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Serotonin systems upregulate the expression of hypothalamic NUCB2 via 5-HT2C receptors and induce anorexia via a leptin-independent pathway in mice

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ABSTRACT

NEFA/nucleobindin2 (NUCB2), a novel satiety molecule, is associated with leptin-independent melanocortin signaling in the central nervous system. Here, we show that systemic administration of *m*-chlorophenylpiperazine (*m*CPP), a serotonin 5-HT1B/2C receptor agonist, significantly increased the expression of hypothalamic NUCB2 in wild-type mice. The increases in hypothalamic NUCB2 expression induced by *m*CPP were attenuated in 5-HT2C receptor mutant mice. Systemic administration of *m*CPP suppressed food intake in db/db mice with leptin receptor mutation as well as lean control mice. On the other hand, the expression of hypothalamic NUCB2 and proopiomelanocortin (POMC) was significantly decreased in hyperphagic and non-obese 5-HT2C receptor mutants compared with age-matched wild-type mice. Interestingly, despite increased expression of hypothalamic POMC, hypothalamic NUCB2 expression was decreased in 5-HT2C receptor mutant mice with heterozygous mutation of β-endorphin gene. These findings suggest that 5-HT systems upregulate the expression of hypothalamic NUCB2 via 5-HT2C receptors, and induce anorexia via a leptin-independent pathway in mice.

NEFA/nucleobindin2 (NUCB2) is a novel satiety molecule that is associated with central melanocortin signaling, and the expression of hypothalamic NUCB2 is reportedly increased by α-MSH, which is released from proopiomelanocortin (POMC) neurons [1]. Intracerebroventricular injection of NUCB2 decreases food intake in Zucker rats with a leptin receptor mutation, and administration of leptin does not induce the expression of hypothalamic NUCB2, suggesting that NUCB2 has a leptin-independent satiety signaling [1]. The regulatory systems of hypothalamic NUCB2 expression, however, remain unknown.

Brain serotonin (5-hydroxytryptamine; 5-HT) systems contribute to the regulation of eating behavior and energy homeostasis [2,3]. Pharmacologic studies using mice with a null mutation of the 5-HT subtype receptor have demonstrated that 5-HT2C receptors and/or 5-HT1B receptors mediate the appetite-suppressing effects of 5-HT drugs, such as *m*-chlorophenylpiperazine (*m*CPP) and D-fenfluramine [2,4–6]. 5-HT drugs such as *m*CPP or fenfluramine directly activate POMC neurons in the arcuate nucleus of the hypothalamus, and 5-HT2C receptors, which are expressed on POMC neurons, may contribute to this effect [7]. The central melanocortin pathway is therefore suggested to be essential for the satiety signaling downstream of 5-HT [7,8].

To determine the role of 5-HT systems in the regulation of hypothalamic NUCB2 expression, we first examined the effects of *m*CPP, a serotonin 5-HT1B/2C receptor agonist, on the expression of hypothalamic NUCB2 in wild-type mice and 5-HT2C receptor mutant mice. In the second experiment, we examined the effects of *m*CPP on food intake in db/db mice with leptin receptor deficiency and control lean mice matched for age. In the third experiment, we examined daily food intake and the basal expression of hypothalamic NUCB2 and POMC in 5-HT2C receptor mutant mice and wild-type mice matched for age. Finally, we examined the basal expression of hypothalamic NUCB2 and the effects of *m*CPP on the expression of hypothalamic NUCB2 in 5-HT2C receptor mutant mice with heterozygous mutation of β-endorphin gene (2CEnd mice) and wild-type mice matched for age.

Materials and methods

Mice. Hemizygous mutant males bearing a null mutation of the X-linked *htr2c* gene (congenic on a C57BL/6J background) and age-matched wild-type mice were used in the experiments. The line has been maintained through a mating of females heterozygous for the *htr2c* gene with C57BL/6J males obtained from the Jackson Laboratory (Bar Harbor, ME). Genotypes were confirmed by South-

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ern blot analysis of BamHl-digested genomic DNA from the tails of littermates as described previously [2]. Blots were hybridized with a 3′ flanking probe. Wild-type and *htr2c* mutant alleles correspond to the 6.0- and 2.5-kb fragments, respectively.

