI[1], Shigenobu MATSUMURA[1], Takeshi YONEDA[1], Teruo KAWADA[2], Satoshi TSUZUKI[1], Kazuo INOUE[1] and Tohru FUSHIKI[1]

[1] Laboratory of Nutrition Chemistry, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502; and [2] Laboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

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ABSTRACT

We investigated the gene expression dynamics of several hypothalamic neuropeptides associated with appetite regulation when rats are anticipating being fed a corn-oil emulsion. For 5 days at the same hour each day, rats were fed 5% corn oil emulsified with 0.3% xanthan gum or the vehicle for 20 min. On Day 6, the 5% corn oil emulsion or the vehicle (Vehicle) was presented to the rats, some of which (Oil-intake) were allowed to eat it and some of which (Oil-anticipation) were kept from eating it. Despite waiting a corn-oil, the mRNA levels of proopiomelanocortin (POMC), a β-endorphin precursor, and orexin showed increases, and high levels of mRNAs of POMC and orexin were maintained for 30 min after the corn-oil was placed before the rats, and only gradually decreased through 150 min. However, the mRNA levels of POMC and orexin in the hypothalamus were decreased within 30 min after starting to ingest the corn-oil emulsion. These results suggest that POMC and orexin mRNA expression was induced by the anticipation in rats after learning the palatability of 5% corn oil emulsion, and the induced mRNA expression based on the anticipation was maintained for at least for 30 min as the rats eagerly waited for ingestion.

Obesity resulting from excessive energy ingestion is a serious health issue. Despite warnings about excessive ingestion of high-fat foods, there currently appears to be no decrease in consumption of such foods (3, 11, 18, 22). It is thought that many mammals prefer to consume high-fat foods due to their pleasant taste, flavor, and texture. In short-term 2-bottle choice tests, mice prefer vegetable oils to sucrose, itself a highly palatable substance (20). In addition, conditioned place preference (CPP) tests have demonstrated a reinforcing effect of corn oil on mice (6). In comparison to rats fed on regular chow diets, rats fed on a high-fat diet show a greater food intake and became overweight as a result (1). Understanding why mammals, including humans, prefer high-fat foods will provide important information for regulating oil ingestion and thus maintaining good health.

Several neuropeptides produced in the hypothalamus mediate feeding behavior. These include proopiomelanocortin (POMC), produced in arcuate nucleus of hypothalamus, and physiologically co-active peptides like beta-endorphin, adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormone (MSH) (4, 17). We discovered that beta-endorphin, an opioid peptide, is released just after dietary oil ingestion in rats (12). Moreover, the gene expression of POMC (a precursor of beta-endorphin) increased just before dietary oil ingestion (12).

Address correspondence to: Takafumi Mizushige, MSc. Laboratory of Nutrition Chemistry, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
Tel: +81-75-753-6263, Fax: +81-75-753-6264
E-mail: takamz@kais.kyoto-u.ac.jp

Considering the fact that POMC gene expression increased prior to substantial oil ingestion, we hypothesized that a learning or anticipation for oil may be related to the initiation of POMC gene expression.

Orexin is produced in lateral hypothalamus area (LHA), and then projected to several regions of brain. Orexin-A administered to rats intracisternally induced relaxation of the proximal stomach, and facilitated phasic contractions in the distal stomach (9). Furthermore, rats administered SB334867A (antagonist of orexin receptor) in ventral tegmental area and prepro-orexin knockout mice suppressed the morphine-induced conditioned place preference (CPP) (5, 13). These results suggested that orexin is involved in preference or palatability and reward effects of food.

In the present study, we measured the gene expression of hypothalamic neuropeptides when rats actually ingested oil emulsion and when rats anticipated eating oil emulsion after learning its palatability through daily regulated feeding.

MATERIALS AND METHODS

Animals. Male Wister rats (Japan SLC, Hamamatsu, Japan) at 9 weeks old were raised in stainless wire mesh cages in a room controlled by a 12-hour light-dark cycle (dark phase: 15 : 00–3 : 00) and constant temperature (24 ± 1°C). They were housed separately for a week for acclimatization to the environment. Animals were fed distilled water and regular chow (MF; Oriental Yeast, Tokyo, Japan) *ad libitum*. This study was conducted in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee and the Japan Neuroscience Society and was in complete compliance with the National Institutes of Health: Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and limit experimentation to what was necessary to produce reliable scientific information.

