Hypothalamic orexin and pro-opiomelanocortin activities are essential for the anorexic effects of m-chlorophenylpiperazine in mice

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Abstract
Hypothalamic pro-opiomelanocortin (POMC) activity is reportedly essential for satiety signalling downstream of serotonin (5-HT). Here we show that food-restricted wild-type mice, which exhibited decreased hypothalamic POMC expression and increased hypothalamic orexin expression, were responsive to m-chlorophenylpiperazine (m-CPP), a 5-HT2C/1B receptor agonist, leading to anorexia, whereas food-restricted Ay mice with decreased hypothalamic POMC and orexin expression, were not. Injection of POMC small interfering RNA (siRNA) oligonucleotide + orexin siRNA oligonucleotide into the third cerebral ventricle was unresponsive to mCPP-induced anorexia, whereas a single injection of POMC or orexin siRNA oligonucleotides elicited a response. The injection of POMC siRNA oligonucleotides suppressed the anorexic effects of sibutramine, a serotonin and noradrenaline re-uptake inhibitor. The injection of orexin siRNA oligonucleotides suppressed the hyperphagia induced by the injection of POMC siRNA oligonucleotides. These findings suggest that functional hypothalamic POMC and orexin activity has a critical role in satiety signalling of mCPP in mice.

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Introduction
The complex serotonin (5-hydroxytryptamine; 5-HT) network systems orchestrate leptin-independent central appetite regulation (Nonogaki et al. 1998; Nonogaki, 2008). m-chlorophenylpiperazine (mCPP), a 5-HT1B/2C receptor agonist, and fenfluramine, a 5-HT reuptake inhibitor and its releaser, both directly activate pro-opiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus (Heisler et al. 2002). 5-HT1C receptors, which are expressed on POMC neurons, reportedly contribute to this effect (Heisler et al. 2002; Xu et al. 2008). These findings suggest that functional hypothalamic POMC activity is essential for satiety signalling downstream of the 5-HT1C receptor.

On the other hand, we previously reported that systemic administration of mCPP or fenfluramine increases hypothalamic POMC, cocaine amphetamine-regulated transcript (CART), and corticotropin releasing hormone (CRH) expression (Heisler et al. 2007; Nonogaki et al. 2006a, 2007a). 5-HT1B receptors, which are located on presynaptic neurons and stimulate 5-HT release, have also been suggested to mediate mCPP and fenfluramine-induced feeding suppression (Lee et al. 2004a, b; Lucas et al. 1998). In addition, CP94253, a selective 5-HT1B receptor agonist, reportedly induces an increase in hypothalamic POMC and CART expression and a decrease in hypothalamic orexin expression, leading to anorectic effect (Nonogaki et al. 2007a).

Moreover, we previously reported that mCPP suppressed food intake in 5-HT1C receptor mutants with or without a decrease in hypothalamic POMC expression (Nonogaki et al. 2009a), suggesting that 5-HT1B receptors are compensatory and contribute to the anorectic effects of mCPP. Thus, it remains unclear whether hypothalamic POMC activity is essential for...
satiety signalling of 5-HT or not, and raises the hypothesis that hypothalamic neuropeptides other than POMC have a critical role in the regulation of satiety signalling of mCPP.

Restricted feeding has no effect on the anorectic effects of mCPP or fenfluramine in wild-type mice while attenuating the anorectic effects in A\(^v\) mice, which overproduce agouti peptide, an endogenous antagonist for melanocortin (MC)-4 and -3 receptors (Nonogaki et al. 2006b), suggesting that agouti peptide and feeding conditions affect the anorectic effects of mCPP or fenfluramine.

To determine whether functional hypothalamic POMC activity is essential for the anorectic effect of mCPP, we examined the expression of hypothalamic 5-HT\(_{1B}\) receptor, 5-HT\(_{2A}\) receptor, NEFA/nucleobindin2 (NUCB2), POMC, CART, neuropeptide Y (NPY), CRH, and orexin genes, all of which are involved in the regulation of feeding behaviour, in relation to the anorectic effects of mCPP, in food-restricted wild-type mice and A\(^v\) mice compared to food-unrestricted animals.

