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GABAergic function in the lateral hypothalamus regulates feeding behavior: Possible mediation via orexin

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Abstract

Aim: The lateral hypothalamus (LH) is known as the hunger center, but the mechanisms through which the LH regulates food intake are unclear. Since GABA neurons are reported to project to the LH, the present study investigated the role of GABAergic function in the LH in the regulation of feeding behavior.

Methods: GABA levels in the LH were measured by in vivo microdialysis. Food intake after drug injection into the LH was measured every 1 hour for 4 hours. The mRNA levels were measured using RT-PCR.

Results: Food intake significantly increased GABA levels in the LH, suggesting that food intake stimulates GABAergic function in the LH. Injection of the $GABA_A$ receptor agonist muscimol into the LH significantly inhibited food intake, whereas injection of the $GABA_A$ receptor antagonist bicuculline into the LH did not significantly affect food intake. The inhibitory effect of muscimol injected into the LH was blocked by co-administration of bicuculline. These results indicate that the stimulation of $GABA_A$ receptors in the LH inhibits food intake. We next examined whether the stimulation of $GABA_A$ receptors affects hypothalamic neuropeptides that are known to regulate feeding behavior. The injection of muscimol significantly decreased preproorexin mRNA in the hypothalamus.

Conclusion: These results indicate that food intake activates GABAergic function in the LH, which terminates feeding behavior by stimulating $GABA_A$ receptors. Moreover, it is suggested that the stimulation of $GABA_A$ receptors in the LH reduces food intake through inhibition of orexin neurons.

KEYWORDS
food intake, $GABA_A$ receptors, hypothalamus, mice, neuropeptides

1 | INTRODUCTION

Previous studies have shown that antipsychotic drugs such as olanzapine cause metabolic disturbances that include hyperglycemia and body weight gain.[1,2] In addition, it is reported that olanzapine increases food intake,[3,4] suggesting that increase in food intake induced by antipsychotics causes body weight gain. Since antipsychotic drugs are thought to act mainly on the central nervous system (CNS), these reports indicate that the CNS plays an important role in the regulation of energy homeostasis, including feeding behavior.

The lateral hypothalamus (LH) is a key brain area in the regulation of feeding behavior. The LH is known as the hunger center subsequent to classical reports showing that lesion of the LH suppresses food intake and electrical stimulation of the LH increases food intake.[5] We have recently reported that opioid and dopamine functions in the LH regulate food intake.[6,7] These reports indicate that neural functions in the LH play an important role in the regulation of food intake.

Over the past 3 decades, many neuropeptides have been isolated from the hypothalamus in relation to regulation of feeding behavior. For instance, neuropeptide Y (NPY) and agouti-related peptide (AgRP) increase food intake,[8,9] and neurons containing these neuropeptides are reported to project from the arcuate nucleus of hypothalamus (ARC) to other nuclei of the hypothalamus, including the LH and the paraventricular nucleus (PVN). In contrast, α-melanocyte-stimulating hormone (α-MSH) inhibits food intake.[5] α-MSH is cleaved from proopiomelanocortin (POMC) and POMC neurons are also known to project from the ARC to other hypothalamic nuclei, including the LH. In the LH, orexin and melanin concentrating hormone (MCH) have been isolated and these are reported to increase food intake.[10,11] Taken together, it is suggested that the LH is a key station in the regulation of feeding behavior. However, since most research has focused on the role of the ARC and the PVN, the role of the LH in the regulation of feeding behavior has received less attention.

GABA neurons are known to distribute across many brain areas, including the hypothalamus. Growing evidence has indicated that neuropeptides and GABA coexist in the same neurons in the hypothalamus,[12] suggesting that GABA neurons in the hypothalamus may regulate feeding behavior. In fact, it is reported that GABA in NPY/AgRP neurons positively regulates feeding behavior together with NPY and AgRP.[13,14] Moreover, other reports indicate that GABA also exists in POMC neurons that are thought to inhibit feeding behavior.[15] Since NPY/AgRP neurons are orexigenic and POMC neurons are anorexigenic, GABA in NPY/AgRP neurons and GABA in POMC neurons might play opposite roles in the regulation of feeding behavior.

