*Am J Physiol Regul Integr Comp Physiol* 306: R265–R272, 2014.
First published January 8, 2014; doi:10.1152/ajpregu.00405.2013.

# δ-Opioid receptor activation stimulates normal diet intake but conversely suppresses high-fat diet intake in mice

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Submitted 23 August 2013; accepted in final form 6 January 2014

**Kaneko K, Mizushige T, Miyazaki Y, Lazarus M, Urade Y, Yoshikawa M, Kanamoto R, Ohinata K.** δ-Opioid receptor activation stimulates normal diet intake but conversely suppresses high-fat diet intake in mice. *Am J Physiol Regul Integr Comp Physiol* 306: R265–R272, 2014. First published January 8, 2014; doi:10.1152/ajpregu.00405.2013.—The central opioid system is involved in a broadly distributed neural network that regulates food intake. Here, we show that activation of central δ-opioid receptor not only stimulated normal diet intake but conversely suppressed high-fat diet intake as well. [D-Pen$^{2,5}$]-enkephalin (DPDPE), an agonist selective for the δ-receptor, increased normal diet intake after central administration to nonfasted male mice. The orexigenic activity of DPDPE was inhibited by blockade of cyclooxygenase (COX)-2, lipocalin-type prostaglandin D synthase (L-PGDS), D-type prostanoid receptor 1 ($DP_1$), and neuropeptide Y (NPY) receptor type 1 ($Y_1$) for $PGD_2$ and NPY, respectively, suggesting that this was mediated by the $PGD_2$-NPY system. In contrast, DPDPE decreased high-fat diet intake in mice fed a high-fat diet. DPDPE-induced suppression of high-fat diet intake was blocked by antagonists of melanocortin 4 ($MC_4$) and corticotropin-releasing factor (CRF) receptors but not by knockout of the L-PGDS gene. These results suggest that central δ-opioid receptor activation suppresses high-fat diet intake via the MC-CRF system, independent of the orexigenic $PGD_2$ system. Furthermore, orally administered rubiscolin-6, an opioid peptide derived from spinach Rubisco, suppressed high-fat diet intake. This suppression was also blocked by centrally administered naltrindole, an antagonist for the δ-receptor, suggesting that rubiscolin-6 suppressed high-fat diet intake via activation of central δ-opioid receptor.

δ-opioid receptor; food intake; $MC_4$; CRF; $PGD_2$

A NUMBER OF ENDOGENOUS orexigenic and anorexigenic factors and their receptors in the central nervous system (CNS) play a key role in food intake regulation and energy homeostasis (16, 31, 41, 44). Opioids are a series of substances having morphine-like analgesic activities and bind to opioid receptors, which are present in the CNS. In general, activation of the opioid system seems to contribute to stimulation of food intake. Indeed, agonists of μ-receptor, among major opioid receptors μ and δ, stimulated normal and high-fat diet intake (1, 45); however, the role of central δ-receptor in food intake regulation has not been fully elucidated.

Recently, we found that rubiscolin-6, an δ-opioid agonist peptide derived from Rubisco, which is a major protein in green leaves such as spinach, stimulates normal diet intake after oral administration in mice (18, 19). We also reported that this orexigenic activity was mediated by prostaglandin (PG) $D_2$. $PGD_2$ is the most abundant PG in the CNS and has various physiological actions, including sleep induction and hyperthermia (28, 30, 38, 39, 43). We previously reported that $PGD_2$ stimulates food intake via $DP_1$ receptor for $PGD_2$, which was coupled with neuropeptide Y (NPY), the hypothalamic peptide that most potently stimulates food intake (38). The rubiscolin-6-induced orexigenic activity was mediated by the central $PGD_2$-NPY system. In our current study, we used the agonist δ-receptor [D-Pen$^{2,5}$]-enkephalin (DPDPE),which is more potent and selective than that of rubiscolin-6, to demonstrate that central activation of δ-opioid-stimulated normal diet intake via activation of the $PGD_2$-NPY system in mice.

It was also well known that the responsiveness of several endogenous peptides regulating food intake is affected by nutritional status. For example, enterostatin selectively suppressed fat intake in rats fed with high-fat but not normal diet (10). We then investigated the effect of central administration of δ agonist on high-fat intake. Intriguingly, in mice fed a high-fat diet, DPDPE suppressed high-fat diet intake, indicating that central δ activation contributed to decrease in high-fat diet intake. Thus we have found that activation of the δ-receptor in the CNS stimulates normal diet intake and conversely decreases high-fat diet intake in mice. We then focused on this novel action induced by central δ activation and tested whether the anorexigenic activity was associated with the melanocortin (MC) system coupled to corticotropin-releasing factor (CRF), which contributes to decreased food intake, as well as the above-mentioned $PGD_2$ system. We also investigated whether rubiscolin-6 suppressed high-fat intake after oral administration.

## MATERIALS AND METHODS

*Materials.* Rubiscolin-6 was synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc) strategy (4). DPDPE, an agonist for the δ-opioid receptor, and naltrindole, an antagonist for the δ-opioid receptor, were obtained from Sigma-Aldrich (St. Louis, MO). BIBO3304 trifluoroacetate, an antagonist for the NPY $Y_1$ receptor, and HS024, an antagonist for the melanocortin 4 ($MC_4$) receptor, were obtained from Tocris Cookson (Ellisville, MO). MK0524 and BWA868C, antagonists of the $DP_1$ receptor, were obtained from Cayman Chemical (Ann Arbor, MI). SC-560, a cyclooxygenase (COX)-1 inhibitor, was obtained from Alexis Biochemicals (Plymouth Meeting, PA). Celecoxib, a COX-2 inhibitor, was obtained from Toronto Research Chemicals (Ontario, Canada). Astressin, an antagonist for the CRF receptor, was obtained from the American Peptide Company (Sunnyvale, CA).

