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Dopaminergic mechanisms in the lateral hypothalamus regulate feeding behavior in association with neuropeptides

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ABSTRACT

This study investigated dopaminergic function in the lateral hypothalamus (LH) in the regulation of feeding behavior. Refeeding increased dopamine levels in the LH. Glucose injection also increased dopamine levels in the LH. When the retrograde tracer Fluoro-Gold (FG) was injected into the LH, FG-positive cells were found in the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNC), which were mostly tyrosine hydroxylase-positive. Injection of the dopamine D_1 receptor agonist SKF 38393, but not the antagonist SCH 23390, into the LH increased food intake. Similarly, injection of the dopamine D_2 receptor agonist quinpirole, but not the antagonist l-sulpiride, into the LH increased food intake. The effect of each agonist was blocked by its respective antagonist. Furthermore, injection of quinpirole, but not SKF 38393, decreased the mRNA level of preproorexin. In addition, injection of SKF 38393 decreased the mRNA levels of neuropeptide Y and agouti-related peptide, whereas the injection of quinpirole increased the mRNA level of proopiomelanocortin. These results indicate that food intake activates dopamine neurons projecting from the VTA/SNC to the LH through an increase in blood glucose levels, which terminates food intake by stimulation of dopamine D_1 and D_2 receptors. It is also possible that stimulation of dopamine D_1 and D_2 receptors in the LH inhibits feeding behavior through different neuropeptides.

1. Introduction

Dopaminergic function in the central nervous system (CNS) is thought to regulate feeding behavior, especially in relation to its rewarding value. Mesolimbic dopamine neurons that project from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) are a key player in the regulation of food reward. For instance, it is reported that dopamine levels in the NAcc are increased when mice consume a preferred diet [1,2], and that the stimulation of dopamine receptors in the NAcc drives intake of palatable food. Moreover, the increase of dopamine levels in the NAcc induced by a preferred diet accelerates its intake through dopamine D_1 and D_2 receptors [3,4]. Since increase in intake of palatable food is thought to reflect its rewarding value, mesolimbic dopamine neurons positively regulate food reward and the stimulation of these neurons increases intake of palatable food.

The hypothalamus is a key player in the regulation of energy homeostasis, including feeding behavior. Recent evidence has shown that hypothalamic neuropeptides have essential roles in the control of food intake. Neuropeptide Y (NPY) and agouti-related peptide (AgRP) work orexigenically [5,6], whereas alpha melanocyte-stimulating hormone (α-MSH), which is synthesized by cleavage of proopiomelanocortin (POMC), is an anorexigenic peptide [7]. NPY/AgRP neurons and POMC neurons are located in the arcuate nucleus of the hypothalamus (ARC) and mainly project to the lateral hypothalamus (LH) and the paraventricular nucleus of hypothalamus (PVN) [8]. In the LH, there are neurons containing other important neuropeptides, orexin and melanocortin

Abbreviations: AgRP, agouti-related peptide; ARC, arcuate nucleus of hypothalamus; CNS, central nervous system; LH, lateral hypothalamus; MCH, melanin-concentrating hormone; NAcc, nucleus accumbens; NPY, neuropeptide Y; POMC, proopiomelanocortin; PPORX, preproorexin; PVN, paraventricular nucleus of hypothalamus; SNC, substantia nigra pars compacta; VTA, ventral tegmental area; α-MSH, α-melanocyte-stimulating hormone.

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concentrating hormone (MCH), which stimulates feeding behavior [9,10]. Thus, it is suggested that the LH is one of the key stations in regulation of feeding behavior.

Several studies show that dopamine receptors are present in the hypothalamus, including the LH [11,12]. A previous study has indicated that dopamine levels in the LH are increased by food intake [13]. In addition, blockade of dopamine D_2 receptors in the LH increases food intake [14]. We have recently reported that blockade of both dopamine D_1 and D_2 receptors in the LH increases food intake in mice [15]. Thus, it is likely that dopamine D_1 and/or D_2 receptors in the LH might play an inhibitory role in the regulation of feeding behavior. Moreover, it is possible that dopaminergic function in the LH regulates feeding behavior through neuropeptides.

