Milnacipran, a serotonin and norepinephrine reuptake inhibitor, induces appetite-suppressing effects without inducing hypothalamic stress responses in mice

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Milnacipran, a selective serotonin (5-HT) and norepinephrine (NE) reuptake inhibitor, increases extracellular 5-HT and NA levels equally in the central nervous system, was originally prescribed to treat depression (1, 18, 24). Milnacipran has no effect on dopamine reuptake and does not interact with any known neurotransmitter receptors, especially noradrenergic, muscarinic, and histaminergic receptors, unlike tricyclic antidepressants (1). Milnacipran is also recommended as a treatment for binge eating in bulimia nervosa (6). The effects of milnacipran on appetite, obesity, plasma corticosterone, and glucose levels, and the expression of hypothalamic neuropeptide genes that are involved in regulating feeding and energy homeostasis, however, are unknown.

To determine whether milnacipran suppresses appetite and increases blood glucose and plasma corticosterone levels, we investigated the effects of milnacipran on food intake in relation to 5-HT2C and 5-HT1B receptors and on blood glucose and plasma corticosterone levels compared with fenfluramine. We also examined the effects of milnacipran on the gene expression of hypothalamic neuropeptides that are involved in regulating feeding and on food intake and body weight in Ay mice. Ay mice display hyperphagia due to ectopic expression of agouti protein, which interferes with the binding of endogenous ligands to MC-4 and MC-3 receptors (5, 8, 17). Hyperphagic Ay mice are responsive to mCPP and fenfluramine (23).

MATERIALS AND METHODS

Animals and drug treatment. Four-week-old male C57BL/6J mice, Ay mice, and wild-type mice were purchased from JapanCLEA. Mice were individually housed in cages with free access to water and chow pellets in a light- (lights on: 0800, lights off: 2000) and temperature (20–22°C)–controlled environment. Animals were acclimatized to the laboratory environment for 1 wk before the experiment. Drugs were administered between 1000 and 1200, as described previously (22, 23). Milnacipran was a kind gift from Asahi Kasei Pharma, (Tokyo, Japan). SB242084 dihydrochloride, SB22489 hydrochloride, and CP94253 hydrochloride were purchased from Sigma Chemical (Tokyo, Japan). Milnacipran and CP94253 hydrochloride were dissolved in saline. SB242084 was dissolved in distilled water and suspended in saline. SB22489 was suspended in saline.

In experiments 1 and 2, separate groups of 5-wk-old C57BL6J mice were deprived of food for 23 h. The following morning, the animals were injected intraperitoneally with saline or milnacipran (3–60 mg/kg) 30 min before food presentation. Intake of chow pellets was measured every hour for the next 1 h (experiment 1) or 6 h (experiment 2).

In experiment 3, 5-wk-old C57BL6J mice were deprived of food for 23 h. The following morning, animals were injected intraperitoneally with saline, SB242084 (2 mg/kg), or SB22489 (5 mg/kg).

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BRAIN SEROTONIN (5-HYDROXYTryptamine; 5-HT) systems contribute to regulate mood, feeding, autonomic outflow, and the hypothalamic-pituitary-adrenal (HPA) axis through complex neural mechanisms. 5-HT drugs, such as m-chlorophenylpiperazine (mCPP), a 5-HT2C/1B receptor agonist, and fenfluramine, which inhibits 5-HT reuptake and stimulates 5-HT release, suppress feeding via 5-HT2C receptors and/or 5-HT1B receptors and the central melanocortin (MC) pathway (12, 16, 27, 28, 29). These drugs are also likely to stimulate the HPA axis and the hypothalamic-sympathetic adrenal medullary axis (3, 4, 9, 14, 19, 20, 26).

Milnacipran, a selective 5-HT and norepinephrine (NE) reuptake inhibitor that increases extracellular 5-HT and NE levels equally in the central nervous system, was originally prescribed to treat depression (1, 18, 24). Milnacipran has no effect on dopamine reuptake and does not interact with any known neurotransmitter receptors, especially noradrenergic, muscarinic, and histaminergic receptors, unlike tricyclic antidepressants (1). Milnacipran is also recommended as a treatment for binge eating in bulimia nervosa (6). The effects of milnacipran on appetite, obesity, plasma corticosterone, and glucose levels, and the expression of hypothalamic neuropeptide genes that are involved in regulating feeding and energy homeostasis, however, are unknown.

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Thirty minutes later, the animals were injected intraperitoneally with saline or milnacipran (30 mg/kg) or CP94253 (5 mg/kg), a selective 5-HT1B receptor agonist or mCPP (5 mg/kg), a 5-HT2C/1B receptor agonist. Thirty minutes later, they were given chow pellets, and pellet intake was measured for the next 1 h.