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Carboxymethyl cellulose (CMC) was obtained from Nacalai Tesque (Kyoto, Japan).

*Animals and diets.* Male ddY or C57BL/6 mice of 7 wk of age were obtained from Japan SLC (Shizuoka, Japan) and used in these experiments. Lipocallin-type (L-) prostaglandin D synthase (PGDS) knockout (KO) mice were also used (18, 28, 30, 38, 39, 43). Each mouse was individually housed under regulated conditions (23 ± 1°C on a 12-h light-dark cycle with lights on at 7 AM) and had free access to water unless otherwise indicated. After a 1-wk acclimation period, we began the food intake experiment. During the acclimatization phase for the normal diet intake experiment, mice were allowed ad libitum access to normal pelleted chow. The normal diet consisted of 12.0% fat, 59.1% carbohydrate, and 28.9% protein and had an energy density of 3.5 kcal/g (CE-2; CLEA Japan, Tokyo Japan). During the high-fat diet intake experiment, mice were allowed ad libitum access to the high-fat diet, which consisted of 60.0% fat, 20.0% carbohydrate, and 20.0% protein and had an energy density of 5.2 kcal/g (D12492; Research Diets, New Brunswick, NJ).

*Cannula implantation.* Central administration was performed as described previously (18, 33, 34, 36–38). Briefly, mice were anesthetized with pentobarbital (80–85 mg/kg ip) and placed in a stereotaxic instrument. A 24-gauge cannula beveled at one end over a distance of 3 mm (Safelet-Cas; Nipro, Osaka, Japan) was implanted 0.9 mm posterior and 0.9 mm to the bregma in the third cerebral ventricle. Animals were tested 1 wk or more after implantation.

*Food intake experiment.* The food intake experiment was performed as previously described (17–19, 33–38). The experiment started at 11 AM. DPDPE at a dose of 0.1–1 nmol/mouse in 4 μl artificial cerebrospinal fluid (aCSF: 138.9 mM NaCl, 3.4 mM KCl, 1.3 mM $CaCl_2$, 4.0 mM $NaHCO_3$, 0.6 mM $NaH_2PO_4$, 5.6 mM glucose, pH 7.4) or vehicle was intracerebroventricularly administered to nonfasted mice. In this study, we used nonfasted male mice, unless otherwise indicated. DPDPE (0.3 nmol/mouse icv) and naltrindole (10 nmol/mouse icv) or BIBO3304 (5 nmol/mouse icv) in 4 μl aCSF were coadministered to ddY mice. A combination of DPDPE (0.3 nmol/mouse icv) and MK0524 or BWA868C (1.6 nmol/mouse icv) in 4 μl 5% dimethylsulfoxide aCSF was coadministered. A combination of DPDPE (0.3 nmol/mouse icv) and SC-560 (8.5 μmol/kg ip) or celecoxib (7.9 μmol/kg ip) in saline containing 0.5% CMC was also coadministered. DPDPE (1 nmol/mouse icv) was administered to L- or hematopoietic (H-) PGDS KO or wild-type C57BL/6 mice. The weight of the food pellets in each cage was measured at 0 min, 20 min, and 1, 2, and 4 h after administration, and the cumulative food intake was calculated.

In the high-fat diet intake experiment, we used 2-wk high-fat diet-fed mice. DPDPE (0.3 nmol/mouse icv) and HS024 (0.1 nmol/mouse icv) (20) or astressin (6 nmol/mouse icv) (14) in 4 μl ACSF were coadministered to C57BL/6 mice. Rubiscolin-6 at a dose of 0.13–1.3 μmol/kg in saline was orally administered to mice. Rubiscolin-6 (0.39 μmol/kg po) in saline was administered just after injection of naltrindole (10 nmol/mouse icv) or HS024 (0.1 nmol/mouse icv). DPDPE (1 nmol/mouse icv) or rubiscolin-6 (0.39 μmol/kg po) was administered to L-PGDS KO or wild-type C57BL/6 mice fed a high-fat diet. DPDPE was administered repeatedly 1, 3, and 7 days after the diet was changed from normal to high-fat diet.The other experiments were performed independently, unless otherwise indicated. All experiments were approved by Kyoto University Ethics Committee for Animal Research Use. After food intake experiments, cannula placement was confirmed by dye (18, 35). The third ventricle and the lateral ventricle of mouse brain were colored after intracerebroventricular administration of dye. All mice were killed by an overdose of anesthesia drugs after the experiment.

*Method of RNA preparation from hypothalamus and quantitative RT-PCR.* Each mouse hypothalamus was excised after decapitation under deep anesthesia and kept in RNA Stabilization Reagent (QIAGEN Sciences, Germantown, MD) until RNA extraction. Total RNA was extracted from the hypothalamus using the RNeasy Lipid Tissue Kit (QIAGEN Sciences) and transcribed to cDNA with random primers and oligo-dT by Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). For quantitative PCR, we amplified the cDNA using Applied Biosystems Prism 7000 Sequence Detection System (Foster City, CA) with Platinum SYBER Green qPCR SuperMix-UDG with ROX solution (Invitrogen) and each primer set specific for mouse β-actin, proopiomelanocortin (POMC), and $MC_4$, according to the manufacturer's instructions (Table 1). The reactions were cycled 40 times with denaturation at 95°C for 15 s and with annealing and elongation at 60°C for 30 s. The relative expression level of each mRNA was normalized using the mRNA level of β-actin.

