RESEARCH ARTICLE

Orally administered rubiscolin-6, a δ opioid peptide derived from Rubisco, stimulates food intake via leptomeningeal lipocallin-type prostaglandin D synthase in mice

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Scope: We found that rubiscolin-6, a δ opioid agonist peptide derived from D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a major protein of green leaves, stimulates food intake after oral administration in mice. We therefore investigated its mechanism.

Methods and results: Orexigenic activity after oral administration of rubiscolin-6 was blocked by central administration of naltrindole, an antagonist for δ opioid receptor, suggesting that orally administered rubiscolin-6 stimulates food intake via central δ opioid receptor activation. The orexigenic activity of rubiscolin-6 was inhibited by celecoxib, a cyclooxygenase (COX)-2 inhibitor. The hypothalamic mRNA expression of COX-2 and lipocallin-type (L) prostaglandin D synthase (PGDS) was elevated in response to rubiscolin-6 administration. Rubiscolin-6 stimulated food intake in wild-type and hematopoietic (H)-PGDS knockout (KO), but not L-PGDS KO mice. Interestingly, rubiscolin-6 stimulated food intake in L-PGDS^{flox}/Nescre mice, which were deficient in L-PGDS in the brain parenchyma, but not leptomeninges. The orexigenic effect of rubiscolin-6 was abolished by genetic deletion of DP_1 receptor for PGD_2, and by MK0524 or BIBO3304, an antagonist of DP_1 receptor or of Y_1 receptor for neuropeptide Y, respectively.

Conclusion: Orally administered rubiscolin-6 may stimulate food intake through COX-2 and leptomeningeal L-PGDS, followed by DP_1 and Y_1 receptors, downstream of the central δ opioid receptor.

Keywords:
Peptide / Orexigenic effect / δ Opioid / L-PGDS / NPY

1 Introduction

It is known that with ageing, there is a decline in food intake [1]. This physiological anorexia of ageing makes the elderly vulnerable to weight loss when they become ill. Sarcopenia is the ageing-associated loss of skeletal muscle mass that leads to loss of strength and function. Cachexia is a metabolic syndrome related to illness, including cancer, and is characterized by the excess loss of muscle. Anorexia nervosa in young women is an important issue. It is known that food intake is controlled by a number of endogenous substances, including neuropeptides [2, 3]. An ageing-associated alteration in the opioid system was previously reported [4].

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Abbreviations: CMC, carboxymethyl cellulose; **CNS**, central nervous systems; **COX**, cyclooxygenase; **KO**, knockout; **NPY**, neuropeptide Y; **PG**, prostaglandin; **PGDS**, prostaglandin D synthase

Endogenous opioid peptides, such as enkephalins, β-endorphins, dynorphins, and endomorphins, and their receptors have been found in the peripheral and central nervous systems (CNS). Various opioid peptides have also been isolated from enzymatic digests of food proteins [5–12]. Among them, rubiscolin-6 (Tyr-Pro-Leu-Asp-Leu-Phe) is an 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. Rubisco is also known to be the most abundant protein on earth, since it consists of approximately 30–50% soluble protein in the green leaves of plants [13]. We have reported that rubiscolin-6 had selective affinity for the δ opioid receptor among opioid receptors μ, δ, and κ, and has memory consolidation enhancing and anxiolytic-like activities via δ opioid receptor [9,10]. In the current study, we found that orally administered rubiscolin-6 (0.3–1 mg/kg), a δ opioid agonist peptide derived from natural protein, potently stimulates food intake in nonfasted mice. Rubiscolin-6 having orexigenic activity as well as memory consolidation enhancing and anxiolytic-like activities might be developed for elderly people.

Prostaglandin (PG) D_2, the most abundant PG in the CNS, has a variety of physiological actions, such as the induction of sleep [14] and hypothermia [15]. Recently, we have found that PGD_2 stimulates food intake in mice [16]. PGD_2 is produced by lipocalin-type PGD synthase (L-PGDS) in the CNS where L-PGDS is dominantly expressed in the meninges surrounding the brain and oligodendrocytes [17]. To clarify whether rubiscolin-6-induced orexigenic activity was mediated by L-PGDS, we used L-PGDS knockout (KO) mice. Furthermore, we investigated whether L-PGDS present in the parenchyma or leptomeninges is critical for the orexigenic activity of rubiscolin-6, using conditional L-PGDS KO mice. The orexigenic activity of PGD_2 was coupled to neuropeptide Y (NPY), the most potent orexigenic peptide in the hypothalamus [16,18–21]. Thus, we also investigated whether the orexigenic activity of rubiscolin-6 is involved in the NPY system.

