markedly decreasing peripheral adiposity, increasing energy expenditure, and improving systemic insulin sensitivity [34]. Although there were no differences in peripheral adiposity and energy expenditure between AdNEU3 mice and AdLacZ mice in this study, the AdNEU3 mice had a phenotype of hepatic steatosis and improved systemic insulin sensitivity with enhanced PPARγ expression in liver. Thus, hepatic PPARγ plays important roles not only in the development of liver steatosis but also systemic insulin sensitivity. Increased PPARγ expression in the livers of AdNEU3 mice might be one of the mechanisms underlying increased systemic insulin sensitivity in vivo. This is the first report, to our knowledge, of hepatic *NEU3* expression increasing hepatic PPARγ expression. Further investigation is needed to elucidate the molecular mechanism by which hepatic NEU3 expression increases hepatic PPARγ expression and triglyceride accumulation in the liver.

Fetuin is a natural inhibitor of the insulin-stimulated insulin receptor tyrosine kinase [35,36]. Fetuin KO mice demonstrate increased basal and insulin-stimulated phosphorylation of insulin receptor and the downstream signaling molecules mitogen-activated protein kinase and Akt in liver and skeletal muscle [37]. Glucose and insulin tolerance tests in fetuin KO mice indicate significantly enhanced glucose clearance and insulin sensitivity. Thus, fetuin is a natural regulator of the insulin sensitivity in liver. Reduced expression of fetuin in the AdNEU3 mice fed standard diets and KKAy mice might contribute to improved insulin sensitivity in these mice.

We previously demonstrated *NEU3* transgenic mice to have an insulin-resistant, diabetic phenotype [16]. In contrast, this study demonstrates that *NEU3* overexpression in the liver improves insulin sensitivity and glucose tolerance in C57BL/6 mice fed standard diet and in 2 types of insulin-resistant mice, C57BL/6 mice on a high-fat diet and KKAy mice. Recent accumulating evidence provides evidence that hepatic expression of PPARγ regulates systemic insulin sensitivity [30,32,34]. Increased hepatic expression of PPARγ in the AdNEU3 mice improves insulin sensitivity and glucose tolerance. In contrast, decreased PPARγ expression in the liver might contribute to the insulin resistance and glucose intolerance of the *NEU3* transgenic mice.

The *NEU3* transgene was expressed in a wide range of tissues, but most prominently in muscles, pancreas, and heart in the transgenic mice. Chronically elevated *NEU3* expression in muscle was speculated to contribute to in vivo insulin resistance because intracellular insulin signaling was reduced in muscles of transgenic mice [16]. In contrast, this study demonstrates that acute expression of *NEU3* in the liver improves insulin sensitivity. These discrepancies might be partially explained by the time (acute or chronic) and tissue (liver or skeletal muscle) differences in *NEU3* overexpression. Further studies are needed to investigate the difference in molecular mechanism to regulate systemic insulin sensitivity between the AdNEU3 mice and the *NEU3* transgenic mice.

In conclusion, *NEU3* overexpression in the liver improves insulin sensitivity and glucose tolerance, possibly through the expression of PPARγ and fetuin, and the modification of the ganglioside composition in healthy mice and 2 types of insulin-resistant mice, mice on a high-fat diet and KKAy mice. Our findings also provide further evidence that *NEU3* is an important regulator of insulin sensitivity and glucose tolerance, making it a potential therapeutic target in type 2 diabetes mellitus.

Acknowledgments

This study was supported by Grants-in-Aid for Basic Research Program CREST (Core Research for Evolutional Science and Technology) type from the Japan Science and Technology Agency and Grants-in-Aid for Scientific Research (70216399, 16390259, 16659236) to S. Suzuki from the Ministry of Education, Science, Sports and Culture of Japan.

We gratefully acknowledge the expert assistance of Chitose Suzuki, Takako Suganami, and Setsuko Moriya.

References

[1] Hakomori S, Yamamura S, Handa AK. Signal transduction through glyco (sphingo) lipids. Introduction and recent studies on glyco (sphingo) lipid-enriched microdomains. Ann N Y Acad Sci 1998;845:1-10.

[2] Rampersaud AA, Oblinger JL, Ponnappan RK, Burry RW, Yates AJ. Gangliosides and growth factor receptor regulation. Biochem Soc Trans 1999;27:415-22.

[3] Hakomori S. Traveling for the glycosphingolipid path. Glycoconj J 2000;17:627-47.

[4] Ikonen E, Vainio S. Lipid microdomains and insulin resistance: is there a connection? Sci STKE 2005;268:e3.

[5] Hakomori S. Glycosylation defining cancer malignancy: new wine in an old bottle. Proc Natl Acad Sci U S A 2002;99:225-32.

[6] Allende ML, Proia RL. Lubricating cell signaling pathways with gangliosides. Curr Opin Struct Biol 2002;12:587-92.

[7] Miljan EA, Bremer EG. Regulation of growth factor receptors by gangliosides. Sci STKE 2002;160:RE15.

[8] Nojiri H, Stroud M, Hakomori S. A specific type of ganglioside as a modulator of insulin-dependent cell growth and insulin receptor tyrosine kinase activity. Possible association of ganglioside-induced inhibition of insulin receptor function and monocytic differentiation induction in HL-60 cells. J Biol Chem 1991;266:4531-7.

[9] Tagami S, Inokuchi Ji J, Kabayama K, Yoshimura H, Kitamura F, Uemura S, et al. Ganglioside GM3 participates in the pathological conditions of insulin resistance. J Biol Chem 2002;277:3085-92.

[10] Tsuruoka T, Tsuji T, Nojiri H, Holmes EH, Hakomori S. Selection of a mutant cell line based on differential expression of glycosphingolipid, utilizing anti-lactosylceramide antibody and complement. J Biol Chem 1993;268:2211-6.

[11] Yamashita T, Hashiramoto A, Haluzik M, Mizukami H, Beck S, Norton A, et al. Enhanced insulin sensitivity in mice lacking ganglioside GM3. Proc Natl Acad Sci U S A 2003;100:3445-9.

[12] Kabayama K, Sato T, Kitamura F, Uemura S, Kang BW, Igarashi Y, et al. TNFalpha-induced insulin resistance in adipocytes as a membrane microdomain disorder: involvement of ganglioside GM3. Glycobiology 2005;15:21-9.

[13] Wada T, Yoshikawa Y, Tokuyama S, Kuwabara M, Akita H, Miyagi T. Cloning, expression, and chromosomal mapping of a human ganglioside sialidase. Biochem Biophys Res Commun 1999;261:21-7.
[14] Miyagi T, Wada T, Iwamatsu A, Hata K, Yoshikawa Y, Tokuyama S, et al. Molecular cloning and characterization of a plasma membrane–associated sialidase specific for gangliosides. J Biol Chem 1999;274:5004-11.
[15] Saltiel AR, Pessin JE. Insulin signaling in microdomains of the plasma membrane. Traffic 2003;4:711-6.
[16] Sasaki A, Hata K, Suzuki S, Sawada M, Wada T, Yamaguchi K, et al. Overexpression of plasma membrane–associated sialidase attenuates insulin signaling in transgenic mice. J Biol Chem 2003;278: 27896-902.
[17] Folch J, Lees M, Sloane Stanley GH, Stanley H. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497-509.
[18] Lo S, Russell JC, Taylor AW. Determination of glycogen in small tissue samples. J Appl Physiol 1970;28:234-6.
[19] Ogihara T, Shin BC, Anai M, Katagiri H, Inukai K, Funaki M, et al. Insulin receptor substrate (IRS)-2 is dephosphorylated more rapidly than IRS-1 via its association with phosphatidylinositol 3-kinase in skeletal muscle cells. J Biol Chem 1997;272:12868-73.
[20] Ono H, Shimano H, Katagiri H, Yahagi N, Sakoda H, Onishi Y, et al. Hepatic Akt activation induces marked hypoglycemia, hepatomegaly, and hypertriglyceridemia with sterol regulatory element binding protein involvement. Diabetes 2003;52:2905-13.
[21] Hasegawa T, Yamaguchi K, Wada T, Takeda A, Itoyama Y, Miyagi T. Molecular cloning of mouse ganglioside sialidase and its increased expression in Neuro2a cell differentiation. J Biol Chem 2000;275: 8007-15.
[22] Hasegawa T, Carnero CF, Wada T, Itoyama Y, Miyagi T. Differential expression of three sialidase genes in rat development. Biochem Biophys Res Commun 2001;280:726-32.
[23] Chien J-L, Hogan EL. Novel pentahexosyl ganglioside of the globo series purified from chicken muscle. J Biol Chem 1983;258: 10727-30.
[24] Nakamura K, Hashimoto Y, Yamakawa T, Suzuki A. Genetic polymorphism of ganglioside expression in mouse organs. J Biochem (Tokyo) 1988;103:201-8.
[25] Yki-Jarvinen H, Westerbacka J. The fatty liver and insulin resistance. Curr Mol Med 2005;5:287-95.
[26] Stiles B, Wang Y, Stahl A, Bassilian S, Lee WP, et al. Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity. Proc Natl Acad Sci U S A 2004;101:2082-7.
[27] Koo SH, Satoh H, Herzig S, Lee CH, Hedrick S, Kulkarni R, et al. PGC-1 promotes insulin resistance in liver through PPAR-alpha–dependent induction of TRB-3. Nat Med 2004;10:530-4.
[28] Xu A, Lam MC, Chan KW, Wang Y, Zhang J, Hoo RL, et al. Angiopoietin-like protein 4 decreases blood glucose and improves glucose tolerance but induces hyperlipidemia and hepatic steatosis in mice. Proc Natl Acad Sci U S A 2005;102:6086-91.
[29] Fajas L, Auboeuf D, Raspé E, Schoonjans K, Lefebvre AM, Saladin R, et al. The organization, promoter analysis, and expression of the human PPAR gene. J Biol Chem 1997;272:18779-89.
[30] Burant CF, Sreenan S, Hirano K, Tai TAC, Lohmiller J, Lukens J, et al. Troglitazone action is independent of adipose tissue. J Clin Invest 1997;100:2900-8.
[31] Chao L, Marcus-Samuels B, Mason MM, Moitra J, Vinson C, Arioglu E, et al. Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. J Clin Invest 2000;106:1221-8.
[32] Matsusue K, Haluzik M, Lambert G, Yim SH, Gavrilova O, Ward JM, et al. Liver-specific disruption of PPAR in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. J Clin Invest 2003;111:737-47.
[33] Kim SY, Kim HI, Park SK, Im SS, Li T, Cheon HG, et al. Liver glucokinase can be activated by peroxisome proliferator–activated receptor-gamma. Diabetes 2004;53:S66.
[34] Uno K, Katagiri H, Yamada T, Ishigaki Y, Ogihara T, Imai J, et al. Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity. Science 2006;312:1656-9.
[35] Srinivas PR, Wagner AS, Reddy LV, Deutsch DD, Leon MA, Goustin AS, et al. Serum alpha 2-HS-glycoprotein is an inhibitor of the human insulin receptor at the tyrosine kinase level. Mol Endocrinol 1993;7:1445-55.
[36] Kalabay L, Chavin K, Lebreton JP, Robinson KA, Buse MG, Arnaud P. Human recombinant alpha 2-HS glycoprotein is produced in insect cells as a full length inhibitor of the insulin receptor tyrosine kinase. Horm Metab Res 1998;30:1-6.
[37] Mathews ST, Singh GP, Ranalletta M, Cintron VJ, Qiang X, Goustin AS, et al. Improved insulin sensitivity and resistance to weight gain in mice null for the Ahsg gene. Diabetes 2002;51:2450-8.