*Statistical analysis.* Values are expressed as means ± SE. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's test. Bonferroni's post hoc test comparisons were carried out if significant *F*-values were obtained. *P* values $<0.05$ were considered significant.

## RESULTS

*Centrally administered δ-opioid agonist stimulates normal diet intake but suppresses high-fat diet intake.* We investigated whether DPDPE, a δ-selective agonist, stimulated normal diet intake after central administration to nonfasted male mice. ANOVA indicated that DPDPE has a significant effect [$F(3, 25) = 6.917$, $P < 0.01$; Fig. 1*A*]. The post hoc analysis using Bonfferoni's test indicated that DPDPE (0.3–1.0 nmol/mouse icv) significantly stimulates normal diet intake 60 min after administration to nonfasted male mice. This increase in food intake lasted for 240 min. In contrast, DPDPE (0.1–1 nmol/mouse icv) suppressed high-fat diet intake in mice fed a high-fat diet for 2 wk [$F(2,15) = 3.893$, $P < 0.05$; Fig. 1*B*]. Thus we found that centrally administered DPDPE stimulates normal diet intake but suppresses high-fat intake. Next, we investigated whether DPDPE-induced changes in food intake were mediated via the δ-opioid receptor using naltrindole, an δ antagonist. Central administration of naltrindole (10 nmol/mouse) inhibited both the DPDPE (0.3 nmol/mouse)-induced increase in normal diet intake and the decrease high-fat diet intake [$F(3,20) = 12.310$, $P < 0.001$ and $F(2,14) = 6.829$, $P < 0.01$; Fig. 1, *C* and *D*, respectively]. Naltrindole alone did not change normal (Fig. 1*C*) and high-fat diet intake (Fig. 5*B*).These results suggest that opposing food intake behaviors in mice fed normal and high-fat diets may be mediated through a common δ-opioid receptor. We also investigated whether

Table 1. *Oligonucleotide sequence of PCR primers specific for δ-opioid receptor, POMC, $MC_4$ receptor, and β-actin*

| Gene | Forward | Reverse |
|---|---|---|
| δ-Opioid receptor | GCTCGTCATGTTTGGCATC | AAGTACTTGGCGCTCTGGAA |
| POMC | GGCTTGCAAACTCGACCTCT | TGACCCATGACGTACTTCCG |
| $MC_4$ receptor | TCTCTATGTCCACATGTTCCTG | GGGGCCCAGCAGACAACAAAG |
| β-Actin | CTGCGCAAGTTAGGTTTTGTCA | TGCTTCTAGGCGGACTGTTACTG |

$MC_4$, melanocortin-4; POMC, proopiomelanocortin.