Therefore, the present study investigated the role of dopaminergic function in the LH in the regulation of feeding behavior. In addition, we examined whether stimulation of dopamine D_1 and D_2 receptors affects neuropeptides in the hypothalamus.

2. Materials and methods

2.1. Animals

Experiments were carried out in male ICR mice (6–7 weeks old) and male Wistar rats (6–7 weeks old) obtained from Tokyo Laboratory Animals Science (Tokyo, Japan). Animals were kept under a 12 h light/dark cycle (lights on at 08:00) in temperature-controlled facilities (24 ± 1 °C). Normal chow diet (MF; Oriental Yeast, Tokyo, Japan) and water were available *ad libitum*.

Experiments were conducted in accordance with the guidelines for the care and use of laboratory animals of Hoshi University, in compliance with the Ministry of Education, Culture, Sports, Science and Technology of Japan. The protocol was approved by the Committee on Animal Research of Hoshi University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Each animal was used only once.

2.2. Drugs

The dopamine D_1 receptor agonist SKF 38393 hydrochloride (Sigma-Aldrich, St Louis, MO, USA), the dopamine D_1 receptor antagonist SCH 23390 hydrochloride (Sigma-Aldrich), the dopamine D_2 receptor agonist quinpirole hydrochloride (Sigma-Aldrich), the dopamine D_2 receptor antagonist l-sulpiride (Sigma-Aldrich) and glucose (Wako Pure Chemical Industries, Osaka, Japan) were used. l-Sulpiride was dissolved in a minimum quantity of 1 N HCl, neutralized by 1 N NaOH to reach pH 6 and diluted with saline (0.9 w/v % NaCl solution). Other drugs were dissolved in saline. The doses of these drugs were as described in previous reports [15–19] and were optimized not to affect locomotor activity (Table S2).

2.3. Surgery

Surgery was conducted as described previously [15,20,21]. Under anesthesia with sodium pentobarbital (60 mg/kg, i.p.), guide cannulae (for microinjection: EKC-0504A, Bio Research Center, Aichi, Japan; for microdialysis: AG-6, Eicom, Kyoto, Japan) were implanted into the LH (A 2.58 mm, V 0.80 mm, L 1.10 mm, from the interaural line) according to a mouse brain atlas [22]. To minimize damage at the target site, the tips of the guide cannulae were placed 1.0–1.5 mm above the desired region. Animals were then allowed to recover for a minimum of 3 days.

2.4. Intracerebral microinjection

Mice were held gently by hand, and the injection needle (0.22 mm) connected to a Hamilton syringe was inserted through the guide cannula into the LH. Drugs were then injected in a volume of 0.2 μl over 20 s and the needle left in position for an additional 20 s to avoid reflux of the solution.

2.5. Refeeding test

Measurement in the refeeding test was as described previously [15,20,21]. Mice were deprived of food for 16 h with free access to water, following which the mice eat considerable amounts of food. After drug injection, food intake was measured hourly for 4 h. Locomotor activity during the experiment was measured by a sensor (NS-AS01; Neuroscience, Tokyo, Japan) placed at the center of polycarbonate lid and processed using commercial software (Act-1 Light® activity; Neuroscience).

2.6. In vivo microdialysis

Measurements using *in vivo* microdialysis were as described previously [15,18,20]. An I-shaped removable-type dialysis probe (A-I-6-01, Eicom, Kyoto, Japan) was inserted though the guide cannula. Mice were fasted for 16 h. Ringer's solution was perfused through the probe and the dialysates were collected every 20 min. Dopamine was separated by an Eicompak CA-5ODS column (Eicom). The quantity of dopamine in dialysates was measured by electrochemical detection using a glassy carbon working electrode set at +450 mV against a silver-silver chloride reference electrode (WE-3G; Eicom). Chromatograms were controlled by an integrator (Power Chrom; AD Instruments, NSW, Australia). The mean of the last three samples before feeding or glucose injection was taken to be the baseline level and indicated in the relevant figure. Previous reports using the same techniques have shown that dopamine levels are stable 16 h after probe insertion and that dopamine levels seen at that time are largely dependent on neural activity, since more than 70% of dopamine is tetrodotoxin-sensitive [23].