Psychiatry and Clinical Neurosciences (2007), **61**, 112–119 doi:10.1111/j.1440-1819.2007.01619.x

Regular Article

Reliability and validity of the Japanese version of the World Health Organization-Five Well-Being Index in the context of detecting depression in diabetic patients

SHUICHI AWATA, MD,[1] PER BECH, MD,[2] SUMIKO YOSHIDA, MD,[3] MASASHI HIRAI, MD,[4] SUSUMU SUZUKI, MD,[4] MOTOYASU YAMASHITA, MD,[5] ARIHISA OHARA, MD,[5] YOSHINORI HINOKIO, MD,[4] HIROO MATSUOKA, MD[6] AND YOSHITOMO OKA, MD[4]

[1]*Division of Neuropsychiatry and Center for Dementia, Sendai City Hospital,* [3]*Department of Health and Welfare Science, Sendai College, Funaoka-Minami, Departments of* [4]*Molecular Metabolism and Diabetes, and* [6]*Psychiatry, and* [5]*Division of Psychobiology, Tohoku University Graduate School of Medicine, Sendai, Japan and* [2]*Psychiatric Research Unit, WHO Collaborating Center in Mental Health, Frederiksborg General Hospital, Frederiksborg, Denmark*

Abstract The present study had two aims. The first was to evaluate the reliability and the validity of the Japanese version of the World Health Organization (WHO)-Five Well-Being Index (WHO-5-J) as a brief well-being scale. The second was to examine the discriminatory validity of this test as a screening tool for current depressive episodes in diabetic patients. A sample of 129 diabetic patients completed the WHO-5-J. Of these, 65 were also interviewed by psychiatrists to assess whether they had any current depressive episodes according to DSM-IV. The internal consistency was evaluated using Cronbach's alpha, the Loevinger coefficient of homogeneity, and factor analysis. The external concurrent validity was evaluated by correlations with the external scales potentially related to subjective well-being. Discriminatory validity was evaluated using receiver operating characteristic (ROC) analysis. Cronbach's alpha and the Loevinger coefficient were estimated to be 0.89 and 0.65, respectively. A factor analysis identified only one factor. The WHO-5-J was significantly correlated with a number of major diabetic complications, depression, anxiety, and subjective quality of life. ROC analysis showed that the WHO-5-J can be used to detect a current depressive episode (area under curve: 0.92; 95% confidence interval: 0.85–0.98). A cut-off of <13 yielded the best sensitivity/specificity trade-off: sensitivity, 100%; specificity, 78%. The WHO-5-J was thus found to have a sufficient reliability and validity, indicating that it is a useful instrument for detecting current depressive episodes in diabetic patients.

Key words depressive episode, diabetes, screening, validity, well-being.

INTRODUCTION

There is a high prevalence of depression among patients with diabetes.[1] Recent studies suggest that depression has a significantly adverse effect on diabetes in terms of decreased glycemic control, a reduction in the quality of life, and an increased health-care expenditure.[2] However, depression frequently goes undetected and it remains untreated.[3] It has been demonstrated that primary care patients with major or minor depressive disorders respond well to psychotherapy and/or treatment with antidepressants.[4,5] Therefore, a brief and rapid instrument for detecting depression in primary care settings may play an important role in the improvement of diabetes care.

One candidate for such an instrument is the World Health Organization (WHO)-Five Well-Being Index (WHO-5). It is a short version of the WHO Well-Being Scale, which was initially developed to evaluate the quality of care for diabetic patients.[6,7] The WHO-5 is a self-administered five-item scale. Each item assesses

Correspondence address: Shuichi Awata, MD, Division of Neuropsychiatry and Center for Dementia, Sendai City Hospital, 3-1, Shimizukoji, Wakabayashi-ku, Sendai 984-8501, Japan. Email: awata-thk@umin.ac.jp

Received 16 June 2006; revised 1 September 2006; accepted 9 September 2006.

the degree of positive well-being during the past 2 weeks on a six-point Likert scale graded from 0 (at no time) to 5 (all of the time), and the total score ranges from 0 to 25, with high scores thereby indicating an increased sense of well-being. The scale has been translated into various languages (http://www.who-5.org/) and, in the West, validation data have been published in the context of various health conditions, including depressive disorders,[8–11] anxiety disorders,[8] cognitive impairment and dementia,[12] and psychiatric disorders,[12] in addition to the health-related quality of life.[13] Discriminatory validity as a screening tool for depressive disorders has also been examined.[9–11] However, no validation data have yet been published in the East, including Japan.

The present study had two aims: to evaluate the reliability and validity of the Japanese version of the WHO-5 (WHO-5-J) as a brief well-being scale, and to examine its discriminatory validity as a screening tool for a current depressive episodes in diabetic patients.

METHODS

Adaptation of the WHO-5 for use in Japan

Using a state-of-the-art procedure for test translation,[14] the original English version of the WHO-5 was adapted to Japanese in collaboration with the European WHO office as follows: two independent forward- and two independent back-translations, linguistic panels, pilot testing, and formal assessment of the reliability and validity of the index.

The WHO-5 was translated into Japanese by two bilingual translators who worked independently to produce two different Japanese versions. These versions were then back-translated and discussed by a panel of experts until agreement was reached on the most suitable items to be included in the final version. The final version was tested in a pilot study, which confirmed a high level of item acceptability and comprehension. The data regarding the reliability and validity are shown in the present study. The WHO-5-J can be obtained from the website http://www.who-5.org.

Subjects

The participants were recruited from 197 outpatients with diabetes who received regular treatment at the Diabetes and Metabolism Unit, Tohoku University Hospital. All patients had been diagnosed to have either type 1 or type 2 diabetes mellitus according to the criteria of the American Diabetes Association.[15] Of these patients, a total of 129 were regarded as eligible for the study. These consisted of 71 men and 58 women who gave their written informed consent, had no explicit dementia (Mini-Mental State ≥ 20), and completed the WHO-5-J as well as a set of questionnaires concerning demographic, social, and health-related variables. The most recent clinical data regarding diabetes were collected from the patients' medical records. All data collection was completed in November 2004. Subject characteristics are given in Table 1.

Approval of the research protocol was obtained from the Ethics Committee of Tohoku University Graduate School of Medicine and Tohoku University Hospital.

Table 1. Description of the 129 diabetic patients who completed the WHO-5-J

Variables	Mean ± SD	Range
Age (years)	53.6 ± 10.4	25–73
Sex (Female %)	45.0	
Educational level (years)	19.8 ± 3.2	15–34
Type of diabetes (Type 1%)	16.3	
Use of insulin (%)	55.8	
Duration of diabetes (years)	10.9 ± 10.2	0.1–40
FBS (mg/dL)	149.3 ± 50.5	66.0–503.0
HbA1c (%)	7.0 ± 1.4	4.0–12.0
BMI (g/cm^2)	24.5 ± 4.4	17.0–47.2
Major complication (%)	43.4	
Neuropathy (%)	24.8	
Retinopathy (%)	30.2	
Nephropathy (%)	13.2	
No. complications		
0	56.6	
1	25.6	
2	10.9	
3	7.0	
SDS score	37.6 ± 9.6	22–63
STAI score, state	39.6 ± 10.6	20–67
STAI score, trait	41.5 ± 11.9	21–74
MMSE score	28.2 ± 2.1	20–30
SF-36 subscale		
Physical functioning	82.3 ± 21.2	0–100
Social functioning	83.2 ± 22.9	12.5–100
Role functioning, physical	76.8 ± 37.6	0–100
Role functioning, emotional	78.1 ± 38.4	0–100
Mental health	70.2 ± 21.7	4–100
Vitality	62.9 ± 22.9	5–100
Pain	71.8 ± 25.8	0–100
General health perceptions	49.7 ± 21.0	0–97
WHO-5-J score	15.5 ± 6.1	0–25

BMI, body mass index; FBS, fasting blood sugar; HbA1c, hemoglobin A1c; MMSE, Mini-Mental State Examination; SDS, Zung's Self-Rating Depression Scale; SF-39, Short-Form 36 Health Survey questionnaire; STAI, State–Trait Anxiety Inventory; WHO-5-J, Japanese version of the World Health Organization-Five Well-Being Index.

Measurements

Sociodemographic variables included sex, age, and educational level. Educational levels were assessed by determining the age when schooling was completed. The assessed clinical variables regarding diabetes included the type of diabetes, the duration of diabetes, fasting blood sugar (FBS), glucosylated hemoglobin (HbA1c), body mass index (BMI), and major diabetic complications (retinopathy, neuropathy, and nephropathy).

Zung's Self-Rating Depression Scale (SDS),[16] the State-Trait Anxiety Inventory (STAI),[17] the Mini-Mental State Examination (MMSE),[18] and the Short-Form 36 Health Survey questionnaire (SF-36)[19] were all used as external scales that were potentially related to subjective well-being.

The SDS is an internationally used 20-item self-administered depression scale. Each item is rated on a four-point Likert scale, with a total score ranging from 20 to 80 and high scores indicating increased depression. The Japanese version was developed by Fukuda and Kobayashi[20] and has been well validated by Kitamura *et al.*[21] The STAI comprises 20-item trait and 20-item state anxiety scales. Each item is rated on the four-point Likert scale, with a total score ranging from 20 to 80 for each scale and high scores indicating increased anxiety. The Japanese version was validated by Nakazato and Mizuguchi.[22] The MMSE is a brief test of several cognitive functions, with a total score ranging from 0 to 30. A score of 23 or less indicates a cognitive disorder and has a high degree of validity and reliability in detecting cognitive impairment.[18] The Japanese version was validated by Mori *et al.*[23] The SF-36 consists of eight subscales for subjective health-related quality of life, with scores on each subscale ranging from 0 to 100 and high scores indicating a high quality of life. The Japanese version was developed and validated by Fukuhara *et al.*[24,25] and is widely used in studies on health-related quality of life among Japanese populations.

Subjects with SDS scores ≥40 were selected to undergo an interview with psychiatrists, who were blind to the results of the questionnaires, in order to diagnose any current major or minor depressive episodes using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I).[26] The Japanese version of SCID-I was developed by Takahashi.[27] Minor depressive episodes were defined as clinically relevant depressive syndromes, not fulfilling the rigorous diagnostic criteria for major depressive episode, but containing two–four symptoms of DSM-IV-defined major depressive episode, one of which must be either a depressed mood or anhedonia (loss of interest or pleasure). Almost the same number of subjects who scored <40 on the SDS were recruited to undergo the same interview.