To further determine the role of central POMC and orexin in the regulation of feeding behaviour in relation to satiety signalling of mCPP, we examined the effects of injection of POMC small interfering RNA (siRNA) oligonucleotide, orexin siRNA oligonucleotide, or POMC siRNA oligonucleotide into the third cerebral ventricle on food intake and body weight, and the anorectic effects of mCPP or sibutramine, a serotonin and noradrenaline re-uptake inhibitor (SNRI), in mice.

### Materials and methods

**Mice**

Animals were purchased from Japan CLEA (Tokyo, Japan). Mice were housed in individual cages with free access to water and chow pellets under a 12-h light/dark cycle (lights on 08:00 hours) and a temperature-controlled (20–22°C) environment.

In expt 1, 5-wk-old male wild-type and A\(^v\) mice (KK background) were provided 3.5 g of chow pellets daily for 5 d before the injection of Chow pellets were provided 30 min later. The intake of chow pellets was measured for the next hour after onset of the dark cycle.

In expt 2, POMC, orexin, POMC + orexin, or control siRNA oligonucleotides were injected into the third cerebral ventricle in 5-wk-old C57BL6j mice. Body weight and daily food intake were measured on days 1 and 2 after the injection. On day 2, animals were intraperitoneally injected with saline or mCPP (3 and 5 mg/kg) 30 min before onset of the dark cycle. Chow pellets were provided 30 min later. The intake of chow pellets was measured for the next 1 h and then 2 h after onset of the dark cycle.

The doses of mCPP (3 and 5 mg/kg) were selected based on evidence that mCPP-induced hypophagia was attenuated by a genetic blockade of 5-HT\(_{1B}\) receptors (Tecott et al. 1995). The doses of sibutramine (3 and 5 mg/kg) were selected based on evidence that sibutramine induces hypophagia (Grignaschi et al. 1999). mCPP was purchased from Sigma Chemical Co. (Japan). Sibutramine was purchased from Wako Pure Chemical Industries Ltd (Japan). The drugs were dissolved in 0.2 ml 0.9% saline.

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

### Real-time quantitative reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was extracted from the mouse hypothalamus using the RNeasy Midi kit (Qiagen, Germany) according to the manufacturer’s instructions. cDNA synthesis was performed using a Super Script III First-Strand Synthesis System for the RT–PCR kit (Invitrogen, USA) using 1 μg total RNA. The cDNA synthesized from the total RNA was evaluated in a real-time PCR quantitative system (Light Cycler Quick System 350S; Roche Diagnostics, Germany), as described previously (Nonogaki et al. 2006b, 2007a). The primers used were as follows. For mouse 5-HT\(_{1B}\) receptor, sense: 5'-TGC CTG CTG GTT TCA CAT-3'; 5'-ATA GAT GTG TGG AGC TGG TG-3'; antisense: 5'-GGG CAC TTA AAG CTT ATC ATC-3'; 5-HT\(_{2A}\) receptor, sense: 5'-CTG AGG GAC GAA AGC AAA G-3'; antisense: 5'-CAC ATA GCC AAT CCA AAC AAA C-3'; POMC, sense: 5'-ATA GAT GTG TGG AGC TGG TG-3'; antisense: 5'-GGC TGT TCA TCT CCG TGT-3'; for mouse cocaine- and amphetamine-regulated transcript (CART), sense: 5'-CTG GAC ATC TAC TCT GCC GTG G-3'; antisense: 5'-GGT CCT CGG
GGA CAG TCA CAC AGC-3′; for mouse NPY, sense: 5′-GCT TGA AGA CCC TTC CAT TGG TG-3′; antisense: 5′-GCC CGA GTC CAC CCT AGT GG-3′; for mouse CRH, sense: 5′-CCG GCC AGA GCA GTT AGC-3′; antisense: 5′-CAA CAT TTC ATT TCC CGA TAA TCT C-3′; 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′-GGT GAC TGT TCA TCT CCG TTG-3′; for mouse orexin, sense: 5′-CTC CTT CAG GCC AAC GGT A-3′; antisense: 5′-GTG GTA GTT ACG GTC GGA CA-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 the fold change of the mean value of the control group, which received saline.