Homozygous mutant males bearing a null mutation of the β-endorphin gene (congenic on a C57BL/6J background) and age-matched wild-type mice were used. The line has been maintained through mating of females heterozygous for the β-endorphin gene with heterozygous males obtained from Jackson Laboratory. Genotypes were confirmed by Southern blot analysis of BglII-digested genomic DNA from tails of littermates as described previously [9]. Blots were hybridized with a 5′ flanking probe. Wild-type and β-endorphin mutant alleles correspond to the 2.1- and 3.8-kb fragments, respectively. Female mice homozygous for the *htr2c* mutations and male mice homozygous for the β-*endorphin* mutations were then bred to produce the additional male experimental mice. The line has been maintained through a mating of females homozygous for the *htr2c* gene with male homozygous for the β-*endorphin* mutation.

Male mice mutant for the leptin receptor (db/db mice), bred on a C57BL/6J background, were purchased from Jackson Laboratories. Age-matched male C57BL/6J mice were used as controls.

The animals were all housed with free access to water and chow pellets in a light- (12 h on/12 h off; lights off at 2000 h) and temperature- (20–22 °C) controlled environment.

Animals were acclimatized to the laboratory environment for 1 week before the experiment.

General procedures. In the first experiment, 14-week-old male C57BL/6J wild-type mice were intraperitoneally injected with saline or *m*CPP (2.5–10 mg/kg). They were not fed chow pellets. Sixty minutes later, the animals were decapitated and the hypothalamus was removed for RNA extraction, as described previously [10–13].

In the second experiment, 14-week-old male 5-HT2C receptor mutant mice were intraperitoneally injected with saline or *m*CPP (5 and 10 mg/kg). They were not fed chow pellets. Sixty minutes later, the animals were decapitated and the hypothalamus was removed for RNA extraction.

In the third experiment, 10-week-old male db/db mice and control lean littermates were intraperitoneally injected with saline or *m*CPP (5 mg/kg) 30 min before the onset of the dark cycle. Intake of chow pellets was measured for the next 2 h after the onset of the dark cycle, as described previously [12,13].

In the fourth experiment, daily food and body weight were measured in 14-week-old male 5-HT2C receptor mutant mice and wild-type littermates. Then, the animals were decapitated and the hypothalamus was removed for RNA extraction.

In the fifth experiment, daily food and body weight were measured in 15-week-old male 2CEnd mice and wild-type littermates. Then, the animals were decapitated and the hypothalamus was removed for RNA extraction.

Finally, 15-week-old male 2CEnd mice were intraperitoneally injected with saline or *m*CPP (10 mg/kg). They were not fed chow pellets. Sixty minutes later, the animals were decapitated and the hypothalamus was removed for RNA extraction.

The doses of *m*CPP (10 mg/kg) were selected based on the evidence that *m*CPP-induced hypophagia was attenuated by a genetic blockade of 5-HT2C receptors [2,5]. *m*CPP was purchased from the Sigma Chemical Co., Japan. The drugs were dissolved in 0.2 ml 0.9% saline.

The animal studies were conducted under protocols in accordance with the institutional guidelines for animal experiments at the Tohoku University Graduate School of Medicine.

Real-time quantitative reverse transcription-polymerase chain reaction. Total RNA was extracted from the mouse hypothalamic tissue using the RNeasy Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's directions. cDNA synthesis was performed using a Super Script III First-Strand Synthesis System for the RT-PCR Kit (Invitrogen, Rockville, MD) using 1 μg total RNA. The cDNA synthesized from the total RNA was evaluated in a real-time polymerase chain reaction (PCR) quantitative system (Light Cycler Quick System 350S; Roche Diagnostics, Mannheim, Germany), as described previously [10–13]. The primers used were as follows. For mouse NUCB2, sense, 5′-ACA AAA TGC AGA GGA CGA TA-3′, antisense, 5′-CTC GGT GAA TAA CTG TTG CT-3′; for mouse POMC, sense, 5′-TGC TTC AGA CCT CCA TAG AT-3′, antisense, 5′-GGC TGT TCA TCT CCG TTG-3′, and for mouse β-actin, sense, 5′-TTG TAA CCA ACT GGG ACG ATA TGG-3′, antisense, 5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′. The relative amount of mRNA was calculated using β-actin mRNA as the invariant control. The data are shown as fold change of the mean values of the control group, which received saline.