Statistics

For the evaluation of internal consistency, Cronbach's alpha, the Loevinger coefficient, and a factor analysis were used. Cronbach's alpha is a measure of how well the subsets of the items can be substituted for each other. A high alpha (>0.9) may suggest a high level of item redundancy; therefore, alpha should fall within the range 0.7–0.9. The Loevinger coefficient of homogeneity was used as part of the Mokken analysis.[28] The Loevinger coefficient is an analysis of unidimensionality that tests the extent to which an extra item fits into the structure established by the other items of a scale. According to the Mokken analysis, homogeneity and unidimensionality are acceptable at a Loevinger coefficient of ≥0.40, while a coefficient of 0.30–0.39 is considered to be barely acceptable.[28]

The Loevinger coefficient is thought to be a better indicator of internal consistency than Cronbach's alpha because, in contrast to Cronbach's alpha, it is independent of the number of items in the scale.[7,29] A factor analysis was performed as a standard principal component analysis with orthogonal varimax rotation of the components. If a substantial shift between the first and the second eigenvalues could be observed, then unidimensionality is indicated.[30]

To evaluate the external concurrent validity, the Spearman correlation coefficient was used to correlate the WHO-5-J with health-related variables potentially related to subjective well-being. For the evaluation of discriminatory validity as a screening tool for current depressive episodes, the area under the curve (AUC), sensitivity, and specificity were calculated using a receiver operating characteristic (ROC) analysis. To evaluate the post-test probability, the positive predictive value (PPV) and negative predictive value (NPV) were calculated using a 2×2 contingency table. For group comparisons, Student's *t*-test, the Wilcoxon *U*-test, or χ^2 test were used where applicable. All statistical analyses were performed using SPSS version 11.5 (SPSS, Chicago, IL, USA) and the SAS system, version 9.1.3 (SAS Institute, Cary, NC, USA). Statistical significance was established at $P = 0.05$.

RESULTS

Internal consistency

Cronbach's alpha was found to be 0.89 and the Loevinger coefficient of homogeneity was 0.65. A factor

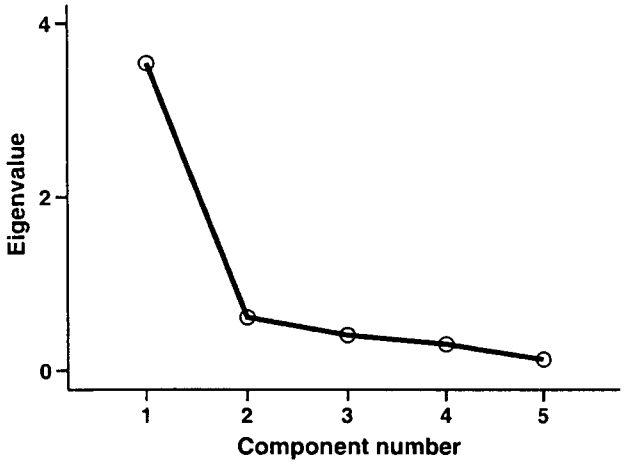

Figure 1. Scree plot for the components of the Japanese version of the World Health Organization-Five Well-Being Index.

Table 2. Factor matrix of the Japanese version of the WHO-5

Item		Factor
Item 1	I have felt cheerful and in good spirits	0.91
Item 2	I have felt calm and relaxed	0.91
Item 3	I have felt active and vigorous	0.83
Item 4	I woke up feeling fresh and rested	0.80
Item 5	My daily life has been filled with things that interest me	0.74

WHO, World Health Organization.
Principle component analysis was used.

analysis identified only one factor when considering eigenvalues >1.0 (Fig. 1, Table 2). The first factor explained 70.8% of the variance. These findings thus indicate that the WHO-5-J has an adequate internal consistency and that the total score has a sufficient degree of statistical validity.

External concurrent validity

No significant differences in the WHO-5-J total scores were observed between the male and female subjects (male *vs* female [mean ± SD], 15.8 ± 6.0 *vs* 15.1 ± 6.3, $t = 0.59$, $P = 0.57$). Neither type of diabetes (type 1 *vs* type 2, 13.9 ± 5.1 *vs* 15.8 ± 6.3, $t = 0.46$, $P = 0.14$) nor status of insulin use (use *vs* no use, 15.9 ± 6.0 *vs* 15.1 ± 6.2, $t = 0.73$, $P = 0.47$) made differences in the WHO-5-J. Table 3 shows correlations between the WHO-5-J and the demographic and health-related variables potentially related to subjective well-being.

Table 3. Correlations between the WHO-5-J and external scales related to subjective well-being

	Spearman correlation coefficients	*P*
Age	0.23	0.01
Education level	0.03	0.77
Duration of diabetes	0.10	0.24
FBS	−0.01	0.88
HbA1c	0.03	0.78
BMI	−0.10	0.25
Number of major complications	−0.21	0.02
SDS score	−0.68	0.00
STAI score, state	−0.73	0.00
STAI score, trait	−0.74	0.00
MMS score	0.11	0.21
SF-36 subscale		
Physical functioning	0.43	0.00
Social functioning	0.40	0.00
Role functioning, physical	0.43	0.00
Role functioning, emotional	0.51	0.00
Mental health	0.70	0.00
Vitality	0.72	0.00
Pain	0.39	0.00
General health perceptions	0.43	0.00

BMI, body mass index; FBS, fasting blood sugar; HbA1c, hemoglobin A1c; MMSE, Mini-Mental State Examination; SDS, Zung's Self-Rating Depression Scale; SF-36, Short-Form 36 Health Survey questionnaire; STAI, State-Trait Anxiety Inventory; WHO-5-J, Japanese version of the WHO-Five Well-Being Index.

The WHO-5-J total score was significantly correlated with age, the number of major complications, and the total scores on the SDS, the STAI, both the state and trait scales, and each subscale of the SF-36. No significant correlations were found between the WHO-5-J total score and educational level, duration of diabetes, FBS, HbA1c, BMI, or the total score on the MMSE.

External discriminatory validity

Table 4 shows the characteristics of the 65 diabetic patients who were interviewed by psychiatrists in order to assess whether they had a current major or minor depressive episode according to the DSM-IV criteria. Seven patients were diagnosed to have a current major depressive episode, while three patients were diagnosed to have a current minor depressive episode. In accordance with our expectations, the patients with current major or minor depressive episodes had a lower mean score on the WHO-5-J as well as higher mean scores on the SDS and the STAI (both the state

Table 4. Description of the 65 diabetic patients who were interviewed for psychiatric diagnosis of a major depressive episode

	DE (−)	DE (+)	*P*
n	55	10	
Age (years)	53.9 ± 10.4	49.7 ± 14.0	0.39
Sex (Female, %)	47.3	60.0	0.51
Educational level (years)	19.8 ± 3.2	18.9 ± 2.2	0.29
Type of diabetes (Type 1%)	16.4	20.0	0.67
Use of insulin	50.9	50.0	1.00
Duration of diabetes (years)	9.9 ± 10.0	5.8 ± 7.5	0.15
FBS (mg/dL)	146.1 ± 38.5	179.0 ± 118.3	0.10
HbA1c (%)	6.8 ± 1.2	6.8 ± 2.1	0.95
BMI (g/cm^2)	23.4 ± 3.5	24.6 ± 4.3	0.40
Major complication	34.6	50.0	0.48
SDS score	37.8 ± 9.3	51.2 ± 6.1	0.00
STAI, state	37.1 ± 9.9	48.9 ± 6.2	0.00
STAI, trait	39.5 ± 10.6	57.4 ± 9.3	0.00
MMSE	28.2 ± 2.0	28.7 ± 1.5	0.41
SF-36 subscale			
Physical functioning	82.0 ± 21.4	71.5 ± 24.5	0.23
Social functioning	84.7 ± 21.9	56.9 ± 24.3	0.01
Role functioning, physical	79.7 ± 35.0	47.2 ± 47.5	0.08
Role functioning, emotional	83.7 ± 33.7	33.3 ± 44.1	0.01
Mental health	76.0 ± 19.2	39.6 ± 17.7	0.00
Vitality	63.4 ± 24.3	35.0 ± 13.0	0.00
Pain	74.6 ± 25.3	56.3 ± 26.4	0.08
General health perceptions	50.8 ± 21.3	34.6 ± 21.5	0.06
WHO-5-J score	16.7 ± 5.8	6.9 ± 3.5	0.00

BMI, body mass index; DE, major or minor depressive episode; FBS, fasting blood sugar; HbA1c, hemoglobin A1c; MMSE, Mini-Mental State Examination; SDS, Zung's Self-Rating Depression Scale; SF-39, Short-Form 36 Health Survey questionnaire; STAI, State–Trait Anxiety Inventory; WHO-5-J, Japanese version of the World Health Organization-Five Well-Being Index.
Statistical analyses were performed using Student's *t*-test, Wilcoxon *U*-test, or χ^2 test.

and trait scales) and lower mean scores on several subscales of the SF-36 (social functioning, emotional role functioning, mental health, and vitality), than patients without a current depressive episode.

Figure 2 shows the ROC curve for the detection of a current major or minor depressive episode according to the WHO-5-J total score. The AUC was estimated to be 0.92 (95% confidence interval [95%CI], 0.85–0.98), which was significantly different from 0.5 ($P < 0.001$). A cut-off of <13 yielded the best sensitivity/specificity trade-off: sensitivity, 100%; specificity, 78.2%; PPV, 45.5%; and NPV, 100%. When associated with a current major depressive episode alone, similar results were obtained: AUC, 0.90; 95%CI, 0.81–0.98; $P < 0.001$; sensitivity, 100%; specificity, 74.1%; PPV, 31.8%; and NPV, 100%.

DISCUSSION

The present report is the first study to evaluate the reliability and validity of the WHO-5 in an Asian region. Similar to the original version, evaluated in Europe,[8–10,12,13] the WHO-5-J was found to have adequate reliability and validity for Japanese diabetic patients.