Experimental Protocols. We first conducted 5-day training sessions with rats, during which daily water deprivation took place at 11 : 00 for 5 h and daily fasting took place at 14 : 00 for 2 h. One hour after the start of dark phase (16 : 00), rats were provided with 5% corn oil suspended in 0.3% xanthan gum aqueous solution, which masked the oil texture (Oil group), or the 0.3% xanthan gum aqueous solution alone (Vehicle group). The amount of intake during a 20-min feeding period was measured for each rat.

On Day 6, after completion of the training sessions, both the Oil and Vehicle groups underwent the test. Water deprivation and fasting took place at the same hour as during the training sessions. The Oil group was divided into two groups based on whether the rats would actually ingest the solution, *i.e*., an Oil-intake and Oil-anticipation group. Rats in Oil-anticipation group were shown a bottle filled with oil but were not given the oil. Rats in Oil-intake group were given oil immediately after being presented the solution.

In order to measure gene expression levels, the hypothalamus was extracted from individuals of all groups −60, 0, 30, and 150 min after presenting the oil emulsion to the rats. The hypothalamus was excised from the basal area of the brain after decapitation and RNA extraction was carried out. Samples were stored at −70°C until the assay.

Measurements of Gene Expression levels. Reverse transcription and real-time RT-PCR were carried out as previously described (19). Total RNA was reverse-transcribed with a MMLV reverse transcriptase (Promega, Madison, Wisconsin, USA) using a thermal cycler (Takara PCR Thermal Cycler SP; Takara, Shiga, Japan). Quantitative PCR to assay gene expression was carried out using a LightCycler (LightCycler System; Roche Diagnostics, Mannheim, Germany). The PCR primers used to amplify each gene are shown in Table 1. PCR was carried out according to the manufacturer's instructions (16). The basic amplification program was set to perform 40 cycles of 0 sec denaturation at 95°C, 5 sec annealing at 55°C, and a 10 sec extension at 72°C, with a slope of 20°C/sec. Fluorescence was recorded at 530 nm during extension. From the hypothalamuses of control rats, mRNAs were extracted, and cDNA were amplified by RT-PCR for 30 cycles, and used as external standards. The external standards and samples were amplified simultaneously.

Statistical Analysis. Data are expressed as mean ± standard errors (SE). For all measurement items, comparisons were separately analyzed by two-way repeated-measure analyses of variance (ANOVA) (group vs. time). Comparisons between the 2 groups were made using an unpaired Student's *t*-test. The comparisons between time with the another time in the same group were made using one-way ANOVA with Sheffé post hoc significance testing. All statistical tests were done with StatView (SAS Institute Inc., Cary, NC). Statistical significance was defined as $p < 0.05$.

Table 1 *Sequences of primers for real time RT-PCR analysis*

Gene		Sequence of primers	Product size (bp)
NPY[1]	sense	AGATACTACTCCGCTCTGCGACAC	286
	antisense	ACAAGGGAAATGGGTCGGAAT	
Galanin	sense	GACCTGCACTAACCAGCTACGC	222
	antisense	AGGCCATGCTTGTCGCTAAAT	
AGRP[2]	sense	AGAGTTCTCAGGTCTAAGTCT	210
	antisense	CTTGAAGAAGCGGCAGTAGCACGT	
Orexin	sense	TTCCTGCCGTCTCTACGAACTG	286
	antisense	ACTAGGACAGGGATAGAAGACGGG	
MCH[3]	sense	GGCTCCAAGCAGAATCTCGTAAC	238
	antisense	ACTTGCCAACAGGGTCGGTAG	
CRH[4]	sense	AGGAAACTGATGGAGATTATCGGG	257
	antisense	AGCGTGAACAATACAAATAACGCTG	
CART[5]	sense	TCCGATCTATGAGAAGAAGTACGGC	241
	antisense	ACGCAAACTTTATTGTTGTTAAAGCG	
POMC[6]	sense	GAAGGTGTACCCCAATGTCGC	248
	antisense	ATGGCGTTCTTGAAGAGCGTC	
Housekeeping gene			
GAPDH[7]	sense	CTCATAGACAAGATGGTGAAGGT	306
	antisense	CGCCAGTAGACTCCACGACATAC	