2 Materials and methods

2.1 Materials

Rubiscolin-6 was synthesized by the Fmoc strategy [22]. δ Opioid receptor antagonist, naltrindole, and dopamine D_2 receptor antagonist, raclopride were purchased from Sigma-Aldrich Co. (St. Louis, MO). NPY Y_1 receptor antagonist, BIBO3304 trifluoroacetate, σ_1 receptor antagonist, α-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazine butanol (BMY14802), and dopamine D_1 receptor antagonist, (R)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH23390), were obtained from Tocris Cookson Inc. (Ellisville, MO). NPY was from the Peptide Institute, Inc. (Osaka, Japan). An antagonist of DP_1 receptor for PGD_2, MK0524 was from Cayman Chemical Company (Ann Arbor, MI), cyclooxygenase (COX) inhibitor, indomethacin, was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), COX-1 inhibitor, SC-560, was obtained from Alexis Biocemicals (Plymouth Meeting, PA), COX-2 inhibitor, celecoxib, was from Toronto Research Chemicals Inc. (Ontario, Canada), and carboxymethyl cellulose (CMC) was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

2.2 Animals

Male ddY or C57BL/6 mice at 7 weeks of age were obtained from Japan SLC (Shizuoka, Japan) and used in experiments. In addition, lipocallin-type (L-) or hematopoietic (H-) prostaglandin D synthase (PGDS) KO and DP_1 receptor KO mice were used [23–26]. Each mouse was individually housed under regulated conditions (23 ± 1°C on a 12 h light–dark cycle with lights on at 7 a.m.), and free access to food pellets and water, unless otherwise indicated. After 1-week acclimation, we performed the food intake experiments.

2.3 Cannula implantation

Central administration was performed as described previously [16,27–30]. Briefly, mice were anesthetized with sodium pentobarbital (80–85 mg/kg i.p.) 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 week or more after implantation.

2.4 Food intake experiment

The food intake experiment was performed as previously described [12, 16, 27–31]. The experiment started at 11 a.m. Rubiscolin-6 at a dose of 1–30 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 control vehicle alone was i.c.v. administered in nonfasted mice. Rubiscolin-6 in saline or vehicle was orally or i.p. injected at a dose of 0.3–1 mg/kg or 0.1–0.3 mg/kg, respectively. Rubiscolin-6 (0.3 mg/kg p.o.) in saline and a δ opioid receptor antagonist naltrindole (10 nmol/mouse i.c.v.) or a NPY Y_1 receptor antagonist BIBO3304 (5 nmol/mouse i.c.v.) in 4 μL ACSF were coadministered. A combination of rubiscolin-6 (0.3 mg/kg p.o.) in saline, an antagonist of DP_1 receptor for PGD_2 MK0524 (1.6 nmol/mouse i.c.v.) or NPY (0.3 nmol/mouse i.c.v.) in 4 μL 5% DMSO ACSF was coadministered. Rubiscolin-6 (0.3 mg/kg p.o.) and σ_1 receptor antagonist BMY14802 (0.5 mg/kg i.p.), dopamine D_1 receptor antagonist SCH23390 (30 μg/kg i.p.) or dopamine D_2 receptor antagonist raclopride (15 μg/kg i.p.) in saline were coadministered. A combination of rubiscolin-6 (0.3 mg/kg p.o.) and COX inhibitor indomethacin (10 mg/kg i.p.),

COX-1 inhibitor SC-560 (3 mg/kg i.p.), or COX-2 inhibitor celecoxib (3 mg/kg i.p.) in saline containing 0.5% CMC was coadministered. The weight of the food pellets in each cage was measured at 0 and 20 min and 1 and 2 h after administration, and the cumulative food intake was calculated. All experiments were approved by Kyoto University Ethics Committee for Animal Research Use. After food intake experiments, cannula placement was confirmed by i.c.v. administration of dye. All mice were killed by an overdose of anesthesia drugs after the experiment.

2.5 RNA preparation from hypothalamus and quantitative RT-PCR

Each mouse hypothalamus was excised after decapitation under deep anesthesia, and kept in RNA later 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 SYBR Green qPCR SuperMix-UDG with ROX solution (Invitrogen) and each primer set specific for mouse β-actin, COX-1, COX-2, H-PGDS, and L-PGDS 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.

2.6 Generation of floxed-L-PGDS mice

A bacterial artificial chromosome (BAC) clone (ID: RP23–310D13) containing the entire L-PGDS gene was purchased from Children's Hospital Oakland Research Institute. Using recombination techniques [32], a loxP-FRT-PGK-Neo-FRT cassette (which was derived from the PL451 plasmid, kindly provided by Dr. N. Copeland, NCI, Frederick) and a loxP sequence were inserted into the BAC L-PGDS gene 217 bp downstream of exon 1 and 119 bp downstream of exon 6, respectively. The targeting construct containing 7.1 kb 5′ upstream and 4.1 kb 3′ downstream region was electroporated into CMTI-2 C57BL/6 embryonic stem cells. Targeted clones were identified by Southern blotting using EcoRI digestion of genomic DNA and a hybridization probe derived from a 13 kb upstream region of exon 1 and then injected into eight-cell stage embryos of ICR mice. The PGK-Neo cassette was removed after germline transmission by crossing with C57BL/6 mice expressing Flp recombinase [33].