In line with the findings of previous studies,[12,13] the scale showed a significant correlation with different indicators of health-related variables including depression (SDS), anxiety (STAI), and subjective quality of life (SF-36). It should also be noted that the WHO-5-J score significantly decreased as the number of major complications increased. Recent studies have indicated that depression has an adverse effect on glycemic control, thus increasing the risk of major diabetic complications, and thereby leading to further reductions in the quality of life among diabetic patients.[2,31] We have reported elsewhere that the presence or absence of major diabetic complications, particularly those of neuropathy, is significantly associated with depression after controlling for potential confounding factors (S. Yoshida *et. al.*, unpub. data). Therefore, the WHO-5-J might be sensitive to decreased subjective well-being in

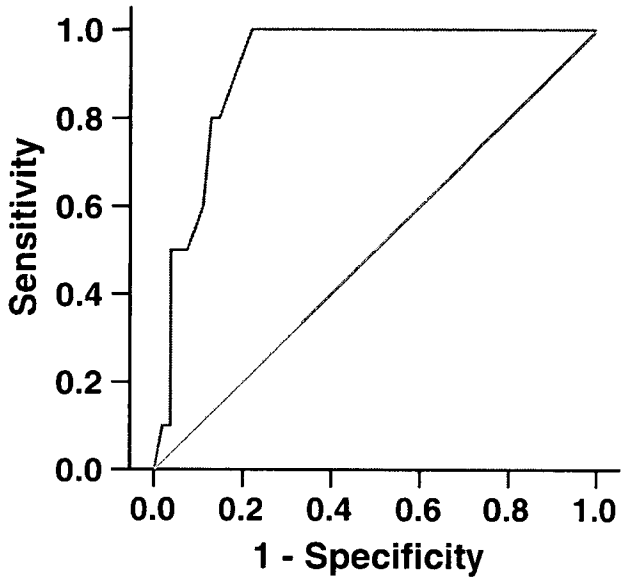

Figure 2. Receiver operating characteristics curve for the Japanese version of the World Health Organization-Five Well-Being Index in association with a current depressive episode as diagnosed according to DSM-IV criteria. The area under the curve was estimated at 0.92 (95% confidential interval: 0.85–0.98), which was significantly different from 0.5 ($P < 0.001$).

patients with major diabetic complications who are prone to develop depression and thus demonstrate a reduction in the quality of life.

However, no significant correlation was observed between the WHO-5-J and cognitive function. The present subjects are comparatively younger and have a higher cognitive functioning than those of a previous study that showed the WHO-5 score to significantly decrease as the MMSE score decreased in a sample of 254 elderly from the general population.[12] This might yield a ceiling effect and conceal the relationship between the cognitive function and subjective well-being. In contrast, the scores on the WHO-5-J significantly decreased as age decreased. In the present study, a decreased age was significantly associated with increases in both state and trait anxiety (state, $r = -0.22$, $P = 0.013$; trait, $r = -0.25$, $P = 0.005$). Younger patients with diabetes might be more likely to feel anxiety regarding an image of his or her future than older patients, and such anxiety might be reflected by the WHO-5-J.

The results of an ROC analysis, in addition to those of comparisons of WHO-5-J scores between the patients with and without a current depressive episode, indicate that the WHO-5-J has a sufficient discriminatory validity as a screening tool for the detection of a current depressive episode. A standard cut-off point of <13 (WHO)[32] had an excellent sensitivity/specificity trade-off. Sensitivity of 100% means that all subjects with depressive episodes were detected by this cut-off criteria (NPV, 100%). However, the specificity of 78% indicates that a considerable number of positive screening subjects had no current depressive episodes (PPV, 45%). Therefore, as Henkel *et al.* suggested,[9,10] this scale might be recommended for use as the first-step screening tool followed by the second-step screening tool such as the Major Depression Inventory to confirm depressive episodes.

The present study had several limitations. First, our sample population may not have been adequately representative of diabetic patients within primary care settings. Because the study population had relatively good glycemic control, the prevalence of depressive episodes in diabetic patients within primary care might have been underestimated. Second, the sample size was relatively small and only 65 patients underwent a diagnostic interview by psychiatrists. To generalize the findings more appropriately, it is necessary to conduct a collaborative multi-institutional study in a larger sample. Third, discriminatory validity for the detection of psychiatric disorders other than depression was not examined in the present study, although it is likely that such psychiatric disorders would influence both the subjective well-being and quality of care of diabetic patients. However, it is evident that depression is highly prevalent and it also has a significantly adverse effect on diabetes. The present study indicates that the WHO-5-J might therefore be a potentially useful screening modality for detecting existing, but unrecognized depression in diabetic patients.

CONCLUSIONS

The WHO-5-J was found to have sufficient reliability and validity as a brief well-being scale. This scale might be a useful instrument for the detection of current major or minor depressive episodes in diabetic patients. A cut-off point of <13 might be recommended for use in the screening of depression.

ACKNOWLEDGMENTS

The authors are grateful to Professor P. Allerup and Mr O. Aaskoven for their valuable advice regarding the statistical analysis, to Professor I. Sora for his close cooperation with research, and to Ms M. Kubota and Ms M. Hino for their valuable assistance. This study was supported, in part, by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Health, Wel-

fare, and Labor and by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 17591194).

REFERENCES

1. Anderson RJ, Freedland KE, Clouse RE, Lustman PJ. The prevalence of comorbid depression in adults with diabetes: A meta-analysis. *Diabetes Care* 2001; **24**: 1069–1078.
2. Lustman PJ, Close RE. Depression in diabetic patients: The relationship between mood and glycemic control. *J. Diabetes Complications* 2005; **19**: 113–122.
3. Gelenberg A. Depression is still underrecognized and undertreated. *Arch. Intern. Med.* 1999; **159**: 1657–1658.
4. Miranda J, Munoz R. Intervention for minor depression in primary care patients. *Psychosom. Med.* 1994; **56**: 136–141.
5. Whooley M, Simon G. Managing depression in medical outpatients. *N. Engl. J. Med.* 2000; **343**: 1943–1950.
6. Bradley C, Lewis KS. Measurements of psychological wellbeing and treatment satisfaction developed from the responses of people with tablet-treated diabetes. *Diabet. Med.* 1990; **7**: 445–451.
7. Bech P, Gudex C, Johansen S. The WHO (ten) well-being index: Validation in diabetes. *Psychother. Psychosom.* 1996; **65**: 183–190.
8. Bonsignore M, Barkow K, Jessen F, Heun R. Validity of the five-item WHO well-being index (WHO-5) in an elderly population. *Eur. Arch. Psychiatry Clin. Neurosci.* 2001; **251** (Suppl. 2): II27–II31.
9. Henkel V, Mergl R, Kohnen R, Maier W, Moller HJ, Hegerl U. Identifying depression in primary care: A comparison of different methods in a prospective cohort study. *BMJ* 2003; **326**: 200–201.
10. Henkel V, Mergl R, Kohnen R, Allgaier A-K, Moller H-J, Hegerl U. Use of brief depression screening tools in primary care: Consideration of heterogeneity in performance in different patient groups. *Gen. Hosp. Psychiatry* 2004; **26**: 190–198.
11. Lowe B, Spitzer RL, Graphe K *et al.* Comparative validity of three screening questionnaires for DSM-IV depressive disorders and physicians' diagnosis. *J. Affect. Disord.* 2004; **78**: 131–140.
12. Heun R, Burkart M, Maier M, Bech P. Internal and external validity of the WHO Well-Being Scale in the elderly general population. *Acta Psychiatr. Scand.* 1999; **99**: 171–178.
13. Bech P, Olsen LR, Kjoller M, Rasmussen NK. Measuring well-being rather than the absence of distress symptoms: A comparison of the SF-36 Mental Health subscale and the WHO-Five Well-Being Scale. *Int. J. Methods Psychiatr. Res.* 2003; **12**: 85–91.
14. Bracken B, Barona A. State of art procedures for translating, validating and using psychoeducational tests in cross-cultural assessment. *School Psychol. Int.* 1991; **12**: 119–132.
15. *Expert Committee on the Diagnosis and Classification of Diabetes Mellitus.* Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 1997; **20**: 1183–1197.
16. Zung W. A self-reported depression scale. *Arch. Gen. Psychiatry* 1969; **126**: 116–121.
17. Spielberger CD, Goursch RL, Lushene RE. *STAI Manual for the State-Trait Anxiety Inventory.* Consulting Psychologists Press, California, 1970.
18. Folstein MF, Folstein SE, McHugh PR. 'Mini-Mental State'. A practical method for grading the cognitive state of patients for the clinician. *J. Psychiatr. Res.* 1975; **12**: 189–198.
19. McHorney CA, Ware JE Jr, Rachel Lu JF, Sherbourne CD. The MOS 36-item Short-Form Survey (SF-36): III. Tests of data quality, scaling assumptions, and reliability across diverse patient groups. *Med. Care* 1994; **32**: 40–66.
20. Fukuda K, Kobayashi S. A study on a self-rating depression scale. *Psychiatr. Neurol. Jpn* 1978; **75**: 673–679.
21. Kitamura T, Shima S, Sugawara M, Toda MA. Temporal validation of validity of self-rating questionnaires: Repeated use of the General Health Questionnaire and Zung's Self-Rating Depression Scale among women during antenatal and postnatal periods. *Acta Psychiatr. Scand.* 1994; **90**: 446–450.
22. Nakazato K, Mizuguchi K. Studies on psychometric characteristics of depression in the field of internal medicine. *Shinshin-Igaku* 1982; **22**: 107–112 (in Japanese).
23. Mori E, Mitani Y, Yamadori A. Usefulness of a Japanese version of the Mini-Mental State test in neurological patients. *Jpn J. Neuropsychol.* 1985; **1**: 82–89 (in Japanese).
24. Fukuhara S, Bito S, Green J, Hsiao A, Kiyoshi K. Translation, adaptation, and validation of the SF-36 Health Survey for use in Japan. *J. Clin. Epidemiol.* 1998; **51**: 1037–1044.
25. Fukuhara S, Ware JE, Kosinski M, Wada S, Gandek B. Psychometric and clinical tests of validity of the Japanese SF-36 Health Survey. *J. Clin. Epidemiol.* 1998; **51**: 1045–1053.
26. First MB, Spitzer RL, Gibbon MSW, Williams JBW. *Structured Clinical Interview for DSM-IV Axis I Disorders.* Biometric Research Department, New York, 1997.
27. Takahashi S. *The Manual for Psychiatric Diagnostic Interview. Structured Clinical Interview For DSM-IV Axis I Disorders.* Nihon Hyoron-sha, Tokyo, 2002 (in Japanese).
28. Mokken RJ. *A Theory and Procedure of Scale Analysis.* Mouton, Paris, 1971.
29. Bech P, Moses R, Gomis R. The effect of prandial glucose regulation with repaglinide on treatment satisfaction, wellbeing and health status in patients with pharmacotherapy-naïve Type 2 diabetes: A placebo-controlled, multicentre study. *Qual. Life Res.* 2003; **12**: 413–425.
30. Licht RW, Qvitzau S, Allerup P, Bech P. Validation of the Bech-Rafaelsen Melancholia Scale and the Hamilton Depression Scale in patients with major depression; is

the total score a valid measure of illness severity? *Acta Psychiatr. Scand.* 2005; **111**: 144–149.
31. de Groot M, Anderson R, Freedland KE, Clouse RE, Lustman PJ. Association of depression and diabetes complications: A meta-analysis. *Psychosom. Med.* 2001; **63**: 619–630.
32. *World Health Organization. World Health Organization Info Package: Mastering Depression in Primary Care.* WHO, Regional Office of Europe, Psychiatric Research Unit, Frederiksborg, 1998.

Am J Physiol Regul Integr Comp Physiol 292: R1775–R1781, 2007.
First published January 11, 2007; doi:10.1152/ajpregu.00527.2006.