Since the LH receives projections from both NPY/AgRP and POMC neurons,[5] GABAergic function in the LH is likely important in the regulation of feeding behavior. Thus, the present study examined the role of GABAergic function in the LH in the regulation of feeding behavior. First, we examined whether GABA levels in the LH were changed by food intake. Next, we examined the role of $GABA_A$

receptors in the LH in the regulation of feeding behavior. Finally, we examined whether neuropeptides were involved in the regulation of feeding behavior by GABAergic function in the LH.

2 | MATERIALS AND METHODS

2.1 | Animals

Male ICR mice (6-7 weeks old; Tokyo Laboratory Animals Science, Tokyo, Japan) were used. The mice were kept in clear polycarbonate cages (20 × 30 × 15 cm) under a 12-hour light/dark cycle (light on at 08:00) at a constant room temperature (24 ± 1°C) with ad libitum access to normal chow diet and water.

The present study was conducted in accordance with the guidelines for the care and use of laboratory animals of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. 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 drugs used in this study were the $GABA_A$ receptor agonist muscimol (Sigma-Aldrich) and the $GABA_A$ receptor antagonist bicuculline (Sigma-Aldrich). The drugs were dissolved in saline immediately before use. Doses of these drugs were selected according to previous reports[16-18] and were optimized to avoid any influence on locomotor activity (Table 1).

2.3 | Surgery

As described previously in detail,[6,7,19] mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and stereotaxically (Narishige) implanted with stainless-steel guide cannulae (for microinjection: EKC-0504A; Bio Research Center; for microdialysis: AG-6; Eicom) into the LH (A 2.58 mm, V 0.80 mm, L 1.10 mm, from the interaural line) according to the stereotaxic atlas of Paxinos and Franklin.[20] The cannulae were secured to the skull with a

TABLE 1 Effect on locomotor activity of the $GABA_A$ receptor agonist muscimol and the $GABA_A$ receptor antagonist bicuculline injected into the lateral hypothalamus during refeeding tests

Treatment	Locomotor activity (counts/4 h)
Saline	1655 ± 339
Muscimol (5 ng)	1766 ± 323
Saline	1440 ± 391
Bicuculline (50 ng)	1923 ± 266
Saline	1759 ± 523
Muscimol (5 ng)	1841 ± 327
Bicuculline (50 ng) + muscimol (5 ng)	2404 ± 337

Note: Each point represents mean ± SEM (n = 6-8).

stainless screw and dental acrylic cement. Guide cannulae were implanted 1.5 mm (microinjection) and 1.0 mm (microdialysis) above the desired injection site to minimize damage at the target site. A wire stylet was placed in the guide cannulae to avoid occlusion. The mice were allowed to recover from surgery for a minimum of 3 days.

2.4 | Intracerebral microinjection

Mice were held manually while the wire stylets were removed. The injection needle (0.22 mm) was connected to a Hamilton syringe and lowered through the guide cannulae to the LH. Drugs were slowly administered by hand in a volume of 0.2 µL over 20 seconds, and the needles were left in place for an additional 20 seconds.

After the experiments, mice were sacrificed by inhalation of ethyl ether (Wako Pure Chemical Industries) and the brain was immediately removed. The brain was fixed in 10% formalin and sectioned at 50 µm thickness. The brain slices were stained with thionin to visualize the injection sites. Only data from mice with correctly placed injections were included in the analysis.

2.5 | Refeeding test

As described previously in detail,[6,7,19] mice were deprived of food for 16 hours (starting at 18:30) with free access to water. The mice were placed individually in a clear polycarbonate cage (20 × 30 × 15 cm) and habituated for 1 hour before the refeeding test. After drug injections, the mice were given food and food intake was measured every 1 hour for 4 hours. Locomotor activity during the refeeding test was measured using a sensor for locomotor activity (NS-AS01; Neuroscience); this was placed at the center of the polycarbonate lid and connected to a computer for processing of locomotor activity (Act-1 Light® activity software; Neuroscience).