2.7 Immunohistochemistry

Under deep anesthesia with pentobarbital, wild-type, L-PGDS^{flox}, or L-PGDS^{flox}/Nescre mice were intracardially perfused with PBS (pH 7.3) and 4% paraformaldehyde phosphate buffer solution followed by Bouin's fluid. The brains were removed and immersed in the same fixative for 5 days at 4°C, embedded into paraffin, and cut into 5-μm sections with a microtome. Paraffin sections were incubated for 1 h with 3% normal donkey serum to mask the nonspecific binding sites and then at 4°C for 2 days were incubated with rabbit anti-L-PGDS antibody (1:10 000) [16]. The sections were then reacted with biotinylated secondary antibody against rabbit IgG (1:1000, Jackson Immuno Researb, West Grove, PA) followed by the avidin–biotin–peroxidase complex kit (Vectastain kit, Vector, Burlingame, CA). These immunoreactivities were visualized by incubation with 3,3′-diaminobenzidine.

2.8 Statistical analysis

Values are expressed as the means ± SEM. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Fisher's test or the unpaired Student's *t*-test. p-values < 0.05 were considered significant.

3 Results

3.1 Orally administered rubiscolin-6 stimulates food intake via the central δ opioid receptor

Rubiscolin-6 dose-dependently stimulated food intake at a dose of 1–30 nmol/mouse 60 min after i.c.v. administration in nonfasted male mice, and this increase in food intake lasted for 120 min (Fig. 1A). When rubiscolin-6 was administered i.p., it also stimulated food intake at a dose of 0.1–0.3 mg/kg

Table 1. Oligonucleotide sequence of PCR primers specific for COX-1, COX-2, L-PGDS, H-PGDS, and β-actin.

Gene name	Forward	Reverse
COX-1	CCTTGCCCAAACCTACGTCTAC	CCAAGAACACCTTACTGCCTTGA
COX-2	CTCTTAGTTCCGTTTCTCGTGGTC	CCCAATCAGCGTTTCTCGTAGTA
H-PGDS	AAACTGGTGTCATTACGGAACAAA	GGCAGAAATGGCAGGGATAG
L-PGDS	ATTCAAGAGTAAACGCAGGTGAGA	CATGTGACCAGCCCTCTGACT
β-actin	CTGCGCAAGTTAGGTTTTGTCA	TGCTTCTAGGCGGACTGTTACTG

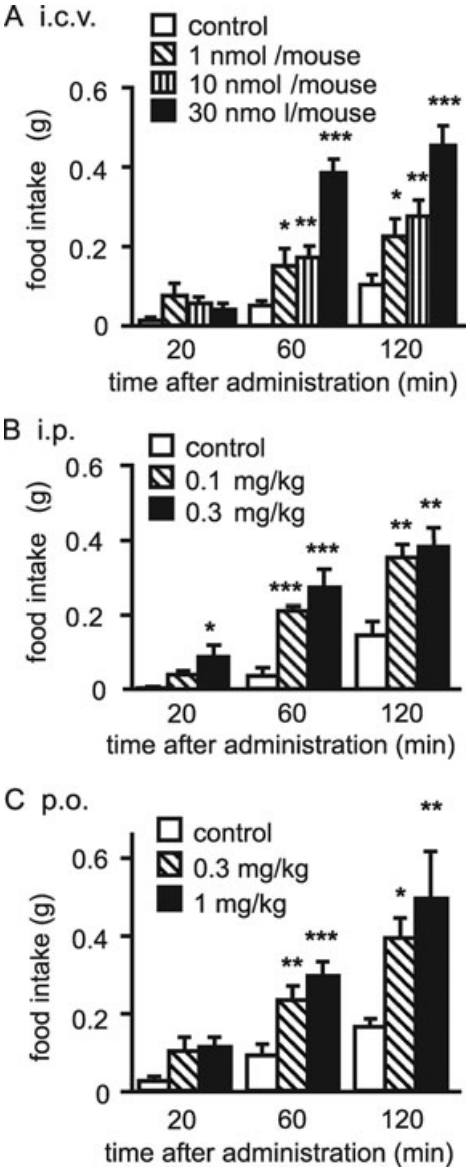

Figure 1. Effect of rubiscolin-6 on food intake after i.c.v. (A), i.p. (B), or oral (C) administration in nonfasted mice. Rubiscolin-6 was administered i.c.v. (1–30 nmol/mouse), i.p. (0.1–0.3 mg/kg), or orally (0.3–1 mg/kg), and food intake was measured. Each column represents the mean ± SEM (A, $n = 15$–16; B and C, $n = 7$). *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$ compared with control group by ANOVA followed by Fisher's test.