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

Katsunori Nonogaki,[1] Kana Nozue,[1] Tomifusa Kuboki,[2] and Yoshitomo Oka[1]

[1]*Center of Excellence, Division of Molecular Metabolism and Diabetes, Tohoku University Graduate School of Medicine; and* [2]*Department of Psychosomatic Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan*

Submitted 22 July 2006; accepted in final form 9 January 2007

Nonogaki K, Nozue K, Kuboki T, Oka Y. Milnacipran, a serotonin and norepinephrine reuptake inhibitor, induces appetite-suppressing effects without inducing hypothalamic stress responses in mice. *Am J Physiol Regul Integr Comp Physiol* 292: R1775–R1781, 2007. First published January 11, 2007; doi:10.1152/ajpregu.00527.2006.—Milnacipran, a selective serotonin (5-HT) and norepinephrine (NE) reuptake inhibitor, increases extracellular 5-HT and NA levels equally in the central nervous system. Here, we report that systemic administration of milnacipran (20–60 mg/kg) significantly suppressed food intake after fasting in C57BL6J mice. The appetite-suppressing effects of milnacipran were sustained for 5 h. Neither SB242084, a selective 5-HT2C receptor antagonist, nor SB224289, a selective 5-HT1B receptor antagonist, reversed the appetite-suppressing effects of milnacipran. Milnacipran suppressed food intake and body weight in wild-type mice and in A^y mice, which have ectopic expression of the agouti protein. Moreover, milnacipran significantly increased hypothalamic proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) mRNA levels, while having no effect on hypothalamic neuropeptide Y, ghrelin, corticotropin-releasing hormone (CRH), and suppressor of cytokine signaling-3 mRNA levels. Interestingly, milnacipran did not increase plasma corticosterone and blood glucose levels, whereas fenfluramine, which inhibits 5-HT reuptake and stimulates 5-HT release, significantly increased plasma corticosterone and blood glucose levels in association with increased hypothalamic CRH mRNA levels. The appetite-suppressing effects of milnacipran had no effects on food intake in food-restricted, wild-type mice and A^y mice. On the other hand, fenfluramine suppressed food intake in food-restricted wild-type mice, but it had no effects in food-restricted A^y mice. 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 increasing plasma corticosterone and blood glucose levels in mice.

5-hydroxytryptamine; norepinephrine; food intake; corticosterone; glucose

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.

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 A^y mice. A^y 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 A^y mice are responsive to mCPP and fenfluramine (23).

MATERIALS AND METHODS

Animals and drug treatment. Four-week-old male C57BL/6J mice, A^y mice, and wild-type mice were purchased from Japan CLEA. 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. SB224289 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 SB224289 (5 mg/kg).

Address for reprint requests and other correspondence: K. Nonogaki, Center of Excellence, Division of Molecular Metabolism and Diabetes, Tohoku Univ. Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, 980-8575, Japan (E-mail: knonogaki-tky@umin.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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^y 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^y 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^y 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^y 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′-GGC 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′-GGC GGA GTC CAG CCT AGT GG-3′; for mouse ghrelin, sense, 5′-GAA AGG 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 GGC AGA GCA GTT 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 ACC TTT CTT ATC C-3′, antisense, 5′-TCC CCG ACT GGG TCT TGA C-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 with 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.

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 ± 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. 1*A*). 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. 1*B*).

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. 1*C*). SB224289 (5 mg/kg) reversed the feeding suppression induced by CP94253 (5 mg/kg; Fig. 1*D*). SB242084 (2 mg/kg) partially but significantly reversed the feeding suppression induced by mCPP (5 mg/kg) (Fig. 1*E*). 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^y mice. To determine the effects of milnacipran on food intake on A^y mice, we administered milnacipran to nonobese (5-wk-old) A^y mice and wild-type mice. Milnacipran (30 mg/kg) significantly reduced food intake in wild-type mice and A^y mice (to ~68% and 48% that of controls,

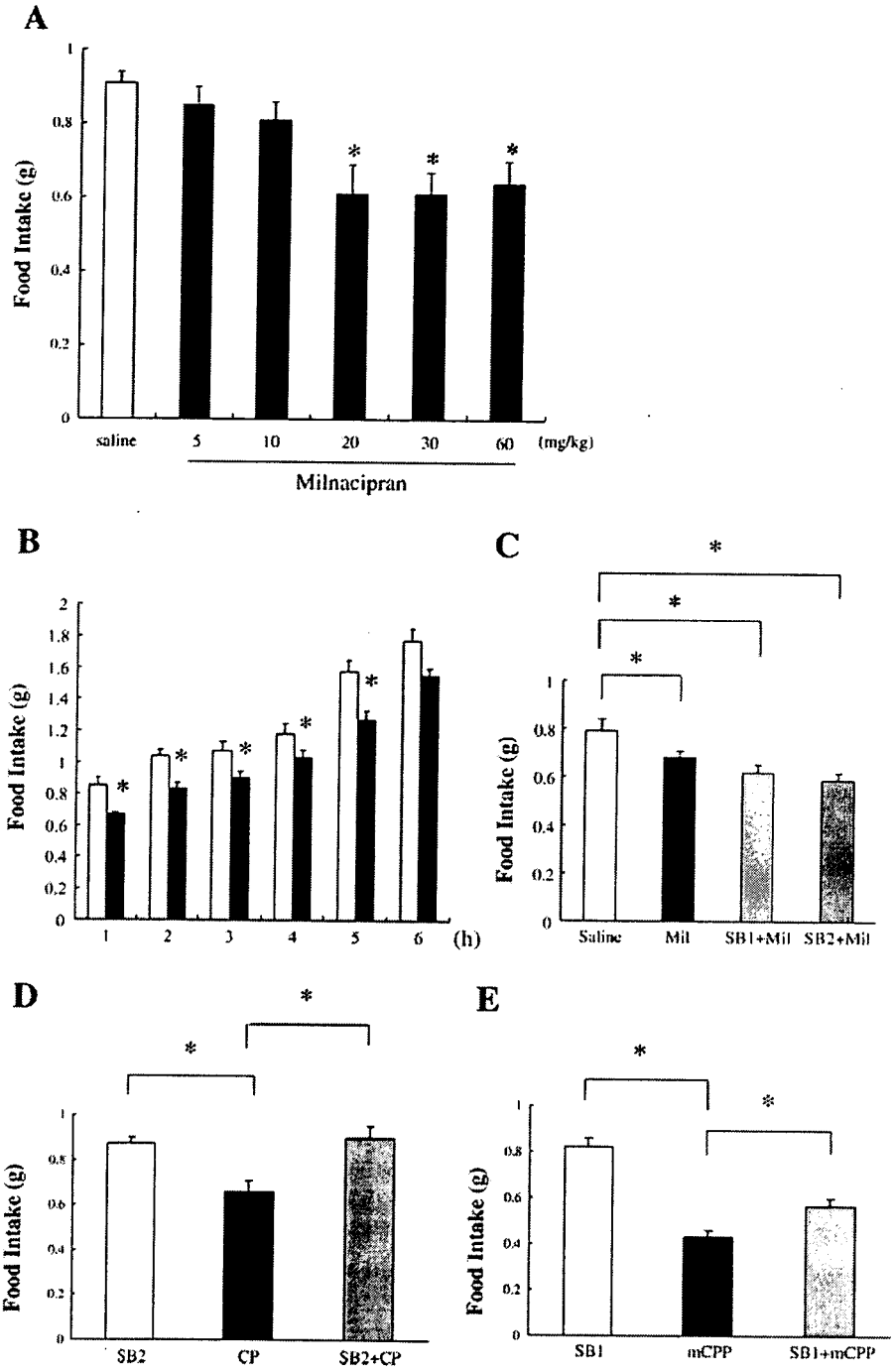

Fig. 1. Dose-response effects of milnacipran on food intake (*A*), time course of the effects of milnacipran (30 mg/kg) on food intake (*B*), and the effects of a selective 5-HT2C receptor antagonist, SB242084, and a selective 5-HT1B receptor antagonist, SB224289, on the feeding suppression induced by milnacipran (*C*), the effects of SB224289 on the feeding suppression induced by CP94253 (*D*), and the effects of SB242084 on the feeding suppression induced by m-chlorophenylpiperazine (mCPP; *E*) in C57BL6J mice. After a 23-h fast, mice were intraperitoneally injected with milnacipran, CP94253, mCPP, or saline. Open bar, saline; solid bars, milnacipran (3–60 mg/kg). SB1, SB242084 (2 mg/kg); SB2, SB224289 (5 mg/kg); CP, CP94253 (5 mg/kg); mCPP (5 mg/kg). Values are expressed as the means ± SE of 7 or 8 animals. $^*P < 0.05$ vs. saline.

respectively) during the first hour of the feeding period after a 23-h fast (Fig. 2*A*).

To further confirm these results, we determined the effects of milnacipran on food intake in A^y 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. 2*B*). These results suggest that milnacipran suppresses food intake in A^y mice.

Moreover, we determined the chronic effects of milnacipran on food intake and body weight in obese (8-wk-old) A^y mice and wild-type mice. Mice received milnacipran (30 mg/kg) or saline twice daily for three consecutive days. A^y mice had elevated baseline food intake and body weight relative to levels in wild-type mice (food intake: WT 3.5 ± 0.2 g, A^y 5.1 ± 0.1 g; $P < 0.05$; body weight: WT 29.4 ± 0.5 g, A^y 37.5 ± 0.5 g; $P < 0.05$, $n = 14–18$). Milnacipran administration reduced food intake and body weight in both A^y mice and wild-type mice (Fig. 3, *A–D*).

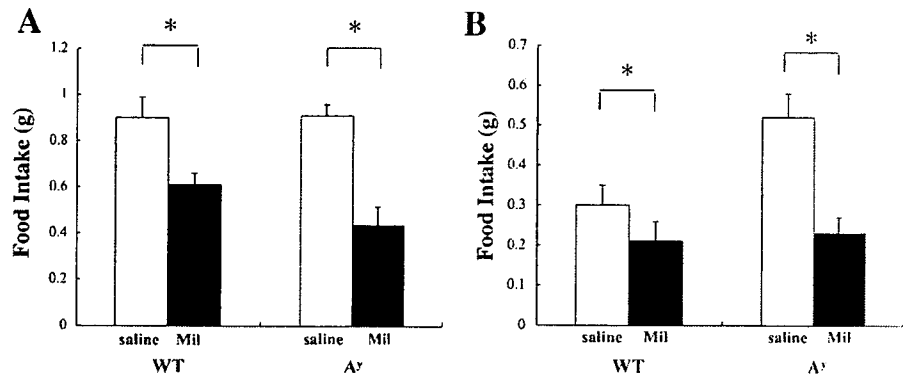

Fig. 2. Effects of milnacipran on food intake in 5-wk-old male A^y mice and wild-type mice. Mice were intraperitoneally injected with milnacipran or saline. Effects of milnacipran (30 mg/kg) on food intake in wild-type mice and A^y mice after a 23-h fast (*A*) and after onset of the dark cycle (*B*). Each column and bar represent the mean ± SE of 7 or 8 mice. Basal body weight: WT saline controls (open bars), 18.8 ± 0.7 g (*A*) and 24.0 ± 0.1 g (*B*); WT milnacipran treatment group (solid bars), 18.7 ± 0.7 g (*A*) and 23.8 ± 0.2 g (*B*); A^y saline controls (open bars), 21.4 ± 0.5 g (*A*) and 24.0 ± 0.3 g (*B*); A^y milnacipran treatment group (solid bars), 21.4 ± 0.3 g (*A*) and 24.1 ± 0.2 g (*B*). WT, wild-type; Mil, milnacipran. *$P < 0.05$.