2.6 | In vivo microdialysis

As described previously in detail,[6,7,21] an I-shaped removable-type dialysis probe (A-I-6-01; Eicom) was used. The stylet was removed from the guide cannulae and the dialysis probe was inserted into the guide cannulae, with the dialysis tubing protruding 1.0 mm from the tip. The probe was secured to the guide cannulae with a screw. After 16 hours, the mouse was placed in a plexiglass box (30 × 30 × 35 cm) and the inlet and outlet tubes were connected to a swivel located on a counterbalanced beam to minimize discomfort. The probe was perfused with modified Ringer's solution (NaCl 147 mmol/L, KCl 4 mmol/L, $CaCl_2$ 1.2 mmol/L, $MgCl_2$ 1.1 mmol/L; pH 7.4) at a rate of 2.0 µL/min, and the outflow was connected to an autosampler (Eicom). The dialysates were collected every 20 minutes. The samples were derivatized with 0.4 mol/L potassium carbonate buffer containing 0.16 N hydrochloric acid, 4 mmol/L o-phthalaldehyde, and 0.04% 2-mercaptoethanol. GABA was separated by a high-performance liquid chromatography system (Eicom) using an Eicompak FA-3ODS column (particle size, 3 µm, 3.0 × 75 mm, Eicom) and phosphate buffer (100 mmol/L, pH 6.0) containing EDTA (13 µmol/L), 13% acetonitrile, and 7% methanol as the mobile phase at a flow rate of 0.50 mL/min. GABA content was quantified by electrochemical detection using a glassy carbon working electrode set at +600 mV against a silver-silver chloride reference electrode (WE-3G; Eicom). After collecting 6 samples (2 hours), we started to measure GABA levels. The mean of GABA levels in three samples was baseline level, and then, we measured GABA levels during food intake. Food intake was measured hourly.

2.7 | Reverse transcription-polymerase chain reaction (RT-PCR)

One hour after drug injection, the hypothalamus was dissected from the brain of mice, immediately frozen in liquid nitrogen and kept at −80°C until use. Nucleospin® RNA kit (Macherey-Nagel) was used to isolate total RNA from the hypothalamus. Reverse transcription was performed using a PrimeScript® RT Master Mix kit (Takara Bio). PCR was conducted using Takara Taq™ Hot Start Version (Takara Bio). The following primers were used: NPY (forward: 5′- CAC GAT GCT AGG TAA CAA G -3′, reverse: 5′- CAC ATG GAA GGG TCT TCA AG -3′), AgRP (forward: 5′- CTG ACT GCA ATG TTG CTG AG -3′, reverse: 5′- CAA CAT CCA TTG GCT AGG TG -3′), POMC (forward: 5′- GCT TGC ATC CGG GCT TGC AAA CT -3′, reverse: 5′- AGC AAC GTT GGG GTA CAC CTT -3′), preproorexin (PPORX; forward: 5′- CCT GAG CTC CAG GCA CCA TGA ACT -3′, reverse: 5′- TGG TTA CCG TTG GCC TGA GGG AGG -3′), pro-MCH (forward: 5′- AAC TCA CGG GCT GCC ACT GAG TC -3′, reverse: 5′- GGT AGA CTC TTC CCA GCA TAC ACC -3′), and β-actin (forward: 5′- CAT CCG TAA AGA CCT CTA TGC CAA C -3′, reverse: 5′- ATG AGC CAC CGA TCC ACA -3′) as an internal standard. PCR was performed on a thermal cycler (TP650; Takara Bio) as follows: initial denaturing at 94°C for 30 seconds, 34 cycles of denaturing at 94°C for 30 seconds, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute, followed by a final extension at 72°C for 6 minutes. PCR products were analyzed by electrophoresis (Mupid®-ex; Advance) on 1.7% agarose (L03; Takara Bio) gels. The agarose gel was stained with ethidium bromide (Sigma-Aldrich) and photographed with UV transillumination. The intensity of the bands was quantified by computer-assisted densitometry using ImageJ image-analysis software (National Institutes of Health). Values for each band were normalized by the respective values for β-actin. The % of control and its standard error were calculated for each sample.