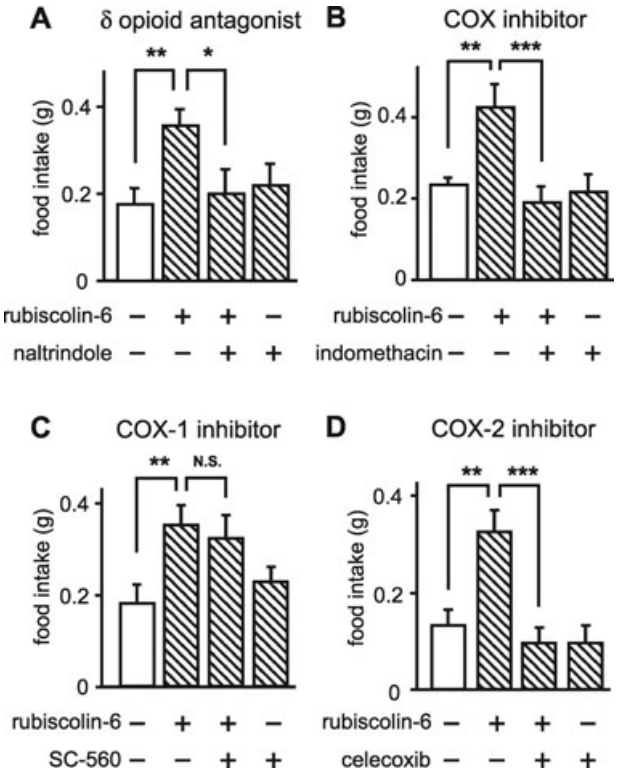

Figure 2. Effect of δ opioid antagonist or COX inhibitors on the orexigenic activity of rubiscolin-6. (A) The orexigenic activity of rubiscolin-6 (0.3 mg/kg p.o.) 120 min after administration was blocked by the δ opioid antagonist naltrindole (10 nmol/mouse i.c.v.) in nonfasted mice. (B) Orexigenic activity of rubiscolin-6 (0.3 mg/kg p.o.) 120 min after administration was blocked by nonselective COX inhibitor indomethacin (10 mg/kg i.p.). The orexigenic effect of rubiscolin-6 (0.3 mg/kg p.o.) 120 min after administration was inhibited by COX-2 inhibitor celecoxib (D, 3 mg/kg i.p.), but not by COX-1 inhibitor SC-560 (C, 3 mg/kg i.p.). Each column represents the mean ± SEM (A, $n = 9$–10; B–D, $n = 7$–9). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with each group by ANOVA followed by Fisher's test. N.S., not significant.

(Fig. 1B). Orally administered rubiscolin-6 stimulated food intake at a dose of 0.3–1 mg/kg, and the minimum effective dose for this orexigenic activity was 0.3 mg/kg (Fig. 1C). Thus, we found that rubiscolin-6 potently stimulates food intake in the CNS after oral administration.

To investigate whether orally administered rubiscolin-6, having affinity for δ opioid receptor, actually stimulates food intake as a δ opioid agonist, we used naltrindole, a selective antagonist for δ opioid receptor. The orexigenic activities of rubiscolin-6 (0.3 mg/kg p.o.) were blocked by i.c.v. administration of naltrindole at a dose of 10 nmol/mouse (Fig. 2A). Taken together, orally administered rubiscolin-6 may stimulate food intake via δ opioid receptor, probably in the CNS. Thus, we demonstrated that rubiscolin-6 acts as a δ opioid agonist peptide having orexigenic activity in addition to previously reported memory consolidation enhancing and anxiolytic-like activities [9, 10].

3.2 Orexigenic activity of rubiscolin-6 is mediated by cyclooxygenase-2 and lipocalin-type PGDS

Next, we tested which mediators are involved in the orexigenic activity of rubiscolin-6, downstream of the central δ opioid receptor. The orexigenic effect of rubiscolin-6 (0.3 mg/kg p.o.)

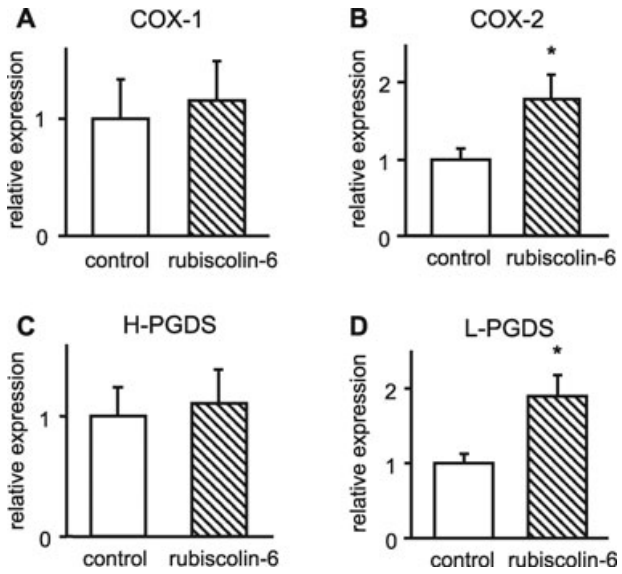

Figure 3. Effect of rubiscolin-6 on hypothalamic mRNA expression of COX-1, COX-2, H-PGDS, and L-PGDS. Hypothalamic mRNA levels of COX-1 (A), COX-2 (B), H-PGDS (C), or L-PGDS (D) 60 min after administration of rubiscolin-6 (30 nmol/mouse i.c.v.) were measured by quantitative RT-PCR. Each column represents the mean ± SEM ($n = 4$–5). $^*p < 0.05$ compared with each group by unpaired Student's *t*-test.