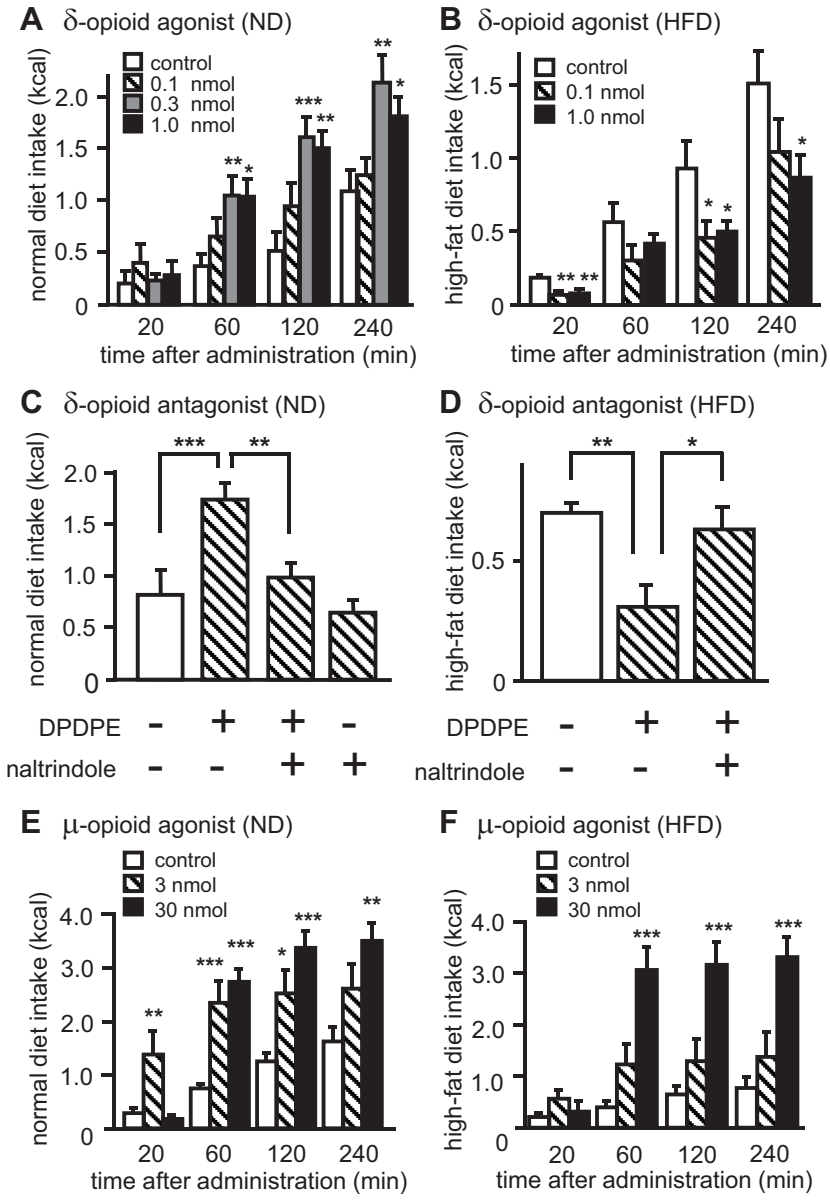


Fig. 1. Effect of δ-opioid agonist on normal diet or high-fat diet intake. [D-Pen$^{2,5}$]-enkephalin (DPDPE) was administered (0.1–1 nmol/mouse icv) and normal diet (ND) intake (*A*) or high-fat diet (HFD) intake (*B*) was measured in nonfasted mice. Normal diet intake stimulation (*C*) or high-fat diet intake suppression (*D*) of DPDPE (0.3 nmol/mouse icv) 120 min after administration was blocked by the δ-opioid antagonist naltrindole (10 nmol/mouse icv) in nonfasted mice. μ-Opioid agonist endomorphin-2 was administered (3–30 nmol/mouse icv) and normal diet intake (*E*) or high-fat diet intake (*F*) was measured in nonfasted mice. Each column represents the means ± SE. One-way ANOVA followed by Bonfferroni's test were used to assess the difference among each groups. (*A*, $n$ = 7–10; *B*, $n$ = 6; *C*, $n$ = 8–9; *D*, $n$ = 6–8; *E*, $n$ = 8; *F*, $n$ = 5). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control or each group.

μ-opioid agonist changes high-fat diet intake. Centrally administered μ-opioid agonist endomorphin-2 stimulates normal diet intake and high-fat diet intake [$F(2,21) = 14.265$, $P < 0.001$ and $F(2,12) = 15.130$, $P < 0.001$; Fig. 1, *E* and *F*, respectively] in nonfasted mice.

*δ-Agonist-induced orexigenic activity in mice fed a normal diet is mediated by the PGD$_2$-NPY system.* We performed experiments to determine which mediators were involved in the orexigenic activity of DPDPE, downstream of the central δ-opioid receptor. The orexigenic effect of DPDPE (0.3 nmol/mouse icv) was blocked by celecoxib, a COX-2 selective inhibitor (7.9 μmol/kg ip), but not by SC-560, a COX-1 selective inhibitor at a dose of 8.5 μmol/kg ip [$F(3,29) = 9.191$, $P < 0.001$ and $F(3,29) = 9.344$, $P < 0.001$; Fig. 2, *A* and *B*, respectively]. Among COX products, PGD$_2$, produced by L-PGDS in the CNS, exhibits orexigenic activity in mice (43); thus we used L-PGDS KO mice to examine whether the orexigenic activity of DPDPE was mediated by L-PGDS. Centrally administered DPDPE (1.0 nmol/mouse) stimulated food intake in wild-type and hematopoietic-PGDS KO but not L-PGDS KO mice, suggesting that the orexigenic activity of DPDPE is mediated by L-PGDS [$F(3,24) = 3.662$, $P < 0.05$ and $F(3,28) = 8.369$, $P < 0.001$; Fig. 2, *C* and *D*, respectively] In addition, normal diet intake in L-PGDS or H-PGDS KO mice did not change significantly compared with wild-type mice after administration of control vehicle in this and a previous study (18).

It was reported that PGD$_2$ stimulates food intake via the DP$_1$ receptor followed by the NPY-Y$_1$ receptor system (43). The orexigenic effect of DPDPE (0.3 nmol/mouse icv) was completely inhibited by MK0524 and BWA868C (1.6 nmol/mouse icv), an antagonist selective for DP$_1$ receptor, or BIBO3304 (5 nmol/mouse), an antagonist for Y$_1$ receptor [$F(5,24) = 9.069$, $P < 0.001$ and $F(3,28) = 9.085$, $P < 0.001$; Fig. 2, *E* and *F*, respectively]. These results suggest that central activation of