Effects of restricted feeding on the responsiveness to milnacipran or fenfluramine in 5-wk-old A^y 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^y 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^y mice and wild-type mice. Both A^y 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^y mice, whereas it significantly suppressed food intake in food-restricted wild-type mice (Fig. 4*A*). In contrast, milnacipran had no effects on food-restricted A^y mice and wild-type mice (Fig. 4*B*).

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 anorexic 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. 6*C*). 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 anorexic 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^y 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^y mice are reportedly resistant to the anorexic 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^y mice are responsive to milnacipran-induced feeding suppression and that chronic treatment with

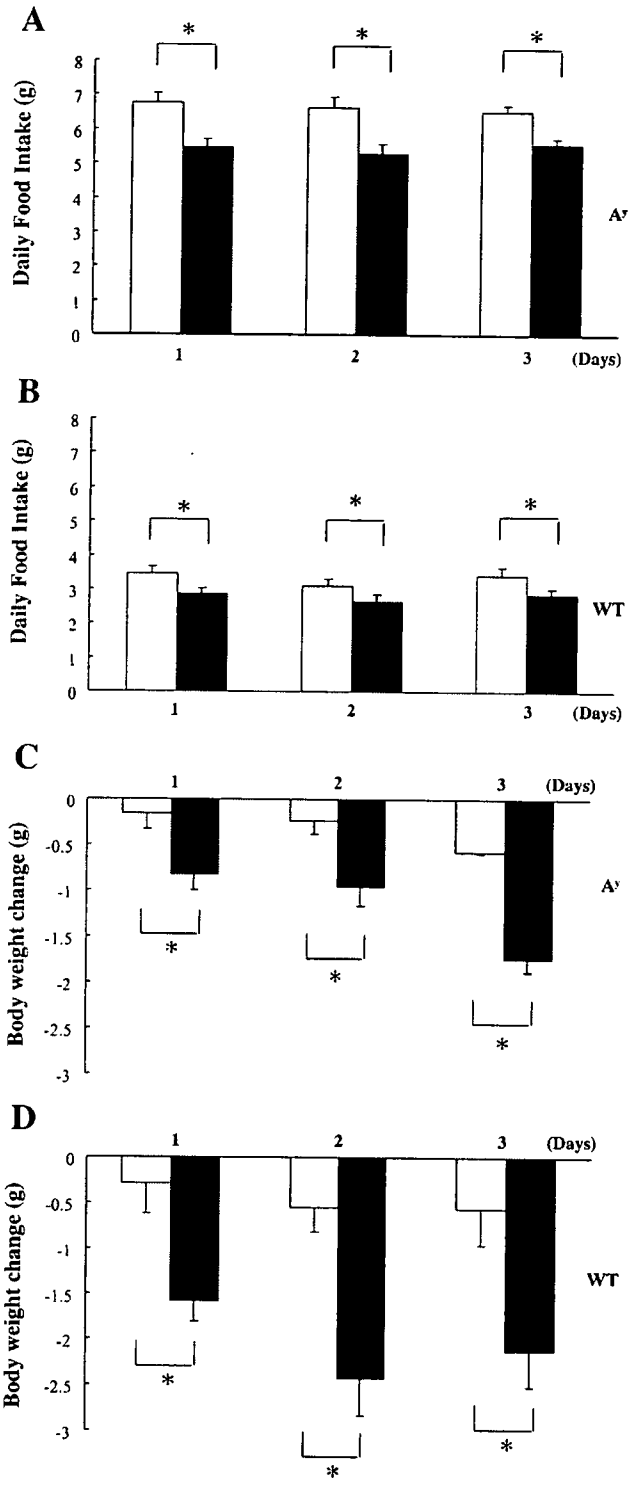

Fig. 3. Effects of chronic administered milnacipran on food intake (*A* and *B*) and body weight (*C* and *D*) in wild-type mice and obese (8-wk-old) A^y mice. Each column and bar represent the mean ± SE of 7–9 mice. Basal body weight: WT saline controls (open bars), 29.0 ± 0.7 g; WT milnacipran treatment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

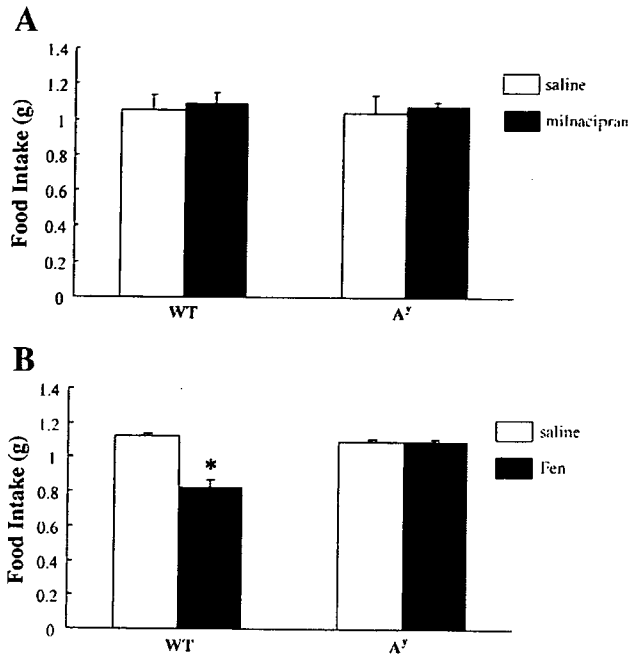

Fig. 4. Effects of restricted feeding on effects of milnacipran (*A*) and fenfluramine (*B*) on food intake in 5-wk-old A^y mice and WT mice. Mice were intraperitoneally injected with milnacipran (30 mg/kg) or fenfluramine (3 mg/kg) or saline. Basal body weight: WT saline controls (open bars), 20.2 ±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

milnacipran suppresses hyperphagia and reduces body weight in obese A^y mice. These findings suggest that milnacipran induces appetite-suppressing effects through different neuronal mechanisms than fenfluramine.

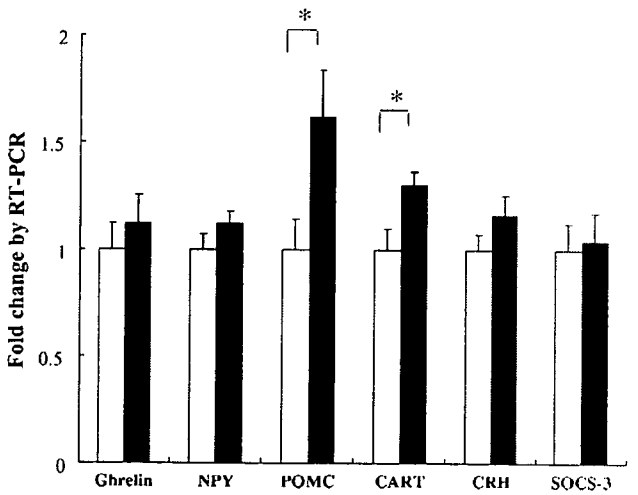

Fig. 5. Effects of milnacipran on hypothalamic ghrelin, neuropeptide Y (NPY), proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), corticotropin-releasing hormone (CRH), and suppressor of cytokine signaling-3 (SOCS-3) mRNA levels in C57BL6J mice. Mice were intraperitoneally 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. $^*P < 0.05$.

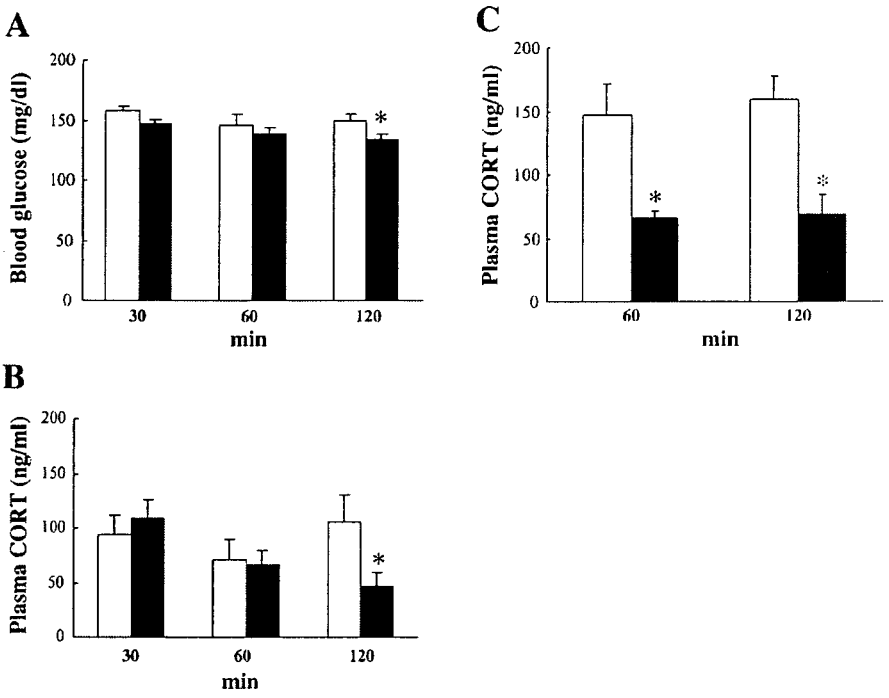

Fig. 6. Effects of milnacipran on and blood glucose levels (*A*) and plasma corticosterone (CORT) levels (*B*) in the light cycle, and plasma corticosterone levels (*C*) in the dark cycle of C57BL6J mice. Mice were intraperitoneally injected with milnacipran or saline. Blood glucose and plasma corticosterone levels were measured as described in the MATERIALS AND METHODS. Each column and bar represent the mean ± SE of 5 mice. Open bar, saline; solid bars, milnacipran (30 mg/kg). $*P < 0.05$.