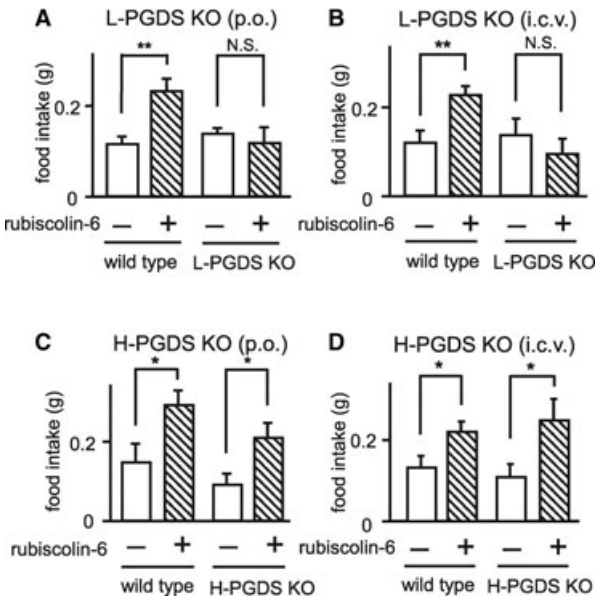

Figure 4. Effect of rubiscolin-6 administration on food intake in wild-type, L-PGDS and H-PGDS KO mice. Rubiscolin-6 was orally (A and C) or centrally (B and D) administered at a dose of 1 mg/kg or 30 nmol/mouse, respectively, in wild-type, L-PGDS KO, or H-PGDS KO mice, and food intake was measured for 120 min. Each column represents the mean ± SEM. ($n = 6$–8). $^*p < 0.05$, $^{**}p < 0.01$ compared with each group by ANOVA followed by Fisher's test. N.S., not significant.

was blocked by indomethacin, a COX inhibitor (10 mg/kg i.p., Fig. 2B).

To clarify if COX-1 or COX-2 is associated with rubiscolin-6-induced food intake, we used SC-560 or celecoxib, which are COX-1- or COX-2-selective inhibitors, respectively. The orexigenic effect of rubiscolin-6 (0.3 mg/kg p.o.) was blocked by celecoxib (3 mg/kg i.p.), but not by SC-560 (3 mg/kg i.p.; Fig. 2C and D). Furthermore, mRNA expression of COX-2 was increased 60 min after central administration of rubiscolin-6 (30 nmol/mouse i.c.v.) compared with COX-1 (Fig. 3A and B). These results suggest that the orexigenic activity of rubiscolin-6 after oral administration is mediated by COX-2.

Next, we investigated the molecular species of PGs activated after rubiscolin-6 administration. We have recently reported that PGD_2 stimulates food intake after central administration [16]. PGD_2, derived from arachidonic acid via PGH_2, is known to be produced by L-PGDS in the CNS. Indeed, hypothalamic mRNA expression of L-PGDS, but not H-PGDS, was also increased after i.c.v. administration of rubiscolin-6 (Fig. 3C and D). Thus, we investigated whether L-PGDS mediated the orexigenic activity of rubiscolin-6, using L- and H-PGDS-deficient mice. Orally (1 mg/kg) or centrally (30 nmol/mouse) administered rubiscolin-6 stimulated food intake in wild-type and H-PGDS, but not L-PGDS KO mice (Fig. 4), suggesting that, the orexigenic activity of rubiscolin-6 is mediated by L-PGDS, but not H-PGDS.

We then generated animals with a genetic construct allowing for the conditional disruption of the mouse L-PGDS gene (Fig. 5A). We inserted loxP sites into the first and sixth intron of the L-PGDS gene to create a mouse line with a L-PGDS gene that is amenable to conditional deletion by Cre recombinase (L-PGDS^{flox}). We then crossed L-PGDS^{flox} mice with mice expressing Cre recombinase under the rat nestin (Nes) promoter, which is expressed selectively in neuronal and glia-cell precursors [34]. In these mice (L-PGDS^{flox}/Nescre), exons 2–6 of the L-PGDS are deleted in the brain parenchyma only, while L-PGDS is expressed in the leptomeninges.