2.7. Histology

After experiments, brains were fixed with 10% formalin and sectioned at 50 μm thickness. Brain sections were stained with thionin to confirm the injection sites. Only data from mice with correctly placed injections (89/160) and probes (30/48) were included in the analysis.

2.8. Immunohistochemistry

Rats were injected with the retrograde tracer Fluoro-gold (FG; 2 μg/0.2 μl) through a guide cannula (EKC-0506A; Bio Research Center) that was implanted into the LH (A 6.44 mm, V 1.50 mm, L 1.80 mm from the interaural line) according to a rat brain atlas [24] and then left for 1 week.

Animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde (pH 7.4; Nacalai Tesque). Brains were removed and fixed with 4% paraformaldehyde. The brains were sectioned (8 μm) coronally using a cryostat (Leica CM1860; Leica Biosystems, Nussloch, Germany). The sections were placed on coated glass slides (Platinum Pro; Matsunami Glass, Osaka, Japan), incubated with 0.3% TritonX-100 (Sigma-Aldrich) in 10 mM phosphate-buffered saline for 1 h at room temperature and then incubated with 10% normal horse serum (NHS; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Sections were incubated

overnight at 4 °C with primary antibody against tyrosine hydroxylase (TH; 1:1000; Millipore) in 10% NHS. Sections were incubated with secondary antibody, Alexa®-488-conjugated anti-rabbit IgG (1:1000; Invitrogen) at room temperature for 1.5 h. Glass slides were sealed with a cover slip and visualized using a light microscope.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was conducted as described previously [20,21]. The hypothalamus was dissected 1 h after drug injections. Total RNA was isolated from the hypothalamus using a Nucleospin® RNA kit (Macherey-Nagel, Düren, Germany). Reverse transcription was carried out using a PrimeScript® RT Master Mix kit (Takara Bio, Shiga, Japan). PCR was performed using Takara Taq™ Hot Start Version (Takara Bio) on a thermal cycler (TP650; Takara Bio). Primers are listed in Table S1. PCR products were analyzed by electrophoresis (Mupid®-ex; Advance, Tokyo, Japan) on 1.7% agarose (Takara Bio). The agarose gels were stained with ethidium bromide (Sigma-Aldrich) and photographed with UV transillumination. The intensity of the band was quantified by computer-assisted densitometry using ImageJ image analysis software (National Institutes of Health, USA). Values of each band were normalized by the respective value for β-actin and % of control and standard error were calculated for each sample.

2.10. Statistical analysis

All data are expressed as means ± S.E.M. Two-way analysis of variance (ANOVA) for repeated measures followed by *post hoc* Bonferroni-corrected tests were used to compare groups. Mann-Whitney *U* test was used to compare two groups, as appropriate. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Effects of food intake and glucose injection on dopamine levels in the lateral hypothalamus

Fig. 1A shows the location of the dialysis probes in the LH. Food intake (0.60 ± 0.11 g/120 min; n = 6) significantly increased dopamine levels in the LH in fasted mice (fasted group, n = 6; treatment: $F_{(1,50)} = 13.07$, $p < 0.01$; Fig. 1B). Glucose (2 g/kg, i.p.; n = 9) also significantly increased dopamine levels in the LH in fasted mice (vehicle, n = 9; treatment: $F_{(1,80)} = 7.57$, $p < 0.05$; Fig. 1C).

3.2. Projections of dopamine neurons from the ventral tegmental area and substantia nigra pars compacta to the lateral hypothalamus

One week after injection of FG into the LH, the distribution of FG-positive cells was examined. The injection sites and locations of the VTA and SNC are shown in Fig. 2A-C. FG-positive cells were found in the VTA and SNC (Fig. 2D and G). TH-positive cells were also located in the VTA and SNC (Fig. 2E and H), and many FG-positive cells were TH-positive (Fig. 2F and I).