2.8 | Statistical analysis

All data are presented as means ± SEM. Two-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni test was used to compare groups. Where appropriate, the Mann-Whitney *U* test was used to compare two groups. Differences were considered statistically significant when $P < .05$.
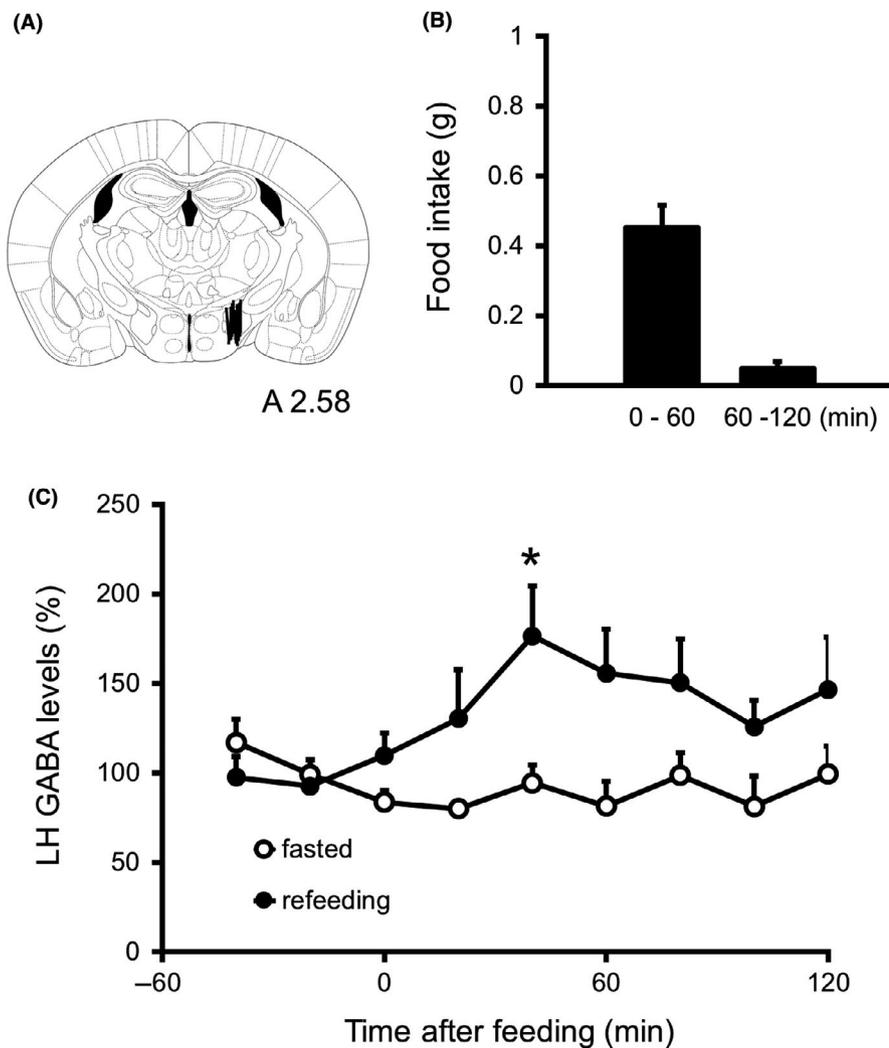
(A)

(B)

FIGURE 1 A, Schematic illustration of the placement of probes in the lateral hypothalamus (LH). B, The amount of food intake during refeeding in fasted mice. C, Effect of food intake on GABA levels in the LH of mice. Each point represents mean ± SEM (n = 7). *P < .05 vs control group

(C)

3 | RESULTS

3.1 | Effect of food intake on GABA levels in the lateral hypothalamus

Figure 1A shows the placements of the dialysis probes in the LH. Figure 1B shows the amount of food intake during the measurement of GABA levels in the LH, indicating that the mice ate food mostly during 1 hour after serving food. Food intake significantly increased GABA levels in the LH in fasted mice (fasted group, n = 6; refeeding group, n = 7; two-way ANOVA, treatment: $F_{(1,55)}$ = 10.29, P < .01; Figure 1C).