Statistical methods. Data are presented as mean values ± SEM (n = 5–8). Statistical significance of difference between two groups was determined using two-tailed unpaired Student's t test. Comparisons among more than two groups were performed with ANOVA using Bonferroni's test. A P value of less than 0.05 was considered statistically significant.

Results and discussion

Effects of mCPP on hypothalamic NUCB2 mRNA levels

Systemic administration of *m*CPP (5 and 10 mg/kg) significantly increased hypothalamic NUCB2 mRNA levels compared with saline controls in C57BL/6J wild-type mice (Fig. 1A). The *m*CPP (5 and 10 mg/kg)-induced increases in hypothalamic NUCB2 mRNA levels in wild-type mice were attenuated in age-matched 5-HT2C receptor mutant mice (Fig. 1B). These results suggest that 5-HT2C receptor stimulation increases the expression of hypothalamic NUCB2 in mice.

Effects of mCPP on food intake in db/db mice

Systemic administration of *m*CPP (5 mg/kg) significantly suppressed food intake in db/db mice as well as control lean mice (Fig. 2). These results suggest that leptin pathway is not essential for the anorexia induced by 5-HT2C/1B receptor stimulation in mice.

Hypothalamic NUCB2 and POMC mRNA levels in 5-HT2C receptor mutant mice

The basal mRNA levels of hypothalamic NUCB2 and POMC were significantly decreased in 5-HT2C receptor mutant mice compared with age-matched wild-type mice (Fig. 3A and B). 5-HT2C receptor mutant mice consume more food than wild-type mice matched for age (Fig. 3C), although there were no genotypic differences in body weight (wild-type mice; 26.2 ± 0.9 g and 5-HT2C receptor mutant mice; 26.6 ± 0.8 g). These results suggest that genetic ablation of 5-HT2C receptor display hyperphagia associated with decreased expression of hypothalamic NUCB2 and POMC in mice.

Hypothalamic NUCB2 and POMC mRNA levels and effects of mCPP on hypothalamic NUCB2 mRNA levels in 2CEnd mice

The basal mRNA levels of hypothalamic NUCB2 were significantly decreased in 2CEnd mice compared with age-matched wild-type mice (Fig. 4A), whereas hypothalamic POMC mRNA levels were increased in 2CEnd mice (Fig. 4B). Although 2CEnd consume more food than wild-type mice matched for age (Fig. 4C), there were no genotypic differences in body weight (wild-type mice; 27.2 ± 0.9 and 2CEnd; 27.2 ± 0.5 g). Moreover, systemic administration of *m*CPP (10 mg/kg) had no effects on hypothalamic