3.3. Effects on food intake of dopamine D_1 and D_2 receptor agonist and antagonist injections into the lateral hypothalamus

Fig. 3A shows the injection sites in the LH. Bilateral injections of SKF 38393 (2 μg/side) into the LH significantly reduced food intake. Decrease in food intake induced by SKF 38393 was inhibited by co-administration of SCH 23390 (200 ng/side), which alone did not significantly change food intake (vehicle, n = 9; SKF 38393, n = 11; SCH 23390, n = 6; SCH 23390 + SKF 38393, n = 6; treatment: $F_{(3,84)} = 6.78$, $p < 0.01$; Fig. 3B). In contrast, SKF 38393 (2 μg/side, n = 8) injected outside the LH did not affect food intake (vehicle, n = 5; Fig. S1). The drug injections did not significantly affect locomotor activity (Table S2).

Quinpirole (2 μg/side) injected bilaterally into the LH significantly decreased food intake. In contrast, injection of l-sulpiride (100 ng/side) into the LH did not significantly affect food intake. Co-administration of l-sulpiride significantly blocked the inhibitory effect of quinpirole injected into the LH (vehicle, n = 10; quinpirole, n = 9; l-sulpiride, n = 9; l-sulpiride + quinpirole, n = 6; treatment: $F_{(3,90)} = 5.11$, $p < 0.01$; Fig. 3C). Iinjection of quinpirole (2 μg/side, n = 5) outside the LH did not significantly change food intake (vehicle, n = 5; Fig. S2). These drug injections did not significantly affect locomotor activity (Table S2).

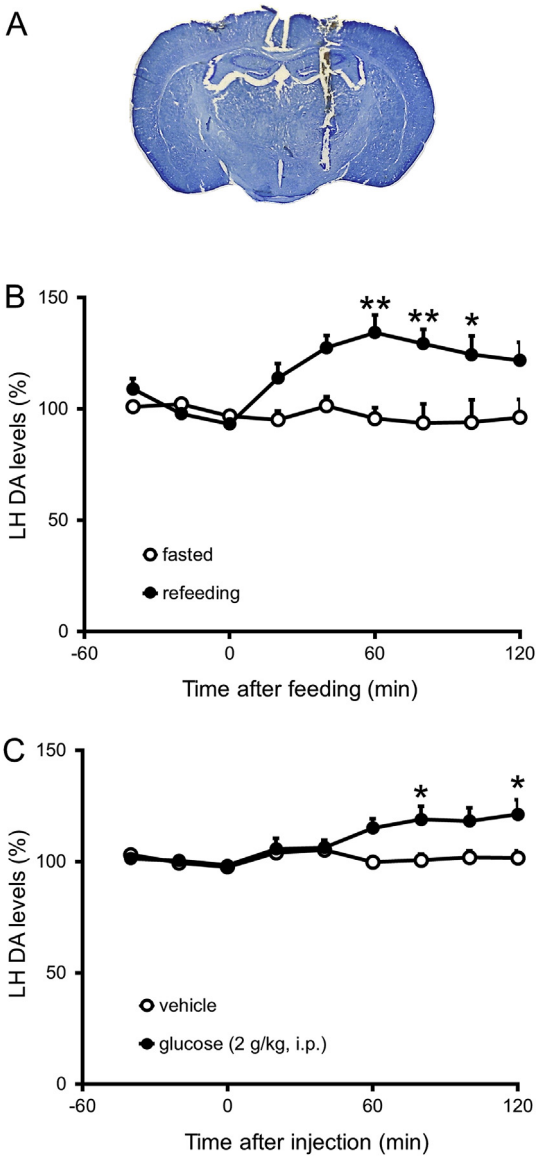

Fig. 1. (A) Representative brain section showing the location of the dialysis probe in the lateral hypothalamus (LH). (B) Effect of food intake on dopamine (DA) levels in the LH. The average food intake was 0.60 ± 0.11 g/120 min. Each point represents mean ± S.E.M. of 6 mice. $^{*}p < 0.05$, $^{**}p < 0.01$ vs fasted group. (C) Effect of glucose (2 g/kg, i.p.) on DA levels in the LH. Each point represents the mean ± S.E.M. of 9 mice. $^{*}p < 0.05$ vs vehicle group.