1. Neuropeptide Y, 2. Agouti-gene related protein, 3. Melanin-concentrating hormone, 4. Corticotropin-releasing hormone, 5. Cocaine- and amphetamine-regulated transcript, 6. Proopiomelanocortin, 7. Glyceraldehyde-3-phosphate dehydrogenase

RESULTS

In the test session after the 5-day training period, Oil-anticipation rats, which had undergone the training with 5% corn oil emulsion, showed high POMC mRNA expression after presentation of the corn oil bottle. The high POMC mRNA expression was maintained for 150 min (*t*-test: $p < 0.05$, Fig. 1) when compared with the Vehicle group. However, Oil-intake group rats, which were given the corn oil immediately upon its presentation (0 time), markedly decreased hypothalamic mRNA levels of POMC within 30 min.

As well as POMC, orexin mRNA expression was significantly greater in the Oil-anticipation group than in the Vehicle group 0, 30 and 150 min after the presentation of their respective solutions (*t*-test: $p < 0.05$, Fig. 2). Orexin mRNA expression was significantly decreased in the Oil-intake group within 30 min after presentation of 5% corn oil emulsion (*t*-test: $p < 0.05$, Fig. 2).

No significant differences in NPY, Galanin, MCH, AGRP, CART or CRH mRNA expressions were observed between the rats trained with oil and the rats trained with vehicle in the test session (Table 2). Galanin mRNA expression was significantly lower in the Oil-intake group than in the Oil-anticipation group 150 min after presentation of 5% corn oil emulsion (*t*-test: $p < 0.05$, Table 2). CRH mRNA expression was significantly lower in the Oil-intake group than in the Oil-anticipation group 30 min after presentation of the 5% corn oil emulsion (*t*-test: $p < 0.05$, Table 2), and tended to be lower in the Oil-intake group than in the Oil-anticipation group 150 min after presentation of the 5% corn oil emulsion (*t*-test: $p = 0.057$, Table 2).

DISCUSSION

In this study, we examined the dynamics of gene expression of several hypothalamic neuropeptides when rats were anticipating being fed corn oil emulsion which they had been fed for 5 days during training. In the previous study, we demonstrated that a daily ingestion of 5% corn oil emulsion led to increased oil intake in rats (12). These results led us to conclude that rats markedly acquired an appetite for 5% corn oil emulsion after 5 days of training. Therefore, we used similar training procedure in this study.

The present study showed that the hypothalamic POMC gene expression just before ingestion of the solution was significantly higher in the group trained with oil than in the group trained with vehicle (Fig. 1). It indicated that anticipation of oil intake made rats increase POMC mRNA expression despite