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^y mice are sensitive to MC agonist-induced feeding suppression (8). In addition, CART might contribute to the milnacipran-induced feeding suppression in A^y 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-

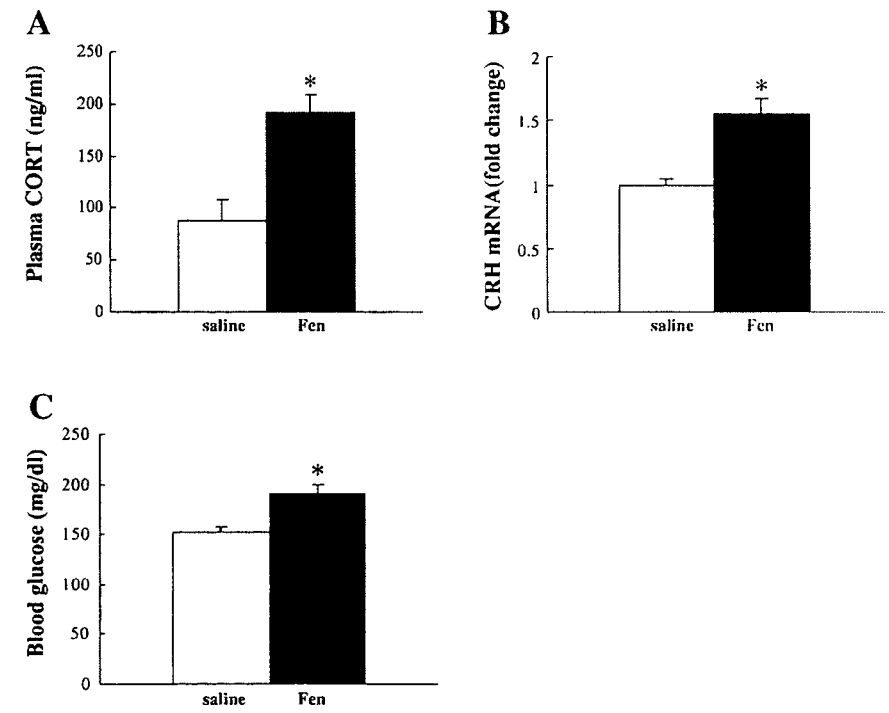

Fig. 7. Effects of fenfluramine (Fen) on plasma corticosterone levels (*A*), hypothalamic CRH mRNA levels (*B*), and blood glucose levels (*C*) in C57BL6J mice. Mice were intraperitoneally injected with milnacipran or saline. Blood glucose and plasma corticosterone levels were measured as described in the MATERIALS AND METHODS. Each column and bar represent the mean ± SE of 5 or 6 mice. Open bar, saline; solid bars, Fen (3 mg/kg). $*P < 0.05$.

sion of milnacipran without stimulating the HPA and sympathetic-adrenal medullary axis.

Other investigators previously reported that A^y mice are resistant to the anorexic effects of fenfluramine (12). Our previous and present studies, however, demonstrated that hyperphagic A^y mice are responsive to mCPP and fenfluramine-induced appetite suppression, whereas food-restricted A^y 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^y 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^y mice.

In addition, our present study demonstrate that hyperphagic A^y mice are responsive to milnacipran-induced appetite suppression, whereas food restricted A^y 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 the HPA axis or increasing blood glucose levels in mice. Restricted feeding can attenuate the milnacipran-induced appetite-suppressing effects.

ACKNOWLEDGMENTS

We thank Youko Hasegawa for the experimental assistance. This work was supported by a Grant-in-Aid for Scientific Research (C2) and Human Science Research (KH21016).

REFERENCES

1. **Boyer P, Briley M.** Milnacipran, a new specific serotonin and noradrenaline reuptake inhibitor. *Drugs Today* 34: 709–720, 1998.
2. **Broberger C.** Hypothalamic cocaine- and amphetamine-regulated transcript (CART) neurons: histochemical relationship to thyrotropin-releasing hormone, melanin concentrating hormone, orexin/hypocretin and neuropeptide Y. *Brain Res* 848: 101–113, 1999.
3. **Chaouloff F, Gunn SH, Young JB.** Central 5-hydroxytryptamine2 receptors are involved in the adrenal catecholamine-releasing and hyperglycemic effects of the 5-hydroxytryptamine indirect agonist D-fenfluramine in the conscious rat. *J Pharmacol Exp Ther* 260: 1008–1016, 1992.
4. **Chaouloff F.** Physiopharmacological interactions between stress hormones and central serotonergic systems. *Brain Res Brain Res Rev* 18: 1–32, 1993.
5. **Ebihara K, Ogawa Y, Katsuura G, Numata Y, Masuzaki H, Satoh N, Tamaki M, Yoshioka T, Hayase M, Matsuoka N, Aizawa-Abe M, Yoshimasa Y, Nakao K.** Involvement of agouti-related protein. An endogenous antagonist of hypothalamic melanocortin receptor, in leptin action. *Diabetes* 48: 2028–2033, 1999.
6. **El-Giamal N, de Zwaan M, Bailer U, Strnad A, Schussler P, Kasper S.** Milnacipran in the treatment of bulimia nervosa: a report of 16 cases. *Eur Neuropsychopharmacol* 13: 73–79, 2003.
7. **Elias CF, Lee CE, Kelly JF, Ahima RS, Kuhar M, Saper CB, Elmquist JK.** Characterization of CART neurons in the rat and human hypothalamus. *J Comp Neurol* 432: 1–19, 2001.
8. **Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD.** Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 385: 165–168, 1997.
9. **Fone KC, Topham IA.** Alteration in 5-hydroxytryptamine agonist-induced behaviour following a corticosterone implant in adult rats. *Pharmacol Biochem Behav* 71: 815–823, 2002.
10. **Friedman JM.** Modern science versus the stigma of obesity. *Nat Med* 10: 563–569, 2004.
11. **Gehlert DR, Dreshfield L, Tinsley F, Benvenga MJ, Gleason S, Fuller RW, Wong DT, Hemrick-Luecke SK.** The selective norepinephrine reuptake inhibitor, LY368975, reduces food consumption in animal models of feeding. *J Pharmacol Exp Ther* 287: 122–127, 1998.
12. **Heisler LK, Cowley MA, Tecott LH, Fan W, Low MJ, Smart JL, Rubinstein M, Tatro JB, Marcus JN, Holstege H, Lee CE, Cone RD, Elmquist JK.** Activation of central melanocortin pathways by fenfluramine. *Science* 297: 609–611, 2002.
13. **Kennet GA, Wood MD, Bright F, Trail B, Riley G, Holland V, Avenell KY, Stean T, Upton N, Bromidge S, Forbes IT, Brown AM, Middlemiss DN, Blackburn TP.** SB242084, a selective and brain penetrant 5-HT2C receptor antagonist. *Neuropharmacology* 36: 609–620, 1997.
14. **Laflamme N, Bovetto S, Richard D, Rivest S.** Effect of dexfenfluramine on the transcriptional activation of CRF and its type 1 receptor within the paraventricular nucleus of the rat hypothalamus. *Br J Pharmacol* 117: 1021–1034, 1996.
15. **Lee MD, Somerville EM, Kennett GA, Dourish CT, Clifton PG.** Tonic regulation of satiety by 5-HT receptors in the mouse: converging evidence from behavioural and c-fos immunoreactivity studies? *Eur J Neurosci* 19: 3017–3025, 2004.
16. **Lee MD, Somerville EM, Kennett GA, Dourish CT, Clifton PG.** Reduced hypophagic effects of D-fenfluramine and the 5-HT2C receptor agonist mCPP in 5-HT1B receptor knockout mice. *Psychopharmacology (Berl)* 176: 39–49, 2004.
17. **Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO, Cone RD.** Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* 371: 799–802, 1994.
18. **Mochizuki D, Tsujita R, Yamada S, Kawasaki K, Otsuka Y, Hashimoto S, Hattori T, Kitamura Y, Miki N.** Neurochemical and behavioural characterization of milnacipran, a serotonin and noradrenaline reuptake inhibitor in rats. *Psychopharmacology (Berl)* 162: 323–332, 2002.
19. **Nonogaki K, Iguchi A.** Role of central neural mechanisms in the regulation of hepatic glucose metabolism. *Life Sci* 60: 797–807, 1997.
20. **Nonogaki K, Iguchi A.** Stress, acute hyperglycemia, and hyperlipidemia: role of the autonomic nervous system and cytokines. *Trends Endocrinol Metab* 8: 192–197, 1997.
21. **Nonogaki K, Ohashi-Nozue K, Oka Y.** A negative feedback system between brain serotonin systems and plasma active ghrelin levels in mice. *Biochem Biophys Res Commun* 341: 703–707, 2006.
22. **Nonogaki K, Nozue K, Takahashi Y, Yamashita N, Hiraoka S, Kumano H, Kuboki T, Oka Y.** Fluvoxamine, a selective serotonin reuptake inhibitor, and 5-HT2C receptor inactivation induce appetite-suppressing effects in mice via 5-HT1B receptors. *Int J Neuropsychopharmacol* In press.
23. **Nonogaki K, Nozue K, Oka Y.** Hyperphagia alters expression of hypothalamic 5-HT2C and 5-HT1B receptor genes and plasma des-acyl ghrelin levels in A^y mice. *Endocrinology* 147: 5893–5900, 2007.
24. **Puech A, Montgomery SA, Prost JF, Solles A, Briley M.** Milnacipran, a new serotonin and noradrenaline reuptake inhibitor: an overview of its antidepressant activity and clinical tolerability. *Int Clin Psychopharmacol* 12: 99–108, 1997.
25. **Redmond AM, Kelly JP, Leonard BE.** The determination of the optimal dose of milnacipran in the olfactory bulbectomized rat model of depression. *Pharmacol Biochem Behav* 62: 619–623, 1999.
26. **Sugimoto Y, Yamada J, Yoshikawa T, Horisaka K.** Effects of the 5-HT2C/2B receptor agonist 1-(3-chlorophenyl) piperazine on plasma glucose levels of rats. *Eur J Pharmacol* 307: 75–80, 1996.
27. **Tecott LH, Sun LM, Akana SF, Strack AM, Lowenstein DH, Dallman MF, Julius D.** Eating disorder and epilepsy in mice lacking 5-HT2C serotonin receptors. *Nature* 374: 542–546, 1995.
28. **Vickers SP, Clifton PG, Dourish CT, Tecott LH.** Reduced satiating effect of D-fenfluramine in serotonin 5-HT(2C) receptor mutant mice. *Psychopharmacology (Berl)* 143: 309–314, 1999.
29. **Vickers SP, Dourish CT, Kennett GA.** Evidence that hypophagia induced by D-fenfluramine and D-norfenfluramine in the rat is mediated by 5-HT2C receptors. *Neuropharmacology* 41: 200–209, 2001.