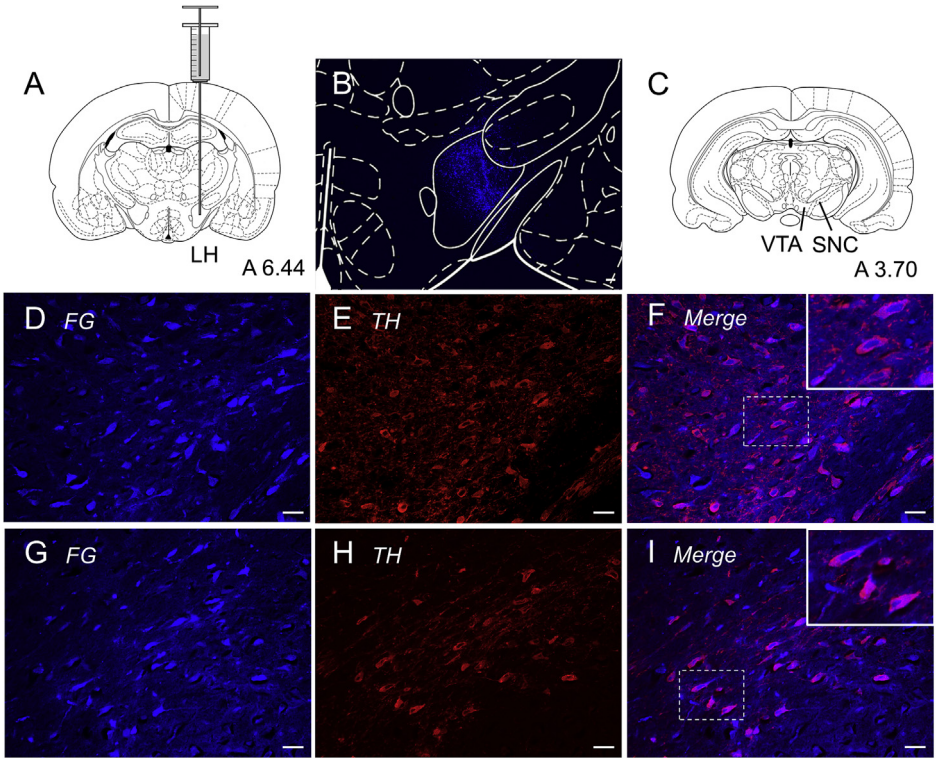

Fig. 2. (A) Schematic illustration showing the injection site of Fluoro-Gold (FG) in the lateral hypothalaus (LH). (B) Localization of the injection site of FG in LH. (C) Schematic illustration showing the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNC). (D–I) Localization of FG-positive cells (D, G), tyrosine hydroxylase (TH)-positive cells (E, H) and merged cells (F, I) in the VTA (D–F) and the SNC (G–I). Scale bar indicates 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Effects of dopamine D_1 and D_2 receptor agonists on mRNA levels of neuropeptides in the hypothalamus

Injection of SKF 38393 (3 mg/kg, i.p., n = 6–9) did not change the mRNA levels of preproorexin (PPORX) and pro-MCH in the hypothalamus (vehicle, n = 6–9; Fig. 4A and B). In contrast, injection of quinpirole (500 μg/kg, i.p., n = 6) significantly decreased the mRNA level of PPORX, but not of pro-MCH, in the hypothalamus (vehicle, n = 6; Mann-Whitney *U* test; Fig. 4C and D).

Injection of SKF 38393 (3 mg/kg, i.p., n = 9) significantly decreased the mRNA levels of NPY and AgRP, but not of POMC (vehicle, n = 9; Mann-Whitney *U* test; Figs. S3A–S3C). Injection of quinpirole (500 μg/kg, i.p., n = 9–12) significantly increased the mRNA levels of POMC, but not of NPY and AgRP (vehicle, n = 9–12; Mann-Whitney *U* test; Figs. S3D–S3F).

4. Discussion

The aim of the present study was to clarify the role of dopaminergic function in the LH in regulating feeding behavior.