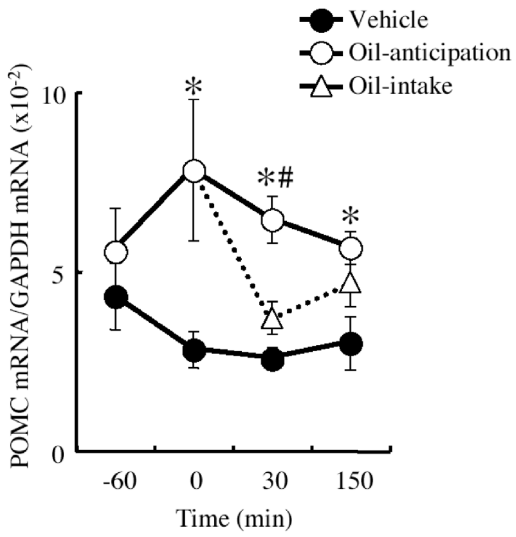

Fig. 1 Effect on hypothalamic POMC gene expression of learned anticipation of corn-oil emulsion feeding in rats. Before the test session, rats were provided with 5% corn oil emulsion (Oil-anticipate group and Oil-intake group) or its vehicle (Vehicle group) for 20 min for 5 days. On Day 6, they were shown bottles of solution for up to 150 min (Oil-anticipation group). Oil-intake and Vehicle groups were given their respective test solutions immediately, at 0 min. Rats were decapitated at different times and their hypothalamuses were extracted and measured for POMC mRNA. POMC gene expression values were normalized to the values of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Each value represents the mean ± standard errors (SE) for 6–8 rats. Asterisks (∗) indicate a significant difference in comparison with the Vehicle control group ($p < 0.05$). Sharp signs (#) indicate a significant difference in comparison with Oil-intake group ($p < 0.05$).

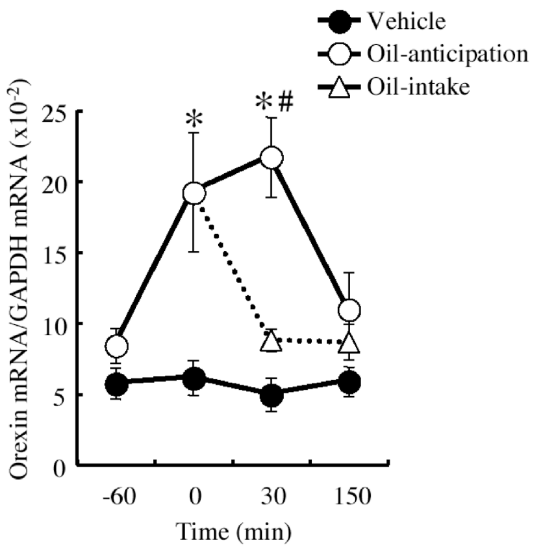

Fig. 2 Effect on hypothalamic orexin gene expression of learned anticipation of corn-oil emulsion feeding in rats. These measurements were conducted by the same method as those of the POMC gene expression above (Fig. 1). Each value represents the mean ± standard errors (SE) for 6–8 rats. Asterisks (∗) indicate a significant difference in comparison with the Vehicle control group ($p < 0.05$). Sharp signs (#) indicate a significant difference in comparison with the Oil-intake group ($p < 0.05$).

substantial oil intake. Instead, five days' daily intake of oil at the same hour produced a sense of expectancy and induced POMC mRNA expression in the hypothalamus. Subsequent corn oil intake resulted in decreased POMC mRNA expression, although the POMC mRNA level was maintained the high level for more than 30 min in rats not fed the oil (Fig. 1). In a previous study, we observed that beta-endorphin was released into the cerebrospinal fluid and the serum just after corn oil ingestion in rats (12). These findings suggested that anticipation of oil intake organizes POMC mRNA expression, and the expression is sustained until substantial ingestion of oil. The oil intake may be a trigger for transient beta-endorphin production and release from hypothalamus. Simultaneously, oil intake may signal an end to POMC mRNA expression.

Orexin as well as POMC increased in the mRNA expression just before ingestion of corn oil in the rats trained with corn oil (Fig. 2). Kobashi M *et al.* reported that administration of orexin-A intracisternally to rats induced relaxation of the proximal stomach, and facilitated phasic contractions in the distal stomach (9). Narita M *et al.* reported that intracerebroventricular (i.c.v.) injection of an antagonist of orexin receptors eliminated the reward effect of drugs in rats (13). These results suggested that orexin is involved in preference, palatability and reward effects of food, corroborating our finding in the present study that orexin is implicated in parts of preference, palatability and reward effect of oil.

Accumulated data support the interaction between orexin and opioid in the reward system in the brain. Clegg *et al.* reported that i.c.v. injection of orexin-A and -B increased food intake for 1 h, and that the effects were inhibited completely by intraperitoneal injection of naloxone, an antagonist of the opioid receptor (2). Furthermore, i.c.v. injection of an antagonist of the orexin receptor eliminated acquisition of CPP induced by injection of morphine, an agonist of the opioid receptor (13). It is possible that the interaction of orexin and opioid affected preference, palatability and reward effect of oil.