3.2 | Effects of GABA$_A$ receptor agonist and antagonist injected into the lateral hypothalamus on food intake

Injection of muscimol (5 ng/side) bilaterally into the LH (Figure 2A) significantly reduced food intake (saline, n = 8; muscimol, n = 7; two-way ANOVA, treatment: $F_{(1,39)}$ = 10.31, P < .05; Figure 2B). In contrast, bilateral injection of bicuculline (50 ng/side) into the LH (Figure 2C) did not significantly change food intake (saline, n = 8; bicuculline, n = 6; Figure 2D). The decrease in food intake induced by

muscimol (5 ng/side) injected into the LH was inhibited by coadministration of bicuculline (50 ng/side; saline, n = 8; bicuculline, n = 8; bicuculline + muscimol, n = 6; two-way ANOVA, muscimol vs bicuculline + muscimol, treatment: $F_{(1,36)}$ = 10.30, P < .01; Figure 2E,F). None of the drugs affected locomotor activity during the measurement of food intake (Table 1).

### 3.3 | Effect of GABA$_A$ receptor agonist on mRNA levels of neuropeptides in the hypothalamus

Injection of muscimol (1 mg/kg, i.p.) significantly decreased the mRNA level of PPORX, but not of NPY, AgRP, POMC, or pro-MCH in the hypothalamus (saline, n = 7-9; muscimol, n = 6-9; Mann-Whitney U test; Figure 3).

4 | DISCUSSION

The present study investigated the role of GABAergic function in the LH in the regulation of feeding behavior.

First, we examined whether GABA levels in the LH were changed by food intake. Our results showed that food intake