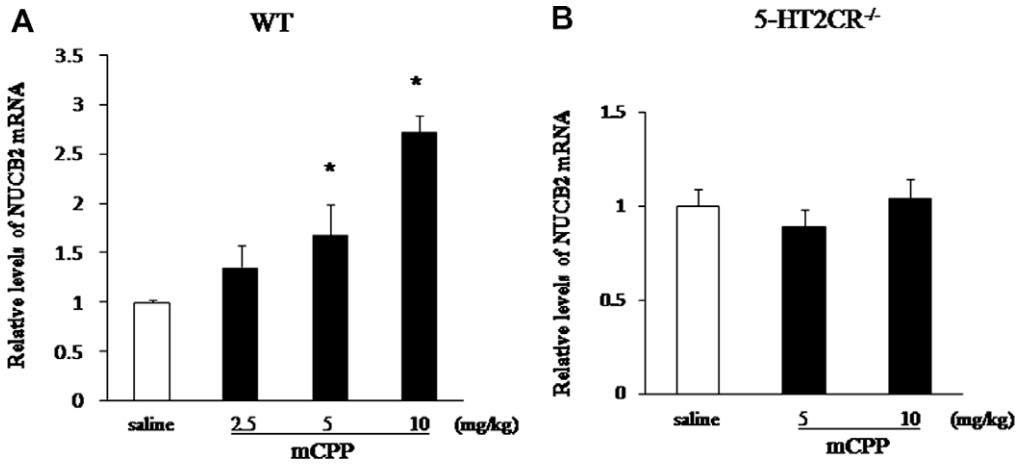

Fig. 1. Effects of *m*CPP (2.5, 5, and 10 mg/kg) or saline on hypothalamic NUCB2 mRNA levels in 14-week-old C57BL/6J wild-type mice (A). Effects of *m*CPP (5 and 10 mg/kg) or saline on hypothalamic NUCB2 mRNA levels in 14-week-old 5-HT2C receptor mutant mice (B). WT, wild-type mice; 5-HT2CR$^{-/-}$, 5-HT2C receptor mutant mice. Data are presented as mean values ± SEM (n = 5–6 for each group of animals). $^{*}P < 0.05$.

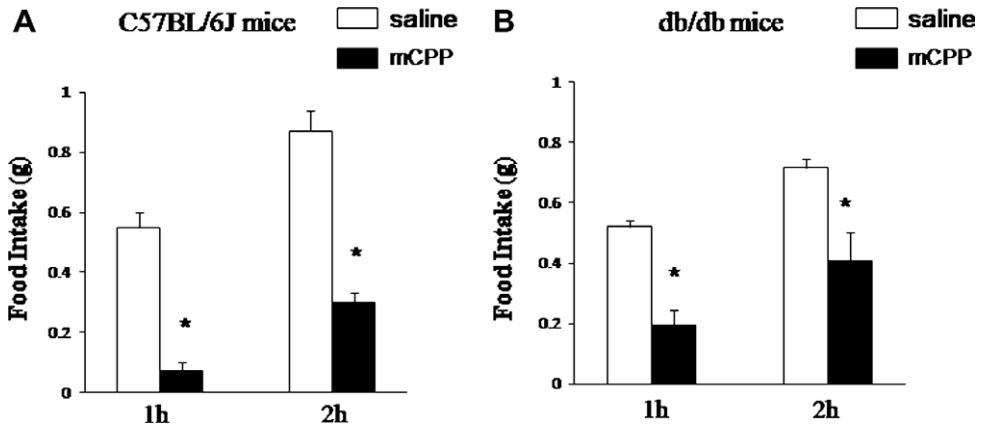

Fig. 2. Effects of *m*CPP (5 mg/kg) or saline on food intake in 10-week-old C57BL/6J mice (A) and db/db mice (B). Data are presented as mean values ± SEM (n = 6–7 for each group of animals). $^{*}P < 0.05$.

NUCB2 mRNA levels in 2CEnd mice (Fig. 4D). These results suggest that the decreased expression of hypothalamic POMC is not always associated with decreased expression of hypothalamic NUCB2 in 2CEnd, and also support that 5-HT systems upregulate the expression of hypothalamic NUCB2 via 5-HT2C receptor in mice.

The present results demonstrate that 5-HT2C receptor stimulation increased the expression of hypothalamic NUCB2 in mice. In addition, genetic deletion of 5-HT2C receptors decreased hypothalamic NUCB2 expression. Moreover, the altered expression of hypothalamic NUCB2 induced by stimulation or ablation of 5-HT2C receptors is associated with changes in feeding behavior and expression of hypothalamic POMC. Because 5-HT2C receptors are expressed on POMC neurons, *m*CPP directly activates POMC neurons in the arcuate nucleus of the hypothalamus [7]. The present study demonstrates that 5-HT systems via 5-HT2C receptors substantially contribute to the maintenance of the basal levels of NUCB2 and POMC expression in the hypothalamus.