**siRNA**

The POMC siRNA oligonucleotide was designed as follows: POMC siRNA (targeting for nucleotides 4–28) has the sequence, antisense: 5′-CUGAGCGAC-UGUAGCAGAAUCUCGG-3′; sense: 5′-CCGAGAU-UCUGCUACAGUCGCUCAG-3′. The orexin (prepro-orexin) siRNA oligonucleotide was designed as follows: orexin siRNA (targeting for nucleotides 342–366) has the sequence, antisense: 5′-UAUACGGGU-GGUAGUUAACGGUCGA-3′; sense: 5′-UCGGACCG-GUAACUACCACCGCUUUA-3′. As control siRNA, Stealth™ RNAi negative control medium GC duplex (Invitrogen, Tokyo) was used. The siRNA particles were resuspended at 0.04 mM in 50 μl RNase-free saline water mixed with 1 μl lipofectamine (Invitrogen, Tokyo), and 10 μl resuspended POMC, orexin, or POMC+orexin siRNA were injected into the third cerebral ventricle of 5-wk-old male C57BL6J mice over 1 min by stereotaxic surgery. Stereotaxic surgery to the mouse was performed under anaesthesia with pentobarbital. For the intracerebroventricular (icv) injection, a microsyringe was placed on a stereotaxic frame into the following coordinates from bregma: anteroposterior −0.5 mm, lateral 0 mm, vertical −2.5 mm, as described previously (Nonogaki et al. 2009b).

**Statistical methods**

Data are presented as mean values ± S.E.M. (n = 6–12). 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 <0.05 was considered statistically significant.

**Results**

**Effects of restricted feeding on hypothalamic gene expression in wild-type and Aβ mice**

Expression of hypothalamic POMC, CART, CRH, and 5-HT1b receptors was significantly decreased in food-restricted wild-type mice compared to food-unrestricted wild-type mice (28%, 22%, 54%, 61%, respectively), whereas hypothalamic orexin expression was increased (4.2-fold) (Fig. 1a). The expression of hypothalamic POMC, CART, and CRH was also significantly decreased in food-restricted Aβ mice compared to food-unrestricted Aβ mice (27%, 44%, 54%, respectively), whereas hypothalamic orexin expression was markedly decreased (16%) (Fig. 1b). In both wild-type and Aβ mice, there were no significant differences in hypothalamic NPY, 5-HT2c receptor, and NUCB2 expression between food-restricted and food-unrestricted animals (Fig. 1a, b). These findings...
demonstrate that the restricted feeding down-regulates expression of hypothalamic POMC, CART, and CRH in both wild-type and Ay mice. In addition, restricted feeding up-regulates expression of hypothalamic orexin in wild-type mice while down-regulating it in Ay mice.

Effects of mCPP on food intake in food-restricted wild-type and Ay mice

Systemic administration of mCPP (3 and 5 mg/kg) significantly suppressed food intake compared to saline controls in food-restricted wild-type mice [saline-treated group: 0.84 ± 0.06 g; mCPP (3 mg/kg)-treated group: 0.63 ± 0.05 g; mCPP (5 mg/kg)-treated group: 0.59 ± 0.05 g, F = 6.54; saline-treated group vs. mCPP (3 mg/kg)-treated group, p < 0.05; saline-treated group vs. mCPP (5 mg/kg)-treated group, p < 0.05], whereas systemic administration of mCPP (3 and 5 mg/kg) had no significant effect on food intake in food-restricted Ay mice (saline-treated group: 1.0 ± 0.02 g; mCPP (3 mg/kg)-treated group: 0.97 ± 0.04 g; mCPP (5 mg/kg)-treated group: 0.92 ± 0.08 g, F = 0.542). These findings demonstrate that mCPP is responsive in food-restricted wild-type mice, leading to feeding suppression, whereas the drug does not elicit a response in food-restricted Ay mice. From these results of altered expression of hypothalamic neuropeptides and the altered anorexic effects of mCPP, hypothalamic orexin activity may be essential for the anorexic effects of mCPP in mice.