FIGURE 2 A, Schematic illustration of the injection sites (stars) of saline and muscimol in the lateral hypothalamus (LH). B, Effect of the GABA$_A$ receptor agonist muscimol injected into the LH on food intake. C, Schematic illustration of the injection sites (stars) of saline and bicuculline in the LH. D, Effect of the GABA$_A$ receptor antagonist bicuculline injected into the LH on food intake. E, Schematic illustration of the injection sites (stars) of saline, muscimol, and bicuculline + muscimol in the LH. F, Effect of bicuculline on the inhibition of food intake induced by muscimol injected into the LH. Each point represents mean ± SEM (n = 6-8). *P < .05, **P < .01, ***P < .001 vs saline group; #P < .05 vs muscimol group
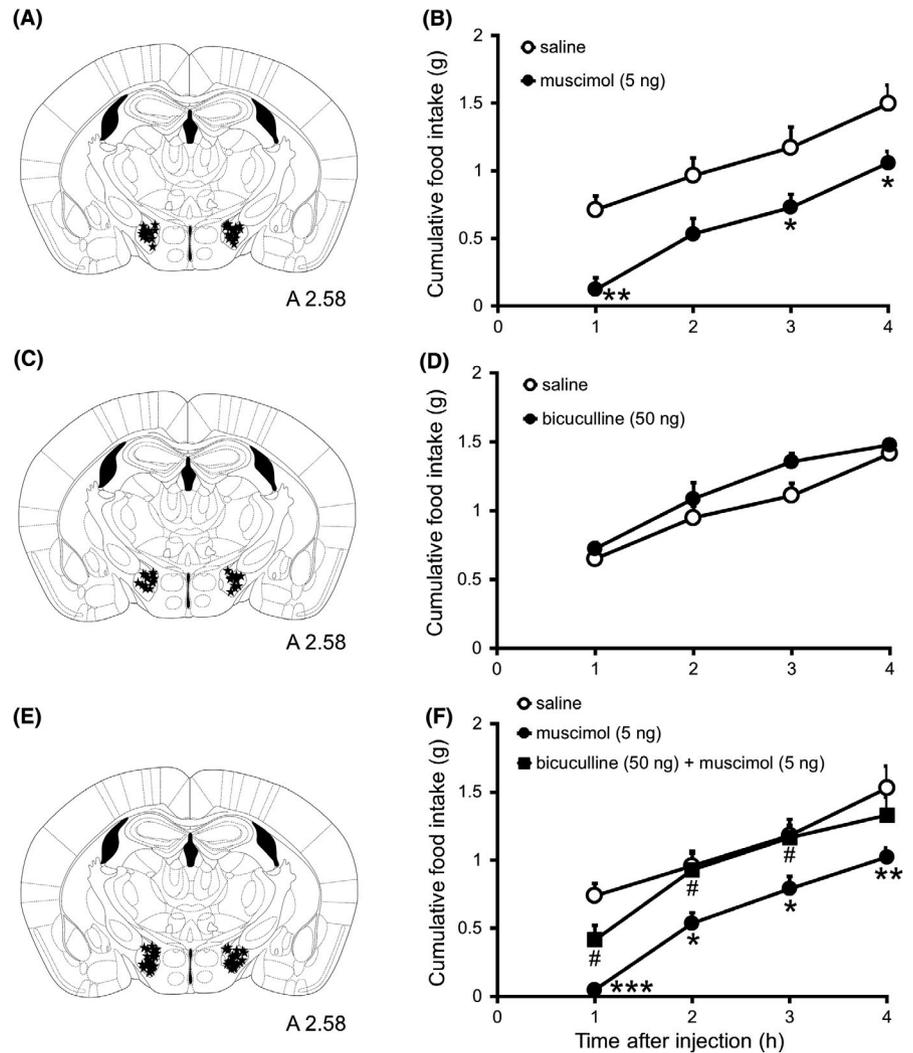
significantly increased GABA levels in the LH. Since previous reports using the similar techniques to us have shown that about 70% of GABA in the dialysates were tetrodotoxin (TTX)-sensitive,[22] it is suggested that GABA neurons projecting to the LH are stimulated by food intake. It is reported that GABA exists in both NPY/AgRP neurons and POMC neurons that project from the ARC to the LH.[12-15] NPY/AgRP neurons are inhibited by food intake, possibly through leptin and insulin, whereas POMC neurons are stimulated by food intake, also possibly through leptin and insulin.[23] Thus, it is possible that the increase of GABA levels in the LH during food intake depends on the activity of POMC neurons. Another possibility is that GABA neurons projecting from other brain areas to the LH are also stimulated by food intake, since previous reports have shown that the LH receives neural inputs from the nucleus accumbens and the ventral pallidum.[24,25] In addition, it is also possible that GABA is released from glial cells, since astrocytes are reported to release GABA,[26] though the role of glial cells in the regulation of feeding behavior is unclear. Taken together, although the present results cannot distinguish between these putative mechanisms, they suggest that food intake stimulates mainly GABA neurons projecting to the LH.