The results showed that refeeding increased dopamine levels in the LH. These data are in line with a previous report showing that food intake increased dopamine levels in the LH in association with meal size [13]. In addition, glucose injection also increased dopamine levels in the LH. It has been reported that neural activities in the CNS are regulated by glucose, with “glucose-responsive” neurons activated by glucose and “glucose-sensitive” neurons inhibited by glucose [25]. Moreover, these neurons are densely located in the hypothalamus [26,27]. Thus, it is likely that dopamine neurons investigated in the present study are glucose-responsive neurons. Moreover, since both food intake and glucose injection increase dopamine levels in the LH, it is suggested that food intake stimulates dopamine release in the LH by increasing blood glucose levels.

To determine the projection of dopamine neurons to the LH, we injected FG into the LH and examined the brain areas that contain FG-positive cells. The results showed that FG-positive cells were in the VTA and SNC, and that most FG-positive cells were TH-positive. Since TH is a precursor of dopamine and a marker of dopamine neurons, it can be concluded that dopamine neurons project from the VTA and SNC to the LH. It is widely known that the cell bodies of dopamine neurons are in the VTA and SNC [28], and it has been reported that glucose applied into the SNC increases dopamine efflux in the striatum, which is the projection area of dopamine neurons in the SNC [29]. Taken together, it is likely that glucose activates dopamine neurons in the VTA and SNC and increases dopamine release in the LH. Further studies are needed to elaborate this possibility.

We next examined the role of dopamine D_1 and D_2 receptors in the LH in the regulation of feeding behavior. Injection of either SKF 38393 or quinpirole into the LH decreased food intake and these effects were abolished by co-injections of SCH 23390 and l-sulpiride, respectively. In addition, SKF 38393 and quinpirole injected outside the LH had no effect on food intake. These results indicate that stimulation of dopamine D_1 and D_2 receptors in the LH suppresses feeding behavior during refeeding. We have recently reported that blockade of both dopamine D_1 and D_2 receptors in the LH increased food intake in non-fasted mice [15]. These findings suggest that the activity of dopamine neurons projecting to the LH was inhibited during hunger, whereas their activity was stimulated during satiation.

We chose the doses of drugs so as not to affect locomotor activity and confirmed this by direct assessment of activity. Thus, our results indicate that inhibition of food intake by SKF 38393 and