We investigated the localization of L-PGDS by immunohistochemistry in the CNS. The L-PGDS-like immunoreactivity was present in the ependymal cells facing the third ventricle and in the parenchyma and oligodendroglial cells of the median eminence, as well as leptomeninges under the hypothalamus, which is an important site for food intake regulation (Fig. 5B). On the other hand, in L-PGDS^{flox}/Nescre mice, the L-PGDS immunoreactivity existed in the leptomeninges, but not oligodendrocytes (Fig. 5C). Thus we demonstrated that the brain parenchymal L-PGDS was conditionally deleted in L-PGDS^{flox}/Nescre mice.

Orally (1 mg/kg) or centrally (30 nmol/mouse) administered rubiscolin-6 stimulate food intake not only in wild-type and L-PGDS^{flox} but also L-PGDS^{flox}/Nescre mice (Fig. 5D and E). These results suggest that L-PGDS in the brain parenchyma is not required for the orexigenic activity of rubiscolin-6, and L-PGDS in the leptomeninges contribute to its orexigenic activity.

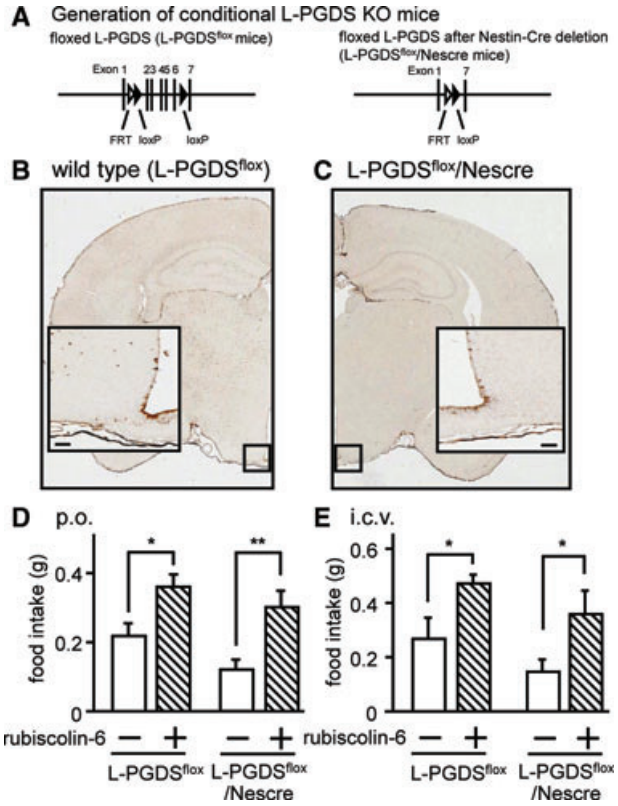

Figure 5. Effect of rubiscolin-6 administration on food intake in L-PGDS^{flox} and L-PGDS^{flox}/Nescre mice. (A) Generation of conditional L-PGDS KO mice. (B) The L-PGDS-like immunoreactivity staining detected leptomeninges and oligodendrocytes of brain sections in wild-type mice or L-PGDS^{flox}/Nescre mice. Rubiscolin-6 was orally (D) or centrally (E) administered at a dose of 1 mg/kg or 30 nmol/mouse, respectively, in L-PGDS^{flox} or L-PGDS^{flox}/Nescre mice, and food intake was measured for 120 min. Each column represents the mean ± SEM. (D, $n = 15$–16; E, $n = 10$–11).*$p < 0.05$, **$p < 0.01$ compared with each group by ANOVA followed by Fisher's test. Scale bars: 50 μm.

3.3 Orexigenic activity of rubiscolin-6 is mediated by the central PGD_2 and NPY system

We previously reported that PGD_2 stimulates food intake via DP_1 receptor among two receptor subtypes for PGD_2 [16]. We tested the involvement of the orexigenic activity of rubiscolin-6 in the central PGD_2-DP_1 system using MK0524, an antagonist for DP_1 receptor, and mice lacking the DP_1 receptor. MK0524 (1.6 nmol/mouse i.c.v.) completely inhibited the orexigenic effect of rubiscolin-6 (0.3 mg/kg p.o.) (Fig. 6A). Furthermore, orally administered rubiscolin-6 (1 mg/kg) stimulated food intake in wild-type, but not DP_1, KO mice (Fig. 6B). These results suggest that rubiscolin-6 increases food intake through activation of central DP_1 receptors.

We have also recently reported that the orexigenic effect of PGD_2 was coupled with hypothalamic NPY, a potent orexigenic peptide that stimulates food intake predominantly via