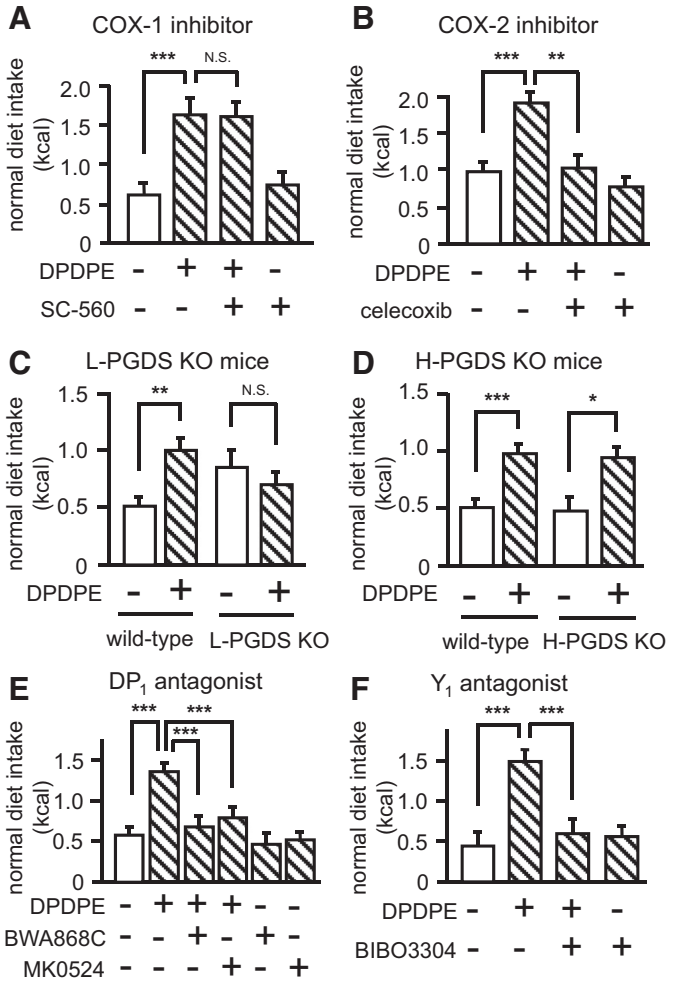


Fig. 2. Involvement of central prostaglandin $D_2$ ($PGD_2$) and neuropeptide Y (NPY) system in the normal diet intake stimulation of δ-opioid agonist. The orexigenic effect of DPDPE (0.3 nmol/mouse icv) 120 min after administration was inhibited by cyclooxygenase (COX)-2 inhibitor celecoxib (*B*, 7.9 μmol/kg ip) but not by COX-1 inhibitor SC-560 (*A*, 8.5 μmol/kg ip) in nonfasted mice. DPDPE was centrally administered at a dose of 1 nmol/mouse to wild-type, lipocalin-type prostaglandin D synthase (L-PGDS) knockout (KO) (*C*) and hematopoietic-PGDS (H-PGDS) KO (*D*) mice, and food intake was measured for 60 min. (*E*, *F*) The orexigenic activity of DPDPE (0.3 nmol/mouse icv) was blocked by $DP_1$ receptor antagonist BWA868C and MK0524 (*E*, 1.6 nmol/mouse icv) or $Y_1$ receptor antagonist BIBO3304 (*F*, 5 nmol/mouse icv) in mice. Each column represents means ± SE. One-way ANOVA followed by Bonferroni's test were used to assess the difference among each groups. (*A* and *B*, $n = 8$–9; *C*, $n = 6$–7; *D*, $n = 7$–9; *E*, $n = 7$–10; *F*, $n = 8$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with each group. NS, not significant.

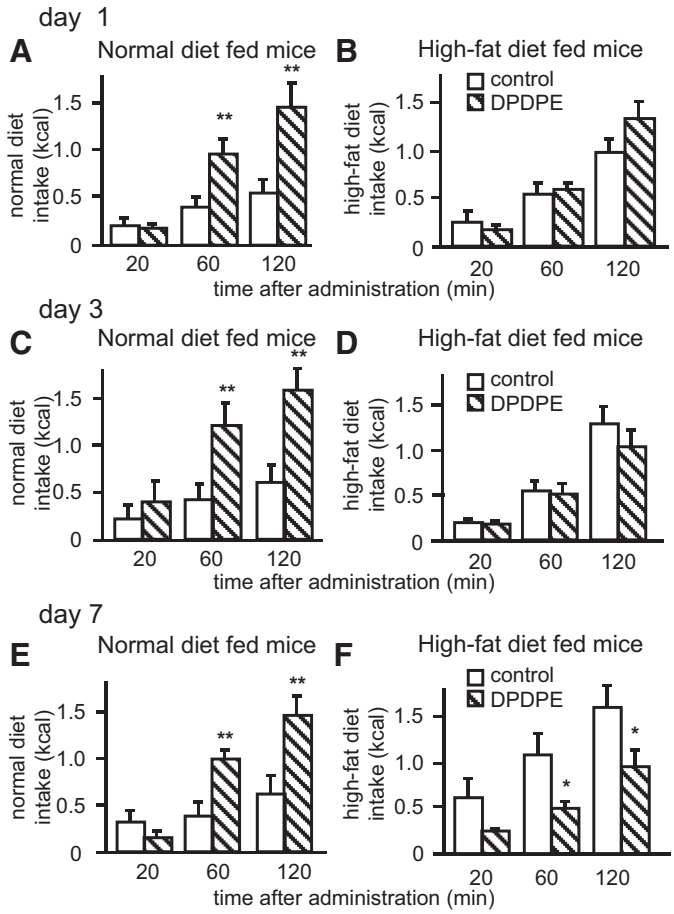


Fig. 3. Effect of feeding periods of high-fat diet on food intake in response to δ-agonist administration. (*B*, *D*) In 1- and 3-day high-fat-diet-fed mice, DPDPE (0.3 nmol/mouse icv) did not change intake of the high-fat diet. (*F*) In 7-day high-fat-diet-fed mice, DPDPE (0.3 nmol/mouse icv) decreased intake of the high-fat diet. (*A*, *C*, *E*). In contrast, DPDPE (0.3 nmol/mouse icv) always stimulated food intake in mice fed normal diet during experimental period. Each column represents means ± SE. One-way ANOVA followed by Bonferroni's test were used to assess the difference among each groups. ($n = 9$). $^*P < 0.05$, $^{**}P < 0.01$ compared with control group.

δ-receptor stimulates food intake via an orexigenic pathway through the L-PGDS-$PGD_2$-$DP_1$ and NPY-$Y_1$ receptor system.