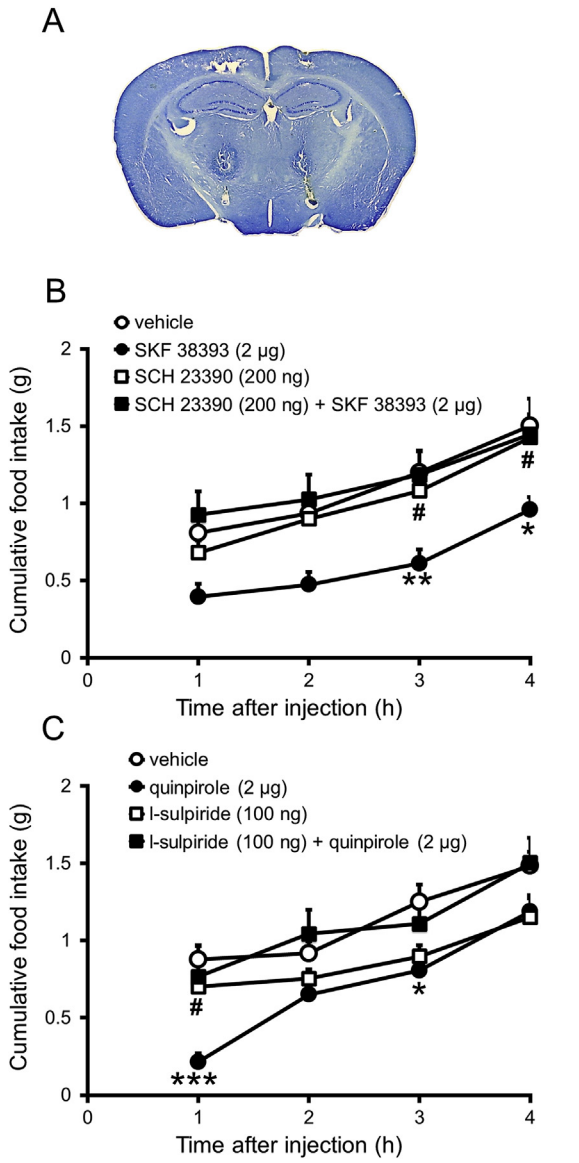

Fig. 3. (A) Representative brain section showing the injection sites in the lateral hypothalamus (LH). (B) Effect of SKF 38393 (2 μg/side) and SCH 23390 (200 ng/side) injected bilaterally into the LH on food intake of mice. (C) Effect of quinpirole (2 μg/side) and l-sulpiride (100 ng/side) injected bilaterally into the LH on food intake of mice. Each point represents the mean ± S.E.M. of 6–11 mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs vehicle group; $^{\#}p < 0.05$ vs SKF 38393/quinpirole group.

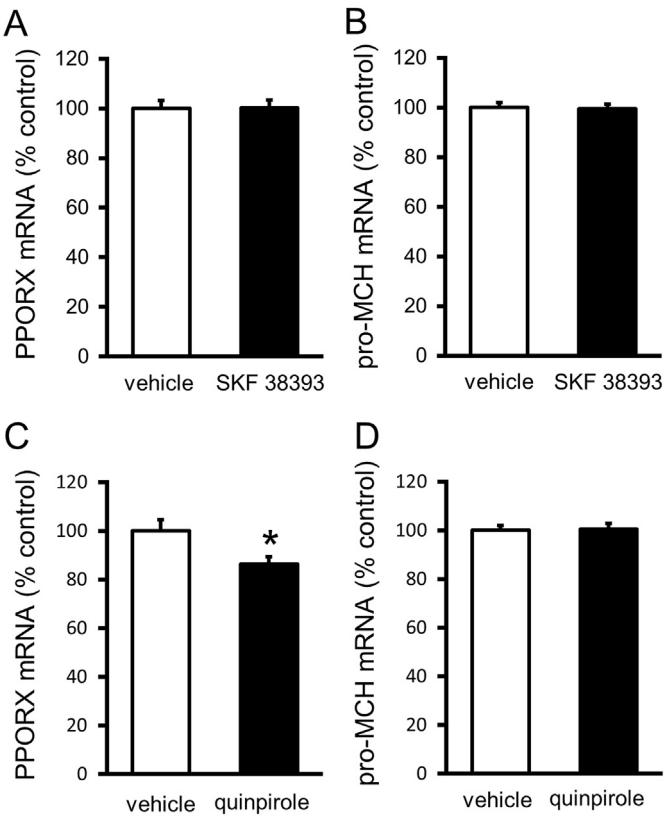

Fig. 4. Effect of SKF 38393 (3 mg/kg, i.p., A, B) and quinpirole (500 μg/kg, i.p., C, D) on mRNA levels of preproorexin (PPORX, A, C) and pro-melanin-concentrating hormone (pro-MCH, B, D) in the hypothalamus. Each bar represents the mean ± S.E.M. of 6–9 mice. $^{*}p < 0.05$ vs vehicle group.

quinpirole is not due to any change of motor function that might disturb feeding behavior.

Orexin and MCH neurons are reported to be located in the LH [9,10]. Thus, we examined whether stimulation of dopamine D_1 and D_2 receptors affects these neuropeptides. Injection of SKF 38393 did not change the mRNA levels of PPORX and pro-MCH, whereas injection of quinpirole decreased the mRNA level of PPORX, but not pro-MCH. Since it has been reported that mRNA expression of neuropeptides correlates with the activity of peptidergic neurons [30,31], stimulation of dopamine D_2 receptors in the LH may inhibit feeding behavior by inhibition of orexin neurons.