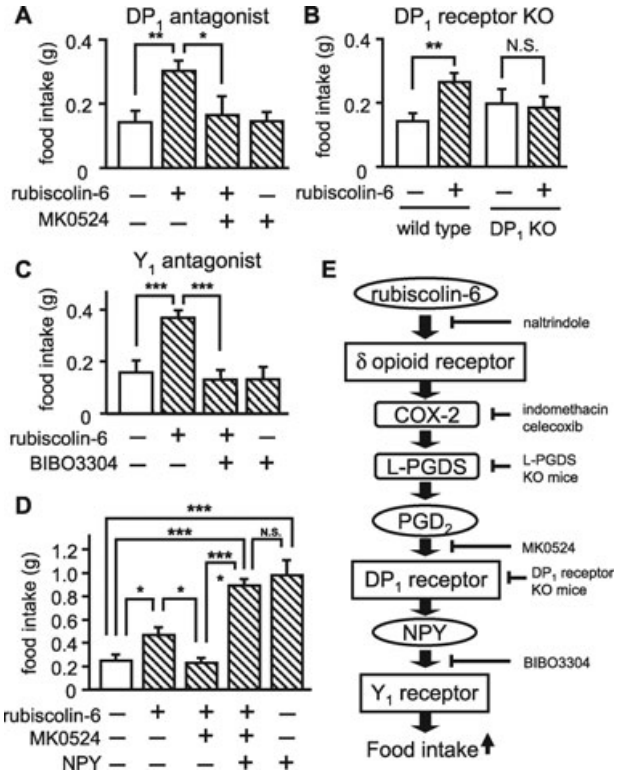

Figure 6. Involvement of central PGD_2-DP_1 and NPY-Y_1 system in the orexigenic activity of rubiscolin-6. (A) Orexigenic activity of rubiscolin-6 (0.3 mg/kg p.o.) 120 min after administration was blocked by DP_1 receptor antagonist MK0524 (1.6 nmol/mouse i.c.v.) in nonfasted mice. (B) Rubiscolin-6 was orally (1 mg/kg) administered to wild-type or DP_1 receptor KO mice, and food intake was measured for 120 min. (C) The orexigenic effect of rubiscolin-6 (0.3 mg/kg p.o.) 120 min after administration was inhibited by NPY Y_1 receptor antagonist BIBO3304 (5 nmol/mouse i.c.v.). (D) The orexigenic effect of rubiscolin-6 was antagonized by MK0524, but the orexigenic effect of NPY (0.3 nmol/mouse i.c.v.) was not inhibited by MK0524. (E) Model of orexigenic activity of rubiscolin-6 after oral administration. Each column represents the mean ± SEM. (A, $n = 10$–11; B, $n = 9$–15; C and D, $n = 8$–9). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with each group by ANOVA followed by Fisher's test. N.S., not significant.

Y_1 receptor among five receptor subtypes for NPY [16]. To investigate whether the orexigenic activity of rubiscolin-6 is also coupled with the NPY–Y_1 system, we used BIBO3304, an antagonist for Y_1 receptor. The orexigenic effect of orally administered rubiscolin-6 (0.3 mg/kg) was blocked by central administration of BIBO3304 (5 nmol/mouse; Fig. 6C). Centrally administered NPY (0.3 nmol/mouse) stimulates food intake in nonfasted mice. Rubiscolin-6-induced food intake stimulation was inhibited by MK0524; however, coadministered rubiscolin-6 and NPY increased food intake, which was not blocked by MK0524 (Fig. 6D). These results suggest that NPY stimulates food intake downstream of δ opioid receptor, followed by DP_1 receptor.

Taken together, orally administered rubiscolin-6 may stimulate food intake via a central orexigenic pathway through the action on δ opioid receptor followed by the L-PGDS–PGD_2–DP_1 and NPY–Y_1 receptor system (Fig. 6E).

4 Discussion

We found for the first time that orally administered rubiscolin-6 stimulates food intake in nonfasted mice. It is known that a variety of endogenous neuropeptides, such as NPY, ghrelin, and orexin, stimulated food intake after central administration [35]; however, they were inactive after oral administration. Rubiscolin-6 was orally active. The minimum effective dose for the orexigenic effect of rubiscolin-6 (YPLDLF, 0.3 mg/kg p.o.) was approximately one-third of rubiscolin-5 (YPLDL, 1 mg/kg p.o., data not shown), which is a peptide truncated with the residue of rubiscolin-6 at the C-terminus, having lower affinity for the δ opioid receptor than rubiscolin-6 [8, 36]. The orexigenic activity of rubiscolin-6 after oral administration was inhibited by central administration of an antagonist for the δ opioid receptor, despite the fact that the antagonist did not affect food intake in the absence of rubiscolin-6 (Fig. 2A) and rubiscolin-6 did not change hypothalamic δ opioid receptor expression (Supporting Information Tables S1 and S2). Our findings indicate the involvement of the central δ opioid receptor, which has previously been reported to be widely expressed in the CNS [37–39]. Rubiscolin-6 may be absorbed from the gastrointestinal tract and cross the blood-brain barrier to activate the central δ opioid receptors, although the possibility that rubiscolin-6 might activate the endogenous δ opioid system in the CNS through signal transduction from the peripheral nervous system, such as the vagal nerves, could not be ruled out.