Effects of icv injection of POMC, orexin, and POMC + orexin siRNA on food intake and body-weight changes in mice

Icv injection of POMC siRNA oligonucleotides significantly increased daily food intake and body weight compared to icv injection of control siRNA oligonucleotides (food intake, day 1: F = 16.21, day 2: F = 17.78; body-weight changes, day 1: F = 4.18, day 2: F = 3.83) (Fig. 2a, b). Icv injection of orexin siRNA or orexin + POMC oligonucleotides had no effect on daily food intake and body-weight changes (Fig. 2a, b). These findings suggest that functional orexin activity in the central nervous system (CNS) is required for hyperphagia induced by impaired POMC signalling in the CNS.

Effects of mCPP on food intake in mice treated with icv injection of POMC, orexin, and POMC + orexin siRNA

Systemic administration of mCPP (3 and 5 mg/kg) significantly suppressed food intake in mice treated with icv injection of control and orexin siRNA oligonucleotides (1 h: F = 20.08, F = 62.46, respectively; 2 h: F = 43.27, F = 33.57, respectively) (Fig. 2c). In addition, systemic administration of mCPP (3 and 5 mg/kg) significantly suppressed food intake in mice treated with icv injection of POMC siRNA oligonucleotides (1 h: F = 7.75; 2 h: F = 9.06) (Fig. 2c). On the other hand, systemic administration of mCPP (3 and 5 mg/kg) had no effect on food intake in mice treated with icv co-injection of POMC + orexin siRNA oligonucleotides (1 h: F = 0.567; 2 h: F = 0.264) (Fig. 2c). These findings suggest that functional POMC + orexin activity in the CNS are required for anorexia induced by mCPP.

Effects of sibutramine on food intake in mice treated with icv injection of POMC siRNA

Systemic administration of sibutramine (3 and 5 mg/kg) significantly suppressed food intake in mice treated with icv injection of control siRNA oligonucleotides (1 h: F = 11.27; 2 h: F = 10.98) (Fig. 2d), while having no significant effect on food intake in mice treated with icv injection of POMC siRNA oligonucleotides (1 h: F = 0.248; 2 h: F = 0.03). Icv injection of POMC siRNA oligonucleotides significantly suppressed expression of hypothalamic POMC compared to control siRNA (58%) (Fig. 2c). These findings suggest that functional POMC activity in the CNS is required for anorexia induced by sibutramine.

Discussion

The present study demonstrates that functional hypothalamic POMC activity is not always essential for satiety signalling of mCPP. In addition, the present study indicates that restricted feeding and agouti peptide affect satiety signalling of mCPP in association with altered expression of hypothalamic orexin in mice. Moreover, the present study using POMC siRNA oligonucleotides and/or orexin siRNA oligonucleotides indicates that hypothalamic orexin activity has a crucial role in satiety signalling of POMC and mCPP in mice.

Orexin, an orexigenic peptide, is localized mainly in the lateral hypothalamic area and the posterior hypothalamus (Shimokawa et al., 2003). The expression of orexin in the lateral hypothalamus is increased in POMC-deficient mice (López, 2007). In an in-vitro study, a reduction of electrical activity of POMC neurons in the arcuate nucleus of the hypothalamus contributes to the appetite enhancing action of orexin (Ma, 2007). The results of the present study demonstrate