5-HT2C receptor mutant mice consume more food than wild-type mice, and have normal responses to exogenous leptin administration, suggesting leptin-independent hyperphagia in 5-HT2C receptor mutant mice [3]. The present study demonstrates that db/db mice have normal responses to administration of *m*CPP, suggesting leptin receptor-independent anorexia induced by 5-HT2C receptor stimulation. Intracerebroventricular injection of NUCB2 decreases food intake in Zucker rats with a leptin receptor mutation, and administration of leptin does not induce the expression of hypothalamic NUCB2, suggesting that NUCB2 has a leptin-independent satiety signaling [1]. Taken together, 5-HT and leptin signaling for satiety can be dissociated, and NUCB2 might be a specific signaling downstream for 5-HT2C receptors in the central nervous system.

It remains unknown whether 5-HT2C receptors are expressed on NUCB2 neurons in the hypothalamus and whether NUCB2 contributes to mediate the anorexia induced by the activation of 5-HT2C receptor. Intracerebroventricular injection of α-MSH, which is released from POMC neurons, reportedly increases the expression of hypothalamic NUCB2 [1]. 5-HT2C receptors are expressed on POMC neurons, and the central melanocortin pathway is reportedly required for the satiety signaling downstream of 5-HT [7,8]. From these evidences, POMC neurons might mediate the 5-HT2C receptor-induced alterations of hypothalamic NUCB2 expression. We, however, cannot rule out the involvement of unknown defects which are related to genetic deletion of 5-HT2C receptor. Interestingly, the present study demonstrates that the decreased expression of hypothalamic NUCB2 was not always associated with the decresaed expression of POMC in 2CEnd mice. 5-HT2C receptor might therefore contribute to the regulation of NUCB2 gene expression via a POMC-independent pathway.

The melanocortin and β-endorphin neuropeptides are processed from POMC. Melanocortin has inhibitory effects on appetite

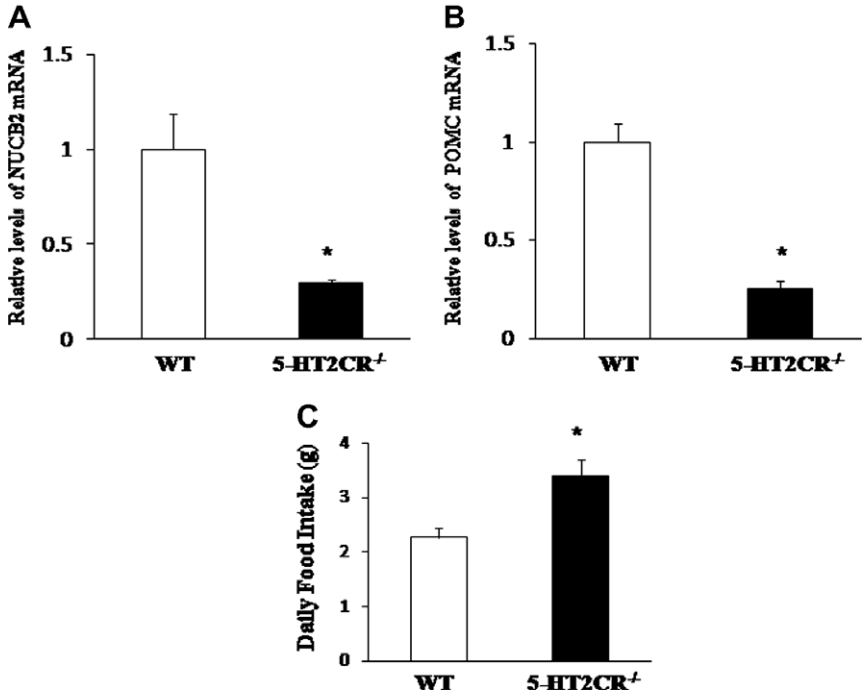

Fig. 3. Basal levels of hypothalamic NUCB2 and POMC mRNA levels (A, B) and daily food intake (C) in 14-week-old 5-HT2C receptor mutant mice (filled bars) and wild-type mice (open bars). WT, wild-type mice; 5-HT2CR$^{-/-}$, 5-HT2C receptor mutant mice. Data are presented as mean values ± SEM (n = 5–6 for each group of animals). $^{*}P < 0.05$.