*δ-Agonist-induced anorexigenic activity in mice fed a high-fat diet is mediated via the MC and CRF system.* In mice fed a high-fat diet, central administration of DPDPE suppressed high-fat diet intake. We then investigated when DPDPE start to decrease high-fat diet intake. One day after changing from a normal to high-fat diet, DPDPE lost orexigenic activity (Fig. 3*B*). Seven days after the change, DPDPE significantly decreased intake of the high-fat diet [$F(1,16) = 5.785$, $P < 0.05$; Fig. 3*F*], suggesting that DPDPE exhibits anorexigenic activity in mice fed a high-fat diet for over 1 wk. We also investigated whether high-fat diet intake affects the mRNA expression of the δ-opioid receptor, proopiomelanocortin (POMC), or $MC_4$ receptor gene in the hypothalamus. Seven days after the change, the mRNA expression of POMC gene increased in high-fat diet-fed mice, but the mRNA expressions of the δ-opioid and $MC_4$ receptor did not change in high-fat diet-fed mice (Table 2).

Table 2. *Effect of 7 days high-fat diet feeding on hypothalamic mRNA levels of δ-opioid receptor, POMC, and $MC_4$ receptor*

| Gene | Relative Expression | |
|---|---|---|
| | Normal diet | High-fat diet |
| δ-Opioid receptor | 1.0 ± 0.13 | 0.73 ± 0.11 |
| POMC | 1.0 ± 0.20 | 1.70 ± 0.19* |
| $MC_4$ receptor | 1.0 ± 0.13 | 1.39 ± 0.30 |

Values are means ± SE ($n = 5$–6). Student's *t*-test was used for comparison of two groups. $^*P < 0.05$ compared with normal diet fed group.

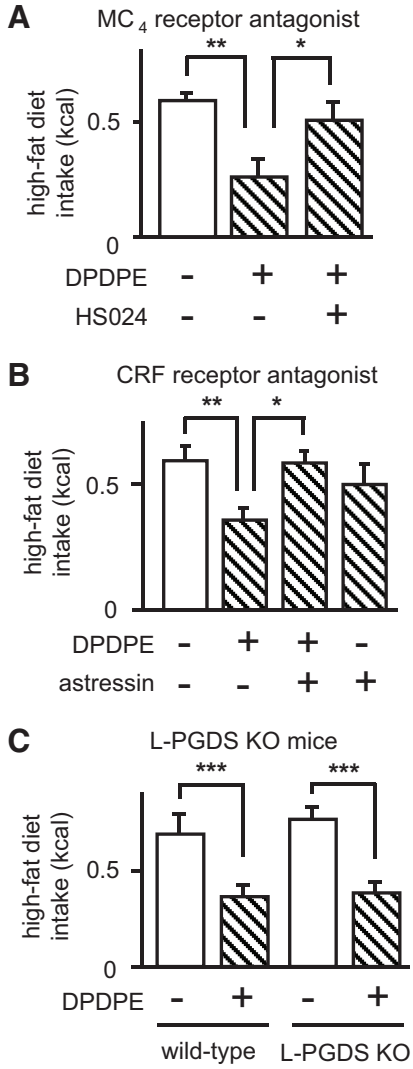


Fig. 4. Involvement of central melanocortin 4 ($MC_4$) and corticotropin-releasing factor (CRF) system in the high-fat diet intake suppression of δ-opioid agonist. The anorexigenic activity of DPDPE (0.3 nmol/mouse icv) was blocked by $MC_4$ receptor antagonist HS024 (*A*, 0.1 nmol/mouse icv) or CRF receptor antagonist astressin (*B*, 6 nmol/mouse icv) in mice. Centrally administered DPDPE (1 nmol/mouse) decrease high-fat diet intake in wild-type and L-PGDS KO mice (*C*). Each column represents means ± SE. One-way ANOVA followed by Bonferroni's test were used to assess the difference among each groups. (*A*, $n = 6–8$; *B*, $n = 8–18$; *C*, $n = 7$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with each group.

Next, we tested which mediators were involved in the suppression of high-fat diet intake by δ-agonist, downstream of the central δ-receptor. HS024, an antagonist for the $MC_4$ receptor, was used to examine the involvement of the $MC_4$ receptor in the δ-agonist-induced effect. The anorexigenic activity of DPDPE (0.3 nmol/mouse icv) was inhibited by coadministration of HS024 (0.1 nmol/mouse), suggesting that DPDPE decreases high-fat intake via the $MC_4$ receptor [$F(2,14) = 6.850$, $P < 0.01$; Fig. 4*A*]. We also investigated the involvement of CRF, which is known to be activated by α-MSH, an endogenous agonist peptide for $MC_4$ receptor, in δ-agonist-induced anorexigenic activity. The DPDPE-induced decrease in food intake (0.3 nmol/mouse) was inhibited by astressin (6 nmol/mouse icv), a CRF receptor antagonist [$F(3,58) = 4.295$, $P < 0.01$; Fig. 4*B*]. These results suggest that central δ-receptor activation decreases high-fat diet intake in mice fed a high-fat diet via an anorexigenic pathway through the $MC_4$ and CRF receptor system.

In addition, we investigated whether food intake suppression of δ-agonist in mice fed a high-fat diet was mediated by L-PGDS, which is coupled to orexigenic activity in mice fed a normal diet. In both L-PGDS KO and wild-type mice fed a high-fat diet, DPDPE decreased food intake [$F(3,28) = 9.338$, $P < 0.001$; Fig. 4*C*], indicating that L-PGDS mediated orexigenic but not anorexigenic activity after central δ-receptor activation. These results suggest that the opposing activities of δ-receptor agonist in food intake in mice fed normal and high-fat diets are associated with different pathways in the CNS, downstream of a common δ-receptor.