In experiment 4, 5-wk-old male A\textsuperscript{v} mice and wild-type littermates were deprived of food for 23 h. The following morning, the animals were intraperitoneally injected with saline or milnacipran (30 mg/kg) 30 min before food presentation. Intake of chow pellets was measured for the next 1 h.

In experiment 5, 5-wk-old male A\textsuperscript{v} mice and wild-type littermates were intraperitoneally injected with saline or milnacipran (30 mg/kg) 30 min before the onset of the dark cycle. Intake of chow pellets was measured for the next hour after onset of the dark cycle.

In experiment 6, 8-wk-old male A\textsuperscript{v} mice and wild-type littermates were intraperitoneally injected with saline or milnacipran (30 mg/kg) twice daily (at 1000 and 1800) for 3 days. Daily food intake and body weight were measured.

In experiment 7, 5-wk-old male A\textsuperscript{v} mice and wild-type littermates, which were provided 3.5 g of chow pellets daily for 5 days before the experiment, were intraperitoneally injected with saline or milnacipran (30 mg/kg) or fenfluramine (3 mg/kg). Chow pellets were provided 30 min later. Intake of chow pellets was measured for the next hour. The experiment was performed between 1000 and 1200.

In experiment 8, 5-wk-old C57BL6J mice were intraperitoneally injected with saline or milnacipran (30 mg/kg) in the light and dark cycle. In the dark cycle, drugs were administered between 2000 and 2030. Animals were not fed chow pellets. After 30, 60, or 120 min, the separate groups of animals were decapitated, and blood was collected for measurement of glucose and corticosterone levels in the light cycle. In a separate group of animals, 60 and 120 min later, the hypothalamus was removed for RNA extraction in the light cycle. Moreover, after 60 or 120 min, the separate groups of animals were decapitated and blood was collected for measurement of corticosterone levels in the dark cycle. Whole blood was mixed with EDTA-2Na (2 mg/ml) to determine plasma corticosterone levels.

Finally, 5-wk-old C57BL6J mice were intraperitoneally injected with saline or fenfluramine (3 mg/kg). They were not fed chow pellets. Sixty minutes later, the animals were decapitated, and the hypothalamus was removed for RNA extraction. Blood was collected for the measurement of glucose and corticosterone levels. Whole blood was mixed with EDTA-2Na (2 mg/ml) to determine plasma corticosterone levels.

The dose of fenfluramine (3 mg/kg) was based on the evidence that fenfluramine-induced hypophagia is attenuated by a genetic blockade of 5-HT2C receptors (28). The dose of SB242084 (2 mg/kg) eliminates 5-HT2C receptor functions in vivo as described previously (13, 22). The dose of SB224289 dihydrochloride (5 mg/kg) eliminates 5-HT1B receptor functions in vivo (15, 16, 22).

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

Real-time quantitative RT-PCR. Total RNA was isolated from mouse hypothalamic tissue using the RNeasy Midi kit (Qiagen, Hilden, Germany), according to the manufacturer's directions, and cDNA synthesis was performed using a Super Script III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Rockville, MD) using 1 µg total RNA. cDNA synthesized from total RNA was evaluated in a real-time PCR quantitative system (Light Cycler Quick System 350S; Roche Diagnostics, Mannheim, Germany). The primers used were as follows. For mouse proopiomelanocortin (POMC), sense, 5'-ATA GAT GTG TGG AGC TGG TG-3'; antisense, 5'-GCC TGT TCA TCT CCG TTG-3'; for mouse cocaine- and amphetamine-regulated transcript (CART), sense, 5'-CTG GACATC TAC TCT GCC GTG G-3'; antisense, 5'-GTT CCT CGG GGA CAG TCA CAC AGC-3'; for mouse neuropeptide Y (NPY), sense, 5'-GCT TGA AGA CCC TTC CAT TGG TG-3'; antisense, 5'-GCC GGA GTC CAG CCT AGT GG-3'; for mouse ghrelin, sense, 5'-GAA GAG AAT CCA AGA AGC CA-3'; antisense, 5'-GCT TGA TGC CAA CAT CGA A-3'; for mouse corticotropin releasing hormone (CRH), sense, 5'-CCG GCC AGA GCA GTC AGC-3'; antisense, 5'-CAA CAT TTC ATT TCC CGA TAA TCT C-3'; for mouse suppressor of cytokine signaling-3 (SOCS-3), sense, 5'-GCG GGC GCC ATT CTT ATC C-3'; antisense, 5'-TCC CCG ACT GGG TCT TGA C-3'; and for mouse \( \beta \)-actin, sense, 5'-TTG TAA CCA ACT GGC ATA TGG-3'; antisense, 5'-GAT CTT GAT CTT GAT GGT GCT AGG-3'. The relative amount of mRNA was calculated with \( \beta \)-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.