Next, we examined the role of GABA$_A$ receptors in the LH in the regulation of feeding behavior. The results showed that the GABA$_A$ receptor agonist muscimol injected into the LH significantly inhibited food intake, an effect that was blocked by coadministration of the GABA$_A$ receptor antagonist bicuculline at a dose that did not itself affect food intake. These results indicate that stimulation of GABA$_A$ receptors in the LH inhibits food intake. Since food intake increased GABA levels in the LH (see above), it is suggested that food intake stimulates GABA neurons projecting to the LH, which terminates food intake by stimulating GABA$_A$ receptors in the LH. Following this mechanism, the injection of bicuculline into the LH should increase food intake by blocking the binding of released GABA to GABA$_A$ receptors. Since we chose the dose of bicuculline, which did not affect food intake to examine whether bicuculline blocked the effect of muscimol, the increase of food intake was not observed. Thus, it is possible that higher dose of bicuculline might increase food intake. There are some reports examining the role of GABA in the LH in the regulation of feeding behavior. However, most of these reports focused on GABA neurons in the LH. For instance, activation of GABA neurons in the LH increased food intake.[27] More specifically, activation of GABA neurons projecting from the LH to
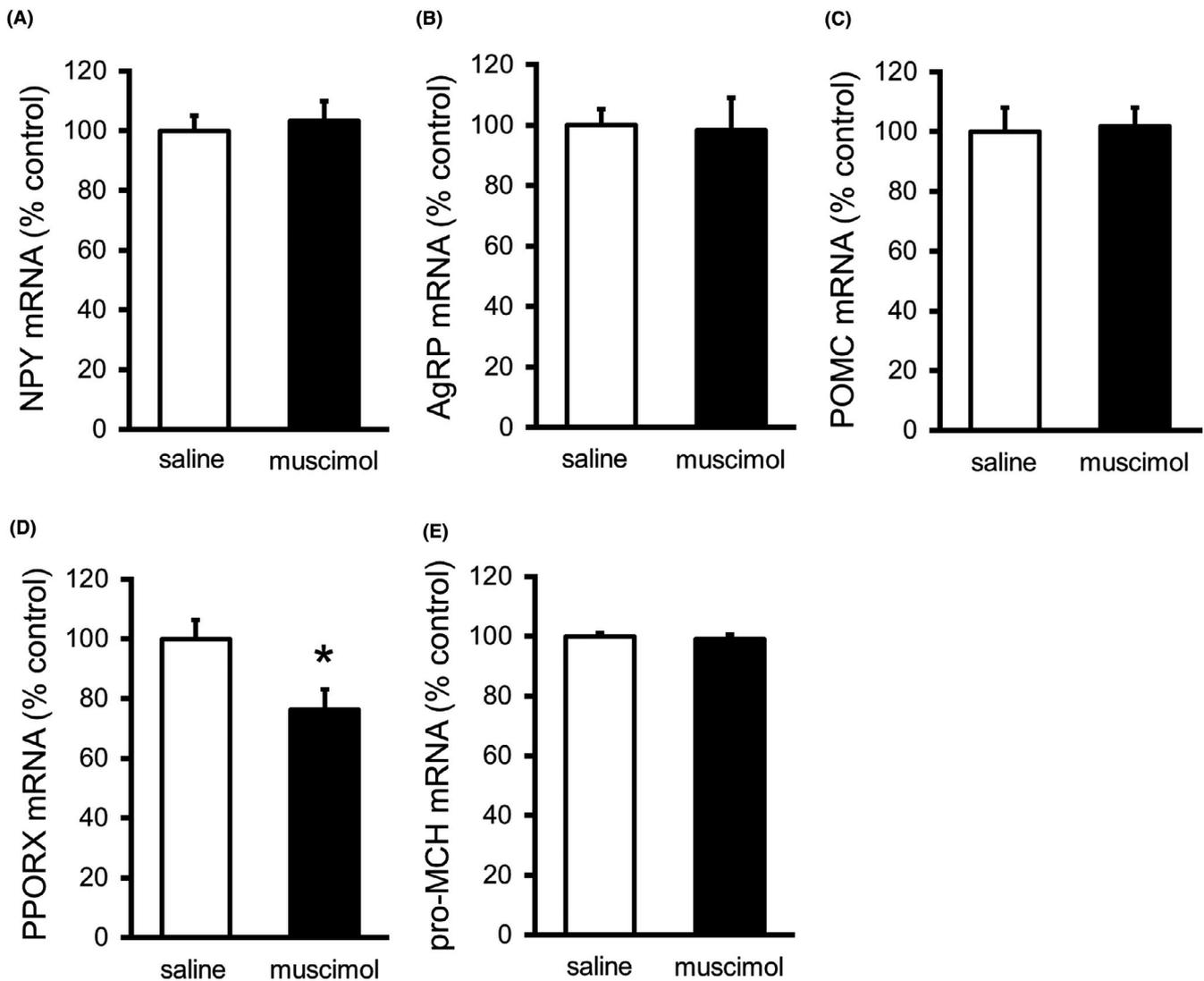
FIGURE 3 Effect of the $GABA_A$ receptor agonist muscimol (1 mg/kg, i.p.) on mRNA levels of neuropeptide Y (NPY; A), agouti-related peptide (AgRP; B), proopiomelanocortin (POMC; C), preproorexin (PPORX; D), and pro-melanin-concentrating hormone (pro-MCH; E) in the hypothalamus. Each column represents mean ± SEM (n = 6-9). *$P < .05$ vs saline group