*Orally administered rubiscolin-6, a δ-opioid agonist peptide derived from food protein, suppresses high-fat diet intake.* Rubiscolin-6 (Tyr-Pro-Leu-Asp-Leu-Phe) is a δ-opioid peptide derived from the large subunit of D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is the key enzyme for carbon dioxide fixation and photorespiration. We found that rubiscolin-6 (0.39–3.9 μmol/kg, corresponding to 0.3–1.0 mg/kg) suppressed high-fat diet intake in mice fed a high-fat diet [$F(3,24) = 10.136$, $P < 0.001$; Fig. 5*A*]. The high-fat diet intake suppression of rubiscolin-6 was blocked by naltrindole [$F(3,21) = 3.351$, $P < 0.05$; Fig. 5*B*], suggesting that orally administrated rubiscolin-6 suppressed high-fat intake via the central δ-opioid receptor. Rubiscolin-6-induced anorexigenic activity (0.39 μmol/kg po) was also inhibited by HS024 (0.1

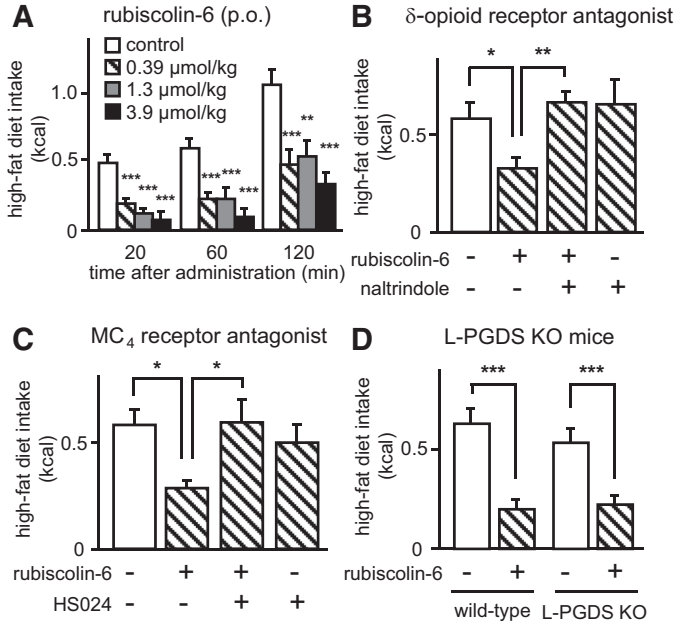


Fig. 5. Effect of rubiscolin-6 oral administration on high-fat diet intake in mice. Rubiscolin-6 was orally administered at a dose of 0.39–3.9 μmol/kg, and food intake was measured in nonfasted mice (*A*). The high-fat diet intake suppression of rubiscolin-6 (0.39 μmol/kg po) 60 min after administration was blocked by naltrindole (*B*, 10 nmol/mouse icv) or HS024 (*C*, 0.1 nmol/mouse icv) in nonfasted mice. Orally administered rubiscolin-6 (1.3 μmol/kg) decreased high-fat diet intake in wild-type and L-PGDS KO mice (*D*). Each column represents means ± SE. One-way ANOVA followed by Bonfferoni's test were used to assess the difference among each groups. ($n = 6–8$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with each group.

nmol/mouse icv) [$F(3,24) = 3.220$, $P < 0.05$; Fig. 5*C*], similarly to DPDPE. In both L-PGDS KO and wild-type mice fed a high-fat diet, orally administered rubiscolin-6 decreased high-fat intake [$F(3,22) = 11.247$, $P < 0.001$; Fig. 5*D*]. Thus orally administered rubiscolin-6 may suppress high-fat diet intake via the central δ-opioid receptor.

## DISCUSSION

We demonstrated that activation of the central δ-opioid receptor stimulated normal diet intake but suppressed high-fat diet intake in mice. These opposing activities, induced by an agonist of the δ-receptor, were blocked by an antagonist of the δ-receptor, suggesting that they are mediated via a common δ-receptor. The δ-opioid system is the first example of one system mediating both the stimulation of normal diet intake and the suppression of high-fat diet intake. In mice fed a normal diet, central administration of DPDPE, a selective δ-agonist, stimulated food intake via δ-receptor. This orexigenic activity was blocked by a COX inhibitor, knockout of the L-PGDS gene, and an antagonist of the $DP_1$ receptor. This was also inhibited by an antagonist of the NPY $Y_1$ receptor. It was reported that the central δ-opioid receptor is widely expressed in the CNS, including the hypothalamus, an important site for food intake regulation, and L-PGDS and NPY-like immunoreactivity was also present in the hypothalamus (18, 38). This suggests that orexigenic activity induced by central δ-activation was mediated by the $PGD_2$-NPY system (Fig. 6).

In mice fed a high-fat diet, DPDPE decreased high-fat diet intake after central administration. In contrast, μ-receptor activation induced by central administration of DAMGO (45) and endomorphin-2 stimulated high-fat diet intake (Fig. 1*F*). It is noteworthy that food intake regulation elicited by activation of central δ is quite different from the μ system. Our results seemed to be consistent with previous reports that whole body deletion of δ-receptor gene increased high-fat intake (9), which was mainly explained by compensation for changes in peripheral energy expenditure. It was also reported that chronic administration of δ-antagonist suppressed intake of normal and cafeteria diet in rats with cafeteria diet-induced or genetic obesity, respectively (6, 7). The possibility that the differences in experimental conditions, including animals, their genetic backgrounds, protocols, terms, and dosages, potentially affects the results cannot be ruled out.