Blood chemistries. Blood glucose levels were measured by glucose strips (Freestyle blood glucose monitoring system; Kasei, Tokyo, Japan). Plasma active ghrelin levels were measured by ELISA (Active ghrelin ELISA kit, Mitsubishi Kagaku Iatron, Tokyo, Japan). Plasma corticosterone levels were measured by radioimmunoassay (Linco, St. Louis, MO).

Statistical methods. Data are presented as the means \( \pm \) SE. Statistical significance of differences between two groups was determined using Student's \( t \)-test. Comparisons among more than two groups were done by analysis of variance using Bonferroni's correction for multiple comparisons. A \( P \) value of less than 0.05 was considered statistically significant.

**RESULTS**

Dose-response effects and time course of the effects of milnacipran on food intake. To determine the dose-response effects of milnacipran on food intake, we examined food intake after the administration of milnacipran (3–60 mg/kg) or saline in C57BL6J mice. Administration of milnacipran (20–60 mg/kg) significantly reduced food intake in C57BL6J mice (to \(~67\%-70\%\) that of saline controls). Lower doses of milnacipran (3–10 mg/kg) had no effect on food intake during a 1-h feeding period after a 23-h fast in C57BL6J mice (Fig. 1A). To further determine the time course of the effects of milnacipran on food intake, we examined food intake after administration of milnacipran (30 mg/kg) or saline in C57BL6J mice. The doses of milnacipran to induce anorexic effects were within the range to induce antidepressive effects in rodents (18, 25). In addition, the anorexic effects of milnacipran (30 mg/kg) were sustained for 5 h (Fig. 1B).

To determine the effects of selective 5-HT2C and 5-HT1B receptor antagonists on the anorexic effects of milnacipran, we intraperitoneally injected SB242084 (2 mg/kg) or SB224289 (5 mg/kg) 30 min before administering the milnacipran (30 mg/kg). Neither SB242084 nor SB224289 reversed the feeding suppression induced by milnacipran (Fig. 1C). SB224289 (5 mg/kg) reversed the feeding suppression induced by CP94253 (5 mg/kg; Fig. 1D). SB242084 (2 mg/kg) partially but significantly reversed the feeding suppression induced by mCPP (5 mg/kg; Fig. 1E). SB242084 and SB224289 alone had no effects on food intake as described previously (22).

Effects of milnacipran on food intake and body weight in A\textsuperscript{v} mice. To determine the effects of milnacipran on food intake on A\textsuperscript{v} mice, we administered milnacipran to nonobese (5-wk-old) A\textsuperscript{v} mice and wild-type mice. Milnacipran (30 mg/kg) significantly reduced food intake in wild-type mice and A\textsuperscript{v} mice (to \(~68\%\) and 48\% that of controls, respectively).
respectively) during the first hour of the feeding period after a 23-h fast (Fig. 2A).

To further confirm these results, we determined the effects of milnacipran on food intake in Aβ mice and wild-type mice for the first hour after onset of the dark cycle. Milnacipran significantly reduced food intake in wild-type mice (to ~70% and 44% that of controls, respectively) during the first hour after onset of the dark cycle (Fig. 2B). These results suggest that milnacipran suppresses food intake in Aβ mice.

Moreover, we determined the chronic effects of milnacipran on food intake and body weight in obese (12-wk-old) Aβ mice and wild-type mice. Mice received milnacipran (30 mg/kg) or saline twice daily for three consecutive days. Aβ mice had elevated baseline food intake and body weight relative to levels in wild-type mice (food intake: WT 3.5 ± 0.2 g, Aβ 5.1 ± 0.1 g; P < 0.05; body weight: WT 29.4 ± 0.5 g, Aβ 37.5 ± 0.5 g; P < 0.05, n = 14–18). Milnacipran administration reduced food intake and body weight in both Aβ mice and wild-type mice (Fig. 3, A–D).
**Effects of restricted feeding on the responsiveness to milnacipran or fenfluramine in 5-wk-old A\(^{\theta}\) mice.** We previously reported that restricted feeding (3.5 g/days for 5 days) decreased hypothalamic 5-HT2C and 5-HT1B receptor gene expression and attenuated the responsiveness of mCPP, a 5-HT2C/1B receptor agonist in food-restricted A\(^{\theta}\) mice compared with wild-type mice (23). To determine whether 5-HT2C and 5-HT1B receptors contribute to the appetite-suppressing effects of milnacipran, we examined the effects of restricted feeding (3.5 g/days for 5 days) on the responsiveness to milnacipran or fenfluramine in 5-wk-old A\(^{\theta}\) mice and wild-type mice. Both A\(^{\theta}\) mice and wild-type mice consumed 3.5 g of food during the light cycle for 5 days and were fasted during the dark cycle. After a 15-h fast, fenfluramine (3 mg/kg) had no effect on food intake in food-restricted A\(^{\theta}\) mice, whereas it significantly suppressed food intake in food-restricted wild-type mice (Fig. 4A). In contrast, milnacipran had no effects on food-restricted A\(^{\theta}\) mice and wild-type mice (Fig. 4B).