Since NPY/AgRP and POMC neurons are known to project from the ARC to the LH [8], we additionally examined whether stimulation of dopamine D_1 and D_2 receptors affects these neuropeptides. Injection of SKF 38393 decreased the mRNA levels of NPY and AgRP, suggesting that stimulation of dopamine D_1 receptors inhibits feeding behavior through inhibition of NPY/AgRP neurons. In contrast, injection of quinpirole increased the mRNA level of POMC, suggesting that stimulation of dopamine D_2 receptors may inhibit feeding behavior by stimulation of POMC neurons. It is unclear how stimulations of dopamine D_1 and D_2 receptors affect NPY/AgRP neurons and POMC neurons, respectively. One possible mechanism is that dopamine D_1 and D_2 receptors affect these neurons through GABA neurons. It has been reported that there are GABA interneurons in the LH [32]. Moreover, GABA neurons in the LH are reported to project to other brain areas that regulate feeding behavior [33,34]. Thus, it is possible that dopamine D_1 receptors inhibit NPY and AgRP neurons by stimulating GABA neurons, and that dopamine D_2 receptors stimulate POMC neurons through inhibition of GABA neurons. Precise mechanisms by which dopamine D_1 and D_2 receptors affect neuropeptides should be investigated.

The present study indicates that food intake stimulates dopamine neurons projecting from the VTA/SNC to the LH, which terminates food intake by stimulation of dopamine D_1 and D_2 receptors in the LH. Moreover, it is suggested that stimulation of dopamine D_2, but not D_1, receptors inhibits food intake through orexin neurons in the LH. In addition, it is possible that dopamine D_1 receptors regulate food intake by inhibition of NPY/AgRP neurons, whereas dopamine D_2 receptors inhibit food intake by stimulation of POMC neurons.

Author contributions

H.I. designed the experiments. N.Y., C.A., L.Y., S.Y. and D.U. performed the experiments. H.I., N.Y., C.A., L.Y., S.Y. and D.U. analyzed

data. H.I. and N.Y. wrote the manuscript. J.L.W. advised on the studies. C.A., L.Y., S.Y., D.U., J.K. and J.L.W. critically read and approved the manuscript.

Conflicts of interest

The authors declare no financial conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.09.037.

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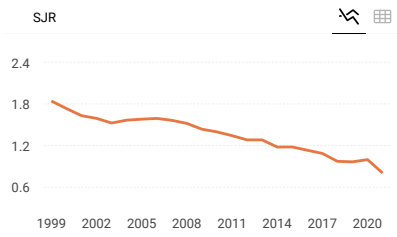

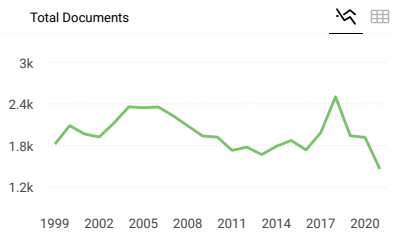

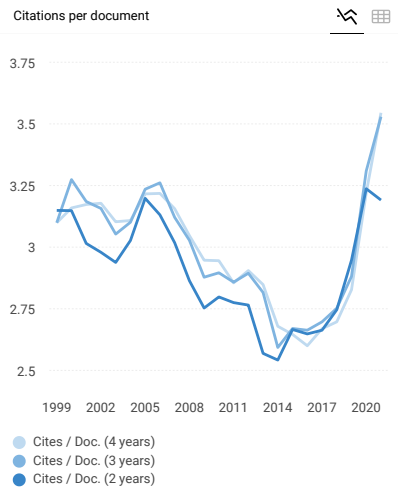

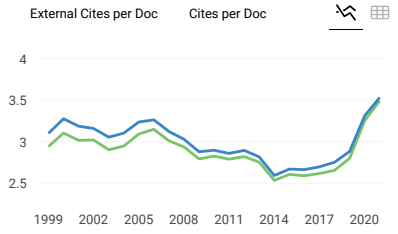

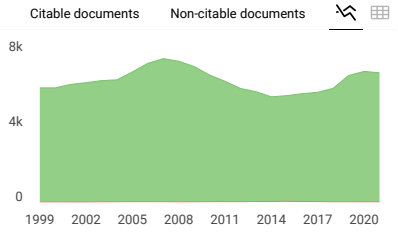

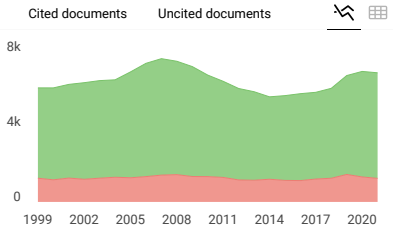

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