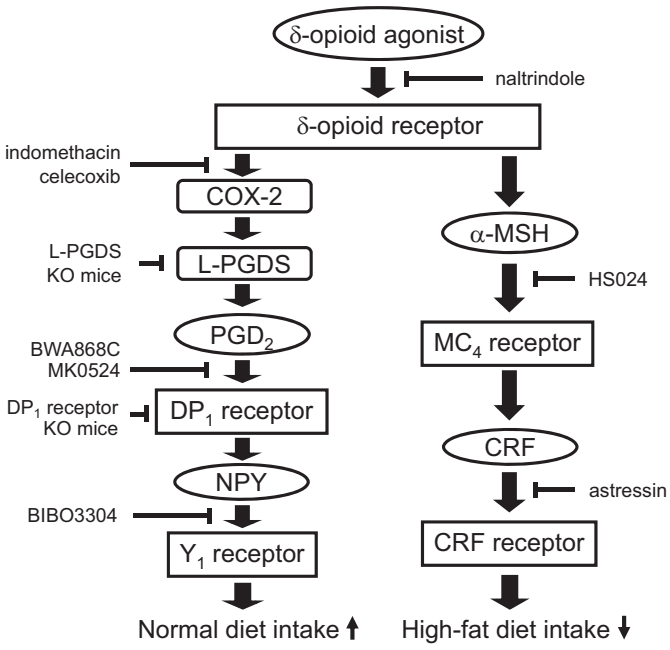


Fig. 6. Model of δ-opioid agonist-induced opposing effects on food intake in mice fed normal and high-fat diets.

The changes in the responsiveness of δ-agonist occurred after short-term feeding with high-fat diet. Indeed, 1 day after changing from a normal to high-fat diet, the orexigenic activity of DPDPE disappeared, and thereafter DPDPE began to decrease intake of the high-fat diet (Fig. 3). Seven days after the change, the mRNA expression of the δ-receptor gene (3, 13, 27, 40) or $MC_4$ receptor gene in the hypothalamus did not change in high-fat diet-fed mice (Table 2). The relative protein expression of δ-receptor also did not change (1.00 ± 0.09% vs. 1.05 ± 0.03%, normal diet vs. high-fat diet fed group, respectively) in the hypothalamus. In contrast, the hypothalamic mRNA expression of POMC gene increased in high-fat diet-fed mice (Table 2). Therefore, the regulation of food intake by the δ-agonist-activated neural pathway might be modulated within a short period in response to dietary fat contents.

We revealed that δ-opioid-induced suppression of high-fat diet intake was mediated by the central $MC_4$ receptor, which is well known to be closely associated with high-fat intake. For example, it was reported that activation of $MC_4$ receptor by α-MSH and the synthetic analog melanotan II (MTII) potently decreased high-fat diet intake (5, 8, 29), and genetic deletion of $MC_4$ receptor resulted in fat-induced hyperphagia and reduced energy expenditure (2, 15, 42). The knockdown of $MC_4$ receptor, highly expressed in the hypothalamus (2, 12, 22–24, 42, 44), increased high-fat diet intake and caused excessive body weight gain (11). Furthermore, it is reported that the $MC_4$ receptor is colocalized with anorexigenic CRF in the hypothalamus (25, 32), and central $MC_4$ activation-induced anorexigenic activities are mediated by the CRF system (21, 26). These results are consistent with our hypothesis that δ-opioid is coupled to the MC-CRF system to decrease fat intake. Further investigations will elucidate the significance of these pathways controlling food intake under physiological and pathophysiological conditions.

We also found that orally administered rubiscolin-6, a δ-opioid peptide derived from Rubisco, a major protein of green leaves, decreased high-fat diet intake via activation of central δ-receptor. Rubiscolin-6 is the first peptide derived from food protein to suppress high-fat diet intake after oral administration. The minimum effective dose was 0.39 μmol/kg comparable to that of conventional pharmaceuticals.

### *Perspectives and Significance*

In conclusion, activation of the central δ-opioid receptor stimulates normal diet intake and conversely decreases high-fat diet intake in mice. These opposing activities were mediated by a common δ-receptor. Furthermore, the δ-opioid receptor agonist activated independent neuronal pathways of orexigenic $PGD_2$-NPY and anorexigenic MC-CRF in mice fed a normal and high-fat diet, respectively, downstream of δ-receptor. We also found that a food-derived peptide suppressed high-fat intake after oral administration.

## GRANTS

This work was supported in part by a Grant-in-Aid for Scientific Research (B) and a Research Fellowship for Young Scientists (DC) from the Japan Society for the Promotion of Science to K. Ohinata and K. Kaneko, respectively.

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

## AUTHOR CONTRIBUTIONS

Author contributions: K.K., T.M., M.Y., and K.O. conception and design of research; K.K., T.M., Y.M., and M.L. performed experiments; K.K., T.M., Y.M., M.L., and K.O. analyzed data; K.K., T.M., M.L., Y.U., M.Y., and K.O. interpreted results of experiments; K.K. and K.O. prepared figures; K.K. and K.O. drafted manuscript; K.K., M.L., Y.U., M.Y., R.K., and K.O. edited and revised manuscript; K.K., T.M., M.L., and K.O. approved final version of manuscript.

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