**Effects of milnacipran on the expression of hypothalamic genes involved in the regulation of feeding and energy homeostasis.** To determine the central mechanisms of the anorectic effects induced by milnacipran, we examined the expression of hypothalamic orexigenic peptides and anorexigenic peptides, which have important roles in the regulation of feeding (10). Milnacipran significantly increased hypothalamic POMC and CART mRNA levels compared with saline controls (1.6-fold and 1.3-fold compared with saline controls, respectively) at 1 h and had no significant effects on hypothalamic ghrelin, NPY, CRH, and SOCS-3 mRNA levels (Fig. 5). Milnacipran had no significant effects on hypothalamic POMC, CART, ghrelin, NPY, CRH, and SOCS-3 mRNA levels at 2 h (data not shown).

**Effects of milnacipran and fenfluramine on plasma corticosterone and blood glucose levels.** Milnacipran (30 mg/kg) had no effect on blood glucose and plasma corticosterone levels at 30 and 60 min after administration, while at 120 min, it significantly reduced both compared with saline controls in the light cycle (Fig. 6, A and B). In the dark cycle, milnacipran significantly reduced plasma corticosterone levels at 60 min and 120 min (Fig. 6C). Fenfluramine (3 mg/kg) significantly increased plasma corticosterone and blood glucose levels and increased hypothalamic CRH mRNA levels compared with saline controls at 1 h (Fig. 7, A–C).

**DISCUSSION**

The previous and present studies demonstrate that fenfluramine and milnacipran induce appetite-suppressing effects and increase hypothalamic POMC and CART gene expression in mice (21). The effects of these drugs on plasma corticosterone and blood glucose levels, however, differed. Activation of brain 5-HT systems increases plasma corticosterone levels associated with activation of hypothalamic CRH neurons, which induce anorectic effects (4, 9, 14). The results of the present study demonstrate that milnacipran did not increase plasma corticosterone levels and hypothalamic CRH gene expression, whereas fenfluramine increased both of them. Because milnacipran had no effect on hypothalamic CRH gene expression, it is reasonable that milnacipran did not increase plasma corticosterone levels. It might also be due to the basic actions of milnacipran, which inhibits 5-HT and NE reuptake but does not directly stimulate 5-HT receptors and 5-HT release.

The present results also demonstrate that milnacipran does not increase blood glucose levels, whereas fenfluramine increases blood glucose levels. At least three different neural pathways, such as increased epinephrine, glucagon, and direct innervation of the liver, are involved in acute hyperglycemia mediated by the CNS (19, 20). Although the relative contribution of these pathways to acute hepatic glucose production can be altered by changes in central neurotransmission, epinephrine secreted from the adrenal medulla has an essential role in the CNS-mediated acute hyperglycemia in rodents (19, 20). Fenfluramine or mCPP-induced hyperglycemia is mediated by increased epinephrine secretion from the adrenal medulla (3, 4, 19, 20, 26). Milnacipran, however, does not seem to induce sympathetic nervous system-mediated acute hyperglycemia. Rather, milnacipran slightly reduced blood glucose levels at 120 min after administration.

The central MC pathway is suggested to mediate satiety signaling downstream of 5-HT2C receptors (12). A\(^{\theta}\) mice have dominant alleles at the agouti locus (A), which produces ectopic expression of the agouti peptide, an antagonist of the hypothalamic MC4 receptors and MC3 receptors (8, 17) and therefore display hyperphagia and obesity. A\(^{\theta}\) mice are reportedly resistant to the anorexie effects of fenfluramine (12) and consumed more food and gained more weight compared with age-matched wild-type mice. The results of the present study demonstrate that A\(^{\theta}\) mice are responsive to milnacipran-induced feeding suppression and that chronic treatment with...
Milnacipran suppresses hyperphagia and reduces body weight in obese Ay mice. These findings suggest that milnacipran induces appetite-suppressing effects through different neuronal mechanisms than fenfluramine.