the PVN increased food intake.[28] These results indicate that activation of GABA neurons projecting from the LH to other brain areas stimulates feeding behavior. Since the present study showed that stimulation of $GABA_A$ receptors in the LH reduced food intake, it is possible that $GABA_A$ receptors regulate GABA neurons projecting from the LH to other brain areas, including PVN.

If the drugs used in the present study were to alter locomotor activity, hypoactivity or hyperactivity might disturb feeding behavior. Thus, we chose doses of drugs that do not affect locomotor activity; direct measurement of locomotor activity in the present study confirmed that none of the drugs used in the present study significantly changed locomotor activity during the measurement of food intake. Thus, the inhibitory effect of muscimol on food intake does not result from changes in locomotor activity.

Finally, we examined whether stimulation of $GABA_A$ receptors inhibited food intake through neuropeptides in the hypothalamus. Injection of muscimol significantly inhibited mRNA level of PPORX,

but not of AgRP, NPY, POMC, or pro-MCH, in the hypothalamus. It has been reported that changes in mRNA levels of neuropeptides correlate with the activity of neurons containing those neuropeptides.[29,30] For instance, the previous reports have shown that fasting and injection of insulin, which increases food intake by hypoglycemia, increased mRNA level of PPORX.[31,32] In addition, it is reported that orexin neurons are activated by fasting.[33] Thus, it is likely that the mRNA level of PPORX reflects activity of orexin neurons. Since orexin is known as an orexigenic neuropeptide,[10] our results suggest that stimulation of $GABA_A$ receptors in the LH inhibits food intake through inhibition of orexin neurons. Further studies are needed to confirm this point.

Previous reports have shown that activation of GABA neurons in the LH increases food intake.[27,28] Since our results showed that activation of $GABA_A$ receptors inhibited food intake, it is possible that stimulation of $GABA_A$ receptors reduces food intake by inhibition of GABA neurons in the LH. In addition, our results

suggest that stimulation of $GABA_A$ receptors reduces food intake by inhibition of orexin neurons in the LH. As discussed above, neuropeptides such as NPY, AgRP, and POMC in the ARC are colocalized with GABA.[12–15] In contrast, there is no report showing that GABA is colocalized with neuropeptides such as orexin and MCH in the LH. Moreover, GABA neurons in the LH regulate physiological functions, including food intake and sleep, through mechanisms are independent of orexin and MCH neurons.[34–36] Thus, it is unclear whether $GABA_A$ receptors in the LH regulate feeding behavior through GABA neurons in the LH. Moreover, since $GABA_B$ receptors are reported to exist in the LH,[37] it is possible that $GABA_B$ receptors in the LH are also involved in regulation of feeding behavior. Further studies are needed to clarify this issue.

In conclusion, the present study demonstrates that food intake stimulates GABA neurons projecting to the LH and that increased GABAergic activity terminates food intake by stimulation of $GABA_A$ receptors in the LH. In addition, it is suggested that stimulation of $GABA_A$ receptors in the LH inhibits food intake through the inhibition of orexin neurons.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

HI designed research. NY, CA, and DU performed research. NY, CA, DU, and HI analyzed data. NY and HI wrote the manuscript. NY, CA, DU, JK, and HI critically read and approved the manuscript.

DATA AVAILABILITY STATEMENT

We have made our data publicly available through direct submission as Supporting Information.

ANIMAL STUDIES

All experiments in the present study were approved by the Committee on Animal Research of Hoshi University.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.