The gene expression of both POMC and orexin increased just before corn oil ingestion in Oil-antici-

Table 2 *The mRNA expression of NPY, Galanin, MCH, AGRP, CART and CRH during the test session*

	NPY	Galanin	MCH	AGRP	CART	CRH
	(target gene mRNA/GAPDH mRNA)					
	($\times 10^{-2}$)	**($\times 10^{-2}$)**	**($\times 10^{0}$)**	**($\times 10^{-2}$)**	**($\times 10^{-1}$)**	**($\times 10^{-2}$)**
Trained with oil but not fed oil in the test session (Oil-anticipation group)						
−60	1.71 ± 0.43	4.35 ± 1.32	2.82 ± 0.81	2.61 ± 0.71	8.15 ± 2.15	3.46 ± 0.82
0	1.38 ± 0.10	3.01 ± 0.37	2.46 ± 0.39	1.95 ± 0.28	6.44 ± 0.85	3.25 ± 0.40
30	1.52 ± 0.07	3.07 ± 0.20	2.20 ± 0.17	1.76 ± 0.14	5.62 ± 0.41	3.42 ± 0.25
150	1.45 ± 0.05	3.43 ± 0.12	2.48 ± 0.15	2.20 ± 0.32	6.12 ± 0.16	3.97 ± 0.34
Trained with oil and fed oil in the test session (Oil-intake group)						
30	1.52 ± 0.31	3.99 ± 0.62	2.64 ± 0.24	2.12 ± 0.38	5.33 ± 0.79	2.61 ± 0.17*
150	1.30 ± 0.16	2.93 ± 0.13*	2.63 ± 0.30	2.02 ± 0.10	5.46 ± 0.62	2.94 ± 0.33
Trained with vehicle and fev vehicle in the test session (Vehicle group)						
−60	1.38 ± 0.19	3.39 ± 0.99	2.35 ± 0.33	2.25 ± 0.34	6.22 ± 0.84	3.03 ± 0.48
0	1.66 ± 0.18	3.76 ± 0.43	3.15 ± 0.40	2.31 ± 0.33	8.60 ± 1.27	3.51 ± 0.42
30	1.41 ± 0.22	3.21 ± 0.56	2.58 ± 0.58	2.25 ± 0.59	7.67 ± 1.49	2.73 ± 0.37
150	1.73 ± 0.37	3.85 ± 0.74	3.20 ± 0.86	2.68 ± 0.60	9.23 ± 2.22	3.47 ± 0.73

Data are the means ± SE, n = 6–8. *$p < 0.05$ (Student's *t*-test), vs. Oil-anticipation group

pate group; however, that of other hypothalamic neuropeptides did not. In the Oil-intake group, these expressions maintained their high levels until rats were given the oil emulsions. The anticipation and/or learning of corn oil ingestion may cause the gene expressions of POMC and orexin. To figure out the mechanisms constituting the anticipation acquired by 5-day training with corn oil, further studies are required. The ingestion of sucrose solution was increased the gene expression of POMC and beta-endorphin (23, 24). POMC and beta-endorphin may strongly associate with preference or palatability of food including sweetener and fat.

Galanin and corticotropin-releasing hormone (CRH) gene expression tended to decrease from corn oil ingestion (Table 2). Several studies have been made on the effects of galanin on the preferential ingestion of fat. Compared to normal rats, galanin-overexpressed rats increased their intake of a high-fat diet, but not of a high-carbohydrate or high-protein diet; further, these effects were eliminated by injection of antagonists of the galanin receptor, galantide and C7, in the cerebral ventricles (14, 15). Moreover, galanin peptide levels, galanin cell density and galanin fiber density in the PVH of rats ingesting a high-fat diet were greater than those of rats ingesting a high-carbohydrate diet (10). Several studies reported that CRH is related to stress response and appetite (7, 8, 21). Galanin and CRH may be related to long-term regulation of oil ingestion.

Overall, we found that POMC and orexin mRNA expression was induced by the anticipation and/or learning of oil ingestion. The anticipation and/or learning of oil ingestion may cause preparation of beta-endorphin and orexin release just after oil ingestion.

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