Fig. 3. Effects of chronic administered milnacipran on food intake (A and B) and body weight (C and D) in wild-type mice and obese (8-wk-old) Ay mice. Each column and bar represent the mean ± SE of 7–9 mice. Basal body weight: WT saline controls (open bars), 29.0 ± 0.7 g; WT milnacipran treatment group (solid bars), 29.8 ± 0.3 g; Ay saline controls (open bars), 37.7 ± 0.5 g; Ay milnacipran treatment group (solid bars), 37.6 ± 0.5 g. *P < 0.05.

Fig. 4. Effects of restricted feeding on effects of milnacipran (A) and fenfluramine (B) on food intake in 5-wk-old Ay mice and WT mice. Mice were intraperitoneally injected with milnacipran (30 mg/kg) or fenfluramine (3 mg/kg) or saline. Basal body weight: WT saline controls (open bars), 20.2 ± 0.2 g; WT milnacipran (30 mg/kg) treatment group (solid bars), 21.3 ± 0.2 g; Ay saline controls (open bars), 21.8 ± 0.3 g; Ay fenfluramine (3 mg/kg) treatment group (solid bars), 22.4 ± 0.2 g. Data are presented as the mean values ± SE of 6 mice. *P < 0.05.

Fig. 5. Effects of milnacipran on hypothalamic ghrelin, neuropeptide Y (NPY), proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), corticotropin-releasing hormone (CRH), and suppressor of cytokine signaling-3 (SOCS-3) mRNA levels in C57BL6J mice. Mice were intraperitoneally injected with milnacipran or saline. Open bar, saline; solid bars, milnacipran (30 mg/kg). Each column and bar represent the mean ± SE of 5 or 6 mice. *P < 0.05.
Milnacipran interacts with POMC neurons in the hypothalamus and could stimulate POMC neurons to release enough alpha-melanocyte-stimulating hormone to overcome agouti blockade of MC receptors, because A^2B mice are sensitive to MC agonist-induced feeding suppression (8). In addition, CART might contribute to the milnacipran-induced feeding suppression in A^2B mice, possibly, because CART and MC neurons do not share a downstream pathway (2, 7). The present study demonstrates that 5-HT2C and 5-HT1B receptors are unlikely to mediate the appetite-suppressing effects of milnacipran. Inhibition of NE reuptake reportedly suppresses food intake and obesity in rats (11). Hypothalamic NE systems might therefore contribute to the appetite-suppressing effects and increased hypothalamic POMC and CART gene expres-
sion of milnacipran without stimulating the HPA and sympathetic-adrenal medullary axis.

Other investigators previously reported that A\(\text{v}\) mice are resistant to the anorexic effects of fenfluramine (12). Our previous and present studies, however, demonstrated that hyperphagic A\(\text{v}\) mice are responsive to mCPP and fenfluramine-induced appetite suppression, whereas food-restricted A\(\text{v}\) mice are resistant to them (23). We previously reported that hypothalamic 5-HT2C and 5-HT1B receptor gene expression was increased in nonobese hyperphagic A\(\text{v}\) mice, whereas it was decreased by food restriction (23). Accordingly, the discrepancy between our results and previous results by other investigators might be due to the differences in feeding states of A\(\text{v}\) mice.

In addition, our present study demonstrate that hyperphagic A\(\text{v}\) mice are responsive to milnacipran-induced appetite suppression, whereas food restricted A\(\text{v}\) mice are resistant to them (23). Moreover, food restricted wild-type mice are resistant to milnacipran-induced appetite suppression, whereas they are responsive to fenfluramine-induced appetite suppression.

These results support that 5-HT2C and 5-HT1B receptors are unlikely to contribute to the appetite-suppressing effects of milnacipran. The appetite-suppressing effects of milnacipran are also different than those of fluvoxamine, a selective 5-HT reuptake inhibitor, because fluvoxamine alone had no effects on feeding, and fluvoxamine and 5-HT2C receptor inactivation exert appetite-suppressing effects via 5-HT1B receptors (22).

In summary, these results suggest that inhibition of 5-HT and NA reuptake induces appetite-suppressing effects independent of 5-HT2C and 5-HT1B receptors and increases hypothalamic POMC and CART gene expression without stimulating 5-HT2C and 5-HT1B receptors (23). Accordingly, the discrepancy between our results and previous results by other investigators might be due to the differences in feeding states of A\(\text{v}\) mice.

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