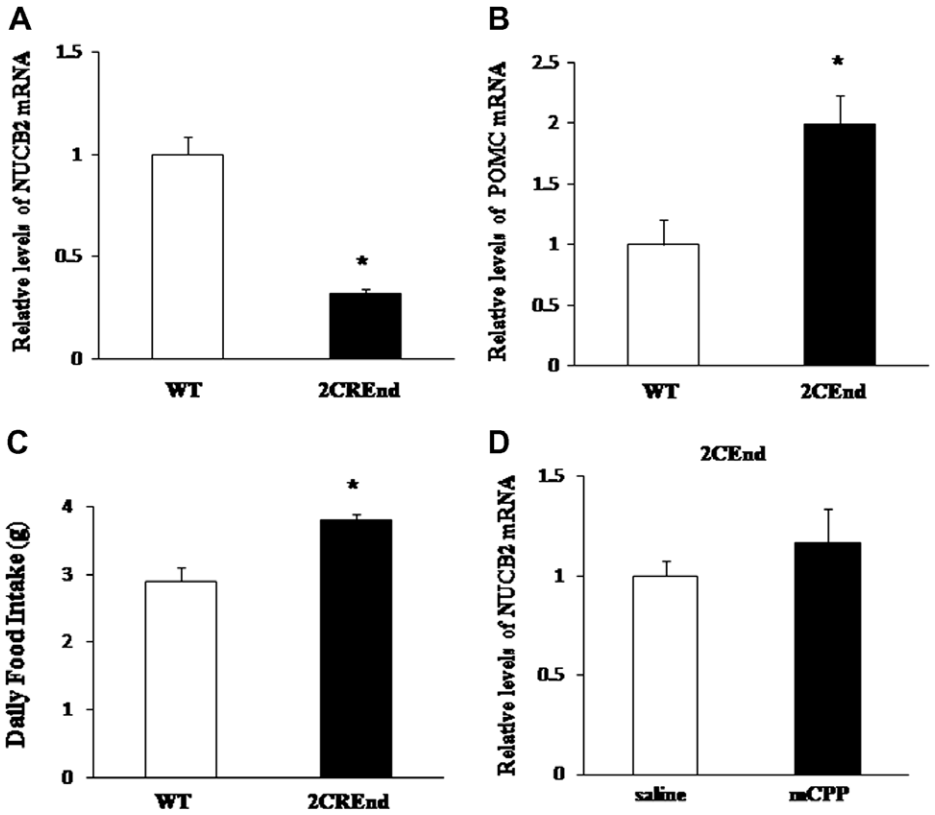

Fig. 4. Basal levels of hypothalamic NUCB2 and POMC mRNA levels (A, B) and daily food intake (C) in 15-week-old 5-HT2C receptor mutant mice with heterozygous mutation of β-endorphin gene (filled bars) and wild-type mice (open bars). Effects of *m*CPP (10 mg/kg) (filled bars) or saline (open bars) on the hypothalamic NUCB2 mRNA levels in 15-week-old 2CEnd mice (D). WT, wild-type mice; 2CEnd, 5-HT2C receptor mutant mice with heterozygous mutation of β-endorphin gene. Data are presented as mean values ± SEM (n = 4–6 for each group of animals). $^{*}P < 0.05$.

and body weight, whereas opioids stimulate food intake. Male mice engineered to selectively lack β-endorphin, but that retained normal melanocortin signaling, however, display hyperphagia and obesity [14]. The increased expression of hypothalamic POMC in

2CEnd mice might be due to the consequence of enhanced gene expression to produce melanocortin as a compensatory response to the partial decreases in β-endorphin. Further studies will be needed to determine the neural linkage between 5-HT2C receptors and NUCB2 neurons in the central regulation of feeding.

In summary, these findings suggest that 5-HT systems via 5-HT2C receptors upregulate the expression of hypothalamic NUCB2 and induce anorexia via a leptin-independent pathway in mice. This is the first report of the role of 5-HT2C receptors in the regulation of hypothalamic NUCB2 expression and leptin pathway-independent satiety signaling in vivo.

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