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Fig. 2. Effects of intracerebroventricular (icv) injection of POMC siRNA oligonucleotides, orexin siRNA oligonucleotides, POMC + orexin siRNA oligonucleotides on (a) daily food intake and (b) body-weight changes in mice. (c) Effects of systemic administration of mCPP (3 and 5 mg/kg) or saline on food intake in mice pretreated with icv injection of control siRNA oligonucleotides, POMC siRNA oligonucleotides, orexin siRNA oligonucleotides, POMC + orexin siRNA oligonucleotides. (d) Effects of systemic administration of sibutramine (3 and 5 mg/kg) or saline on food intake in mice pretreated with icv injection of control siRNA oligonucleotides and POMC siRNA oligonucleotides. (e) Effects of icv injection of control siRNA oligonucleotides and POMC siRNA oligonucleotides on hypothalamic POMC mRNA levels. The intake of chow pellets was measured for the next 1 h and then 2 h after the onset of the dark cycle, as described in the Materials and methods section. C, icv injection of control siRNA oligonucleotides; P, icv injection of POMC siRNA oligonucleotides; O, icv injection of orexin siRNA oligonucleotides; PO, icv injection of POMC + orexin siRNA oligonucleotides; Sib, sibutramine. Data are presented as the mean values ± S.E.M. (n = 6–12 for each group of animals).
that in vivo, icv co-injection of orexin siRNA oligonucleotides suppress feeding signalling induced by icv injection of POMC siRNA oligonucleotides in mice, suggesting that central orexin contributes to hyperphagia induced by impaired central POMC signalling. Thus, there appears to be a neural network between POMC and orexin in the central regulation of feeding. POMC probably down-regulates expression of orexin and its feeding effect.

Despite the interaction between 5-HT systems and POMC via 5-HT₁B/₁C receptors, the results of the present study demonstrate that mice with impaired central POMC activity were responsive to the anorexic effects of mCPP. mCPP might stimulate hypothalamic POMC neurons which were not completely impaired, to release α-MSH, leading to anorexia. Orexin directly interacts with POMC neurons in the arcuate nucleus and decreases [Ca²⁺], POMC neurons in vitro (Muroya, 2004), suggesting that orexin neurons down-regulate POMC neuronal activity. Interestingly, the impaired central orexin and POMC activities attenuated the anorexic effects of mCPP. These findings suggest that central orexin neurons have a crucial role in central POMC-mediated satiety signalling of 5-HT₁B/₁C receptors.

Sibutramine, a SNRI, is used as a treatment for obesity. The initial studies using non-selective receptor antagonists suggested that α₁ adrenoceptors, β₁ adrenoceptors, and 5-HT₂A/₂C receptors contribute to the hypophagic effect of sibutramine (Grignaschi et al. 1999; Jackson et al. 1997). The present study demonstrates that hypothalamic POMC contributes to the anorexic effects of sibutramine, suggesting that sibutramine and mCPP induce the anorexic effects via different central pathways.

Restricted feeding in rats reportedly decreases expression of the orexigenic peptides hypothalamic POMC and CART, whereas it increases expression of the orexigenic peptides hypothalamic NPY and agouti-related peptide (AGRP) (de Rijke et al. 2005; Harrold et al. 1999; Remmers et al. 2008). The results of the present study demonstrate that moderately restricted feeding down-regulates expression of hypothalamic POMC, CART and CRH without affecting the expression of hypothalamic NPY in both wild-type and A<sup>Y</sup> mice.

Moreover, restricted feeding reportedly has no effect on expression of hypothalamic orexin in Sprague–Dawley rats and C57BL/6J mice (Cai et al. 1999; de Rijke et al. 2005; Lutter et al. 2008) but increases it in ob/ob and db/db mice (Yamamoto et al. 2000). However, the results of the present study, demonstrate that moderately restricted feeding down-regulated expression of hypothalamic orexin in A<sup>Y</sup> mice while up-regulating it in wild-type mice. Thus, agouti peptide and leptin signalling have an opposite effect on the expression of hypothalamic orexin under the food-restricted condition. The decreases in hypothalamic POMC and CART expression were found to be the same in the food-restricted animals, although the differences in the effects of restricted feeding on expression of hypothalamic NPY and orexin appear to be due to differences in species, the duration of food restriction, or age.

In summary, the results of the present study suggest that restricted feeding up-regulates expression of hypothalamic orexin, which in turn is down-regulated by agouti peptide. Functional hypothalamic orexin activity is required in addition to POMC activity for satiety signalling of 5-HT in mice. To the best of our knowledge this is the first report of opposing effects of restricted feeding and agouti peptide on hypothalamic orexin expression, and a potential regulator of 5-HT satiety signalling other than POMC activity.

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Statement of Interest
None.

References