Among COX products, PGD_2 has orexigenic activity after central administration [16]. PGD_2, via PGH_2 from arachidonic acid, is known to be produced by L-PGDS in the CNS. The orexigenic effect of rubiscolin-6 was blocked by an inhibitor of COX-2, but not COX-1. It was reported that δ opioid receptor is coupled with COX-2 in delayed cardioprotection [40, 41]. We also found that δ opioid agonist peptide activates the central COX-2 system. Hypothalamic mRNA expression of L-PGDS was increased by centrally administered rubiscolin-6. L-PGDS is reported to be colocalized with COX-2 in not only the leptomeninges and choroid plexus [17, 24, 42, 43] but also the hypothalamus [16]. In the current study, we confirmed that L-PGDS was present in the brain parenchyma as well as leptomeninges. Genetic deletion of L-PGDS in both the brain parenchyma and leptomeninges abolished the orexigenic activity induced by rubiscolin-6. Thus we clearly demonstrated that rubisobolin-6 stimulates food intake via L-PGDS, downstream of the δ opioid receptor. Furthermore, rubiscolin-6 stimulated food intake in mice lacking L-PGDS in the parenchyma, but not in the membrane system surrounding the brain. Taken together, leptomeningeal L-PGDS is required for the orexigenic activity of rubiscolin-6. This is the first example of an orexigenic pathway via the leptomeningeal L-PGDS.

PGD_2 and NPY are also observed in the medial hypothalamus [44–47]. Taken together, the orexigenic activity of rubiscolin-6 is considered to be mediated by PGD_2 derived from the hypothalamus or choroid plexus, which activates the DP_1 receptor coupled to the NPY–Y_1 receptor system in the hypothalamus [16]. Recently, we found that complement C5a stimulates food intake via the PGD_2–NPY system after i.c.v. administration [48]. The fact that C5a also activates the COX-2 system is consistent with our current study. It is necessary to clarify whether the central PGD_2–NPY system in food intake regulation is coupled to other orexigenic system.

PGD_2 is a potent endogenous sleep-inducing substance, whereby PGD_2 produced by L-PGDS stimulates DP_1 receptors on the ventral surface of the basal forebrain and the hypothalamus to inhibit wakefulness [14, 17, 42]. The effect of rubiscolin-6 on sleep–wake regulation mediated by L-PGDS and DP_1 receptor however remains to be clarified. Interestingly, the wakefulness-inducing effect of caffeine depends on the expression of adenosine A_{2A} receptors on striatopallidal neurons in the shell of the nucleus accumbens of the striatum [49], where δ opioid receptors and enkephalin, the endogenous ligand of the δ opioid receptor, are also abundantly expressed. Therefore, a possibility exists that rubiscolin-6 regulates sleep and wakefulness through a PGD_2-independent pathway in the striatum.

Another COX product, PGE_2, which is a positional isomer of PGD_2 produced from the same precursor PGH_2, suppressed food intake via the EP_4 receptor among four receptor subtypes for PGE_2 [28]. The hypothalamic mRNA expression of EP_4 receptor and microsomal PGE synthase, associated with production of anorectic PGE_2 in the CNS, was not affected by central administration of rubiscolin-6 (Supporting Information Tables S1 and S2); however, we also found that the anorexigenic activities of complement C3a, angiotensin II, WPLPR, and novokinin are mediated by activation of the PGE_2–EP_4 system [16, 27, 29, 31, 50]. The physiological significance of the differential effects of PGs and peptides in food intake regulation remain to be further investigated.

To the best of our knowledge, this is the first orally active peptide to stimulate intake of a normal diet. In contrast to rubiscolin-6, it was reported that β-casomorphin, a μ opioid agonist peptide derived from β-casein from bovine milk, increased the intake of a high-fat diet, but not a normal diet in rats [51, 52].

We reported that the memory consolidation enhancing and anxiolytic-like effects of rubiscolin-6 were mediated by σ_1 receptor and dopamine systems, downstream of the δ opioid receptor; however, the orexigenic activity of rubiscolin-6 was not blocked by BMY14802, SCH23390, or raclopride, an antagonist of the σ_1 receptor, dopamine D_1 or D_2 receptor, respectively (data not shown). These results suggest that orexigenic activity was independent of σ_1 and D_1 receptors, which are involved in memory consolidation enhancing and anxiolytic-like activities [9, 10].

It is known that a number of dysfunctions observed in elderly people are facilitated in response to a decrease in appetite and food intake. Since rubiscolin-6 has orexigenic, memory consolidation enhancing, and anxiolytic-like activities, rubiscolin-6 itself and/or functional foods containing rubiscolin-6 might be developed for elderly people. Furthermore, it would be interesting to investigate whether rubiscolin-6 derived from Rubisco, the major leaf protein ubiquitous to all plant species, contributes to food intake regulation in herbivorous animals and humans.

In conclusion, we found that rubiscolin-6 stimulates food intake after oral administration. This orexigenic activity was mediated by COX-2 and L-PGDS in the leptomeninges, downstream of the central δ opioid receptor. The orexigenic activity of rubiscolin-6 was also involved in the hypothalamic orexigenic system via the central PGD_2–DP_1 receptor coupled to the NPY–Y_1 receptor.

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