0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(5):2006–2015

doi: 10.1210/en.2006-1351

Bone Marrow (BM) Transplantation Promotes β-Cell Regeneration after Acute Injury through BM Cell Mobilization

Yutaka Hasegawa,* Takehide Ogihara,* Tetsuya Yamada, Yasushi Ishigaki, Junta Imai, Kenji Uno, Junhong Gao, Keizo Kaneko, Hisamitsu Ishihara, Hironobu Sasano, Hiromitsu Nakauchi, Yoshitomo Oka, and Hideki Katagiri

Division of Advanced Therapeutics for Metabolic Diseases (Y.H., T.O., J.I., K.U., J.G., K.K., H.K.), Center for Translational and Advanced Animal Research, Division of Molecular Metabolism and Diabetes (Y.H., T.Y., Y.I., J.I., K.U., J.G., K.K., H.I., Y.O.), and Department of Pathology (H.S.), Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan; and Laboratory of Stem Cell Therapy (H.N.), Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

There is controversy regarding the roles of bone marrow (BM)-derived cells in pancreatic β-cell regeneration. To examine these roles *in vivo*, mice were treated with streptozotocin (STZ), followed by bone marrow transplantation (BMT; lethal irradiation and subsequent BM cell infusion) from green fluorescence protein transgenic mice. BMT improved STZ-induced hyperglycemia, nearly normalizing glucose levels, with partially restored pancreatic islet number and size, whereas simple BM cell infusion without preirradiation had no effects. In post-BMT mice, most islets were located near pancreatic ducts and substantial numbers of bromodeoxyuridine-positive cells were detected in islets and ducts. Importantly, green fluorescence protein-positive, *i.e.* BM-derived, cells were detected around islets and were CD45 positive but not insulin positive. Then to examine whether BM-derived cell mobilization contributes to this process, we used $Nos3^{-/-}$ mice as a model of impaired BM-derived cell mobilization. In streptozotocin-treated $Nos3^{-/-}$ mice, the effects of BMT on blood glucose, islet number, bromodeoxyuridine-positive cells in islets, and CD45-positive cells around islets were much smaller than those in streptozotocin-treated $Nos3^{+/+}$ controls. A series of BMT experiments using $Nos3^{+/+}$ and $Nos3^{-/-}$ mice showed hyperglycemia-improving effects of BMT to correlate inversely with the severity of myelosuppression and delay of peripheral white blood cell recovery. Thus, mobilization of BM-derived cells is critical for BMT-induced β-cell regeneration after injury. The present results suggest that homing of donor BM-derived cells in BM and subsequent mobilization into the injured periphery are required for BMT-induced regeneration of recipient pancreatic β-cells. (*Endocrinology* 148: 2006–2015, 2007)

SEVERAL LINES OF evidence indicate that bone marrow (BM)-derived cells are capable of transdifferentiating into various cell types, including endothelial cells, arterial smooth muscle cells, myoblasts, myocardium, and epithelia of the gastrointestinal tract (1–6). In the field of regenerative medicine for diabetes treatment, BM cells are seen as promising pancreatic β-cell sources (7–9). However, whether BM cells can transdifferentiate into β-cells and/or stimulate β-cell differentiation is controversial.

A previous study (10) showed that BM-derived cells can directly transdifferentiate into β-cells. In that report, 4–6 wk after BM transplantation (BMT; *i.e.* lethal irradiation of recipient mice and subsequent BM cell infusion from other mice), donor BM-derived insulin-positive cells were detected in 1.7–3% of pancreatic islet cells. However, in subsequent similar studies (11–13), very few or no donor BM-derived insulin-positive cells were detected in recipient islets, suggesting that if direct transdifferentiation from BM-derived cells into β-cells occurs, it would involve only a very small percentage of cells. BM-derived cells also reportedly initiate recipient β-cell regeneration rather than directly transdifferentiating into β-cells (14). In that study, BMT increased recipient β-cells with the appearance of donor-derived endothelial cells in the pancreas, resulting in improvement of hyperglycemia in streptozotocin (STZ)-induced diabetic mice. Other studies also demonstrated that BMT improves hyperglycemia in diabetic animals such as STZ-treated mice (15) and rats (16), E2f1/E2f2 mutant mice (17), and KKAy mice (18). However, several studies obtained contradictory results, *i.e.* no improvement in hyperglycemia after BMT (12, 19). Whether BMT promotes β-cell regeneration and improves hyperglycemia in diabetic mice and, if so, how β-cells are regenerated remains essentially unknown. Herein we attempted to address these questions.

First, we observed that BMT, but not simple BM cell infusion without preirradiation, restored islet numbers and improved hyperglycemia in STZ-treated mice. Donor-derived cells were detected around post-BMT islets and were

First Published Online January 25, 2007

* Y.H. and T.O. contributed equally to this work.

Abbreviations: BM, Bone marrow; BMT, BM transplantation; BrdU, bromodeoxyuridine; eNOS, endothelial nitric oxide synthase; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GFP, green fluorescence protein; MMP, matrix metalloproteinase; PECAM, platelet endothelial cell adhesion molecule; sKitL, soluble kit-ligand; STZ, streptozotocin; WBC, white blood cell.

Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

CD45 (pan-hematopoietic marker) positive, suggesting that mobilization of BM-derived cells to the pancreas induces β-cell regeneration. To examine this hypothesis, we performed BMT experiments using endothelial nitric oxide synthase (eNOS)-deficient ($Nos3^{-/-}$) mice, in which mobilization of BM-derived cells after myelosuppression is impaired (20). In STZ-treated $Nos3^{-/-}$ mice, BMT effects on β-cell regeneration and improvement of hyperglycemia were very limited. Thus, BM-derived cell mobilization is apparently involved in BMT-induced β-cell regeneration after acute injury.

Materials and Methods

Animals

C57BL/6J mice were purchased from Clea Japan, Inc. (Tokyo, Japan). Green fluorescent protein (GFP) transgenic mice with the C57BL/6J background were kindly provided by Dr. M. Okabe (Osaka University, Osaka, Japan) (21). Enhanced GFP is under transcriptional control of the chicken β-actin promoter and the cytomegalovirus enhancer in this strain, resulting in high-level expression in most tissues. $Nos3^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Age- and sex-matched wild-type ($Nos3^{+/+}$) littermates served as controls. These animals were generated and have been maintained with a C57BL/6J background by backcrossing of hemizygous carriers to C57BL/6J for more than six generations. Mice were housed in an air-conditioned environment, with a 12-h light, 12-h dark cycle, and fed a regular unrestricted diet. Hyperglycemia was induced by ip infusion of 35 mg/kg body weight STZ (Sigma-Aldrich, St. Louis, MO) daily for 8 d [modification of method reported by Wang *et al.* (22)]. STZ was solubilized in citrate sodium buffer (pH 4.5) and injected, within 15 min after preparation, into 6-wk-old mice. All animal experiment procedures were approved by our Institutional Review Board, Tohoku University School of Medicine, and conducted according to institutional guidelines for animal experiments.

Measurements

Blood glucose was measured after a 10-h fast and assayed using Antsense II (Horiba Industry, Kyoto, Japan). Plasma insulin was determined with an ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan). Insulin content was measured as described previously (23).

BMT

BM cells were flushed in bulk from the medullary cavities of femurs and tibias. BM donors were young (6 wk old) sex-matched GFP transgenic mice and $Nos3^{-/-}$ or $Nos3^{+/+}$ mice. Recipient mice were lethally irradiated (10 Gy) and reconstituted a single iv infusion of 2×10^6 BM cells, from donor mice through the tail vein. Tissues were analyzed 30–40 d after BMT. The percentage of GFP-positive cells among recipient BM cells was determined by fluorescence-activated cell sorting (FACS), using a FACS Caliber with CellQuest software (BD PharMingen, Franklin Lakes, NJ).

Bromodeoxyuridine (BrdU) in situ detection

To identify proliferating cells in the pancreas, BrdU was injected according to the BrdU *in situ* detection kit protocol (BD Bioscience, San Jose, CA). Mice were injected ip with 1 mg BrdU 24 h before pancreas extraction at 0, 3, 7, 10, 15, or 25 d after BMT. The labeled cells were immunostained with anti-BrdU antibody. To calculate numbers of islets and cells per islet and the percentage of BrdU-positive cells among islet cells, we microscopically examined the whole pancreas in 30-μm sections and counted the numbers of islets, islet cells, and BrdU-positive nuclei in islets.

Immunohistochemistry

Mouse pancreases were excised and fixed overnight in 10% paraformaldehyde. Fixed tissues were processed for paraffin embedding and 3-μm sections were prepared. The streptavidin-biotin method was performed with a Histofine streptavidin-biotin-PO kit (Nichirei, Tokyo, Japan) for immunostaining using antibody against insulin (Sigma-Aldrich) or GFP (Santa Cruz Biotechnology, Santa Cruz, CA). Slides were deparaffinized and immediately exposed to the blocking solution. Sections were incubated for 18 h at 4 C with antibody against human insulin or GFP diluted 1:1000 in PBS. Slides were incubated with the biotinylated IgG for 1 h and then peroxidase-conjugated streptavidin for 30 min at room temperature. Finally, immunoreactivity was visualized by incubation with a substrate solution containing 3,3′-diaminobenzidine tetrahydrochloride. For double staining of insulin and BrdU, the streptavidin-peroxidase method was applied, followed by incubation with Simple stain 3-amino-9-ethyl carboxazole solution (Nichirei).

Fluorescent immunohistochemistry

For double staining of insulin with glucagon, keratin/cytokeratin, or CD45, the 3-μm sections of paraffin-embedded pancreases were incubated overnight with the respective antibodies at 4 C. Antibodies against insulin, glucagon (Dako Corp., Carpinteria, CA), keratin/cytokeratin (Nichirei, and CD45 (Santa Cruz Biotechnology) were diluted 1:1000 in PBS. For platelet endothelial cell adhesion molecule (PECAM)-1 staining, sections were immunostained with rat anti-CD31 (1:10; BD Biosciences). Labeled cells were visualized with a biotin-conjugated secondary antibody with streptavidin, TX red conjugate (Vector Laboratories, Burlingame, CA). For double staining of insulin with glucagon or keratin/cytokeratin, the sections were incubated for 1 h at room temperature in a mixture of Alexa Fluor 488 goat chicken antimouse IgG (Molecular Probes, Eugene, OR) diluted 1:100 and Alexa Fluor 594 donkey antirabbit diluted 1:50 in PBS. For double staining of insulin and CD45, the sections were incubated in a mixture of Alexa Fluor 488 chicken antimouse IgG and Alexa Fluor 546 goat antirabbit IgG diluted 1:1000 in PBS. Sections were observed under a fluorescence microscope, LSM 5 PASCAL (Carl Zeiss, Oberkochen, Germany) and the image was analyzed using the PASCAL system.

Statistical analysis

Data are expressed as means ± SE. Differences between experimental groups were evaluated using the unpaired Student's t test for several independent observations. $P < 0.05$ was considered significant.

Results

Recipient BM was replaced with donor cells after irradiation followed by BM cell infusion but not after simple BM cell infusion without preirradiation

Six-week-old C57BL/6J mice were given STZ daily for 8 d, followed by lethal irradiation and subsequent infusion of BM cells (STZ+BMT mice). In these experiments, BM cells were obtained from GFP transgenic mice (Fig. 1A). A group of STZ-treated mice was simply infused with the same number (2×10^6) of BM cells without preirradiation (STZ+BM-infused mice). First, we confirmed replacement of recipient BM with that of donor mice using fluorescence microscopy and FACS analysis. As shown in Fig. 1B, there were no GFP-positive cells in the BM of C57BL/6J mice, whereas nearly all BM cells from GFP mice were GFP positive. BM cells of STZ+BMT mice showed high donor chimerism, indicating the recipient BM to have essentially been replaced with donor BM cells. In contrast, STZ+BM-infused mice had no donor-derived GFP cells in their BM, suggesting that preirradiation is necessary for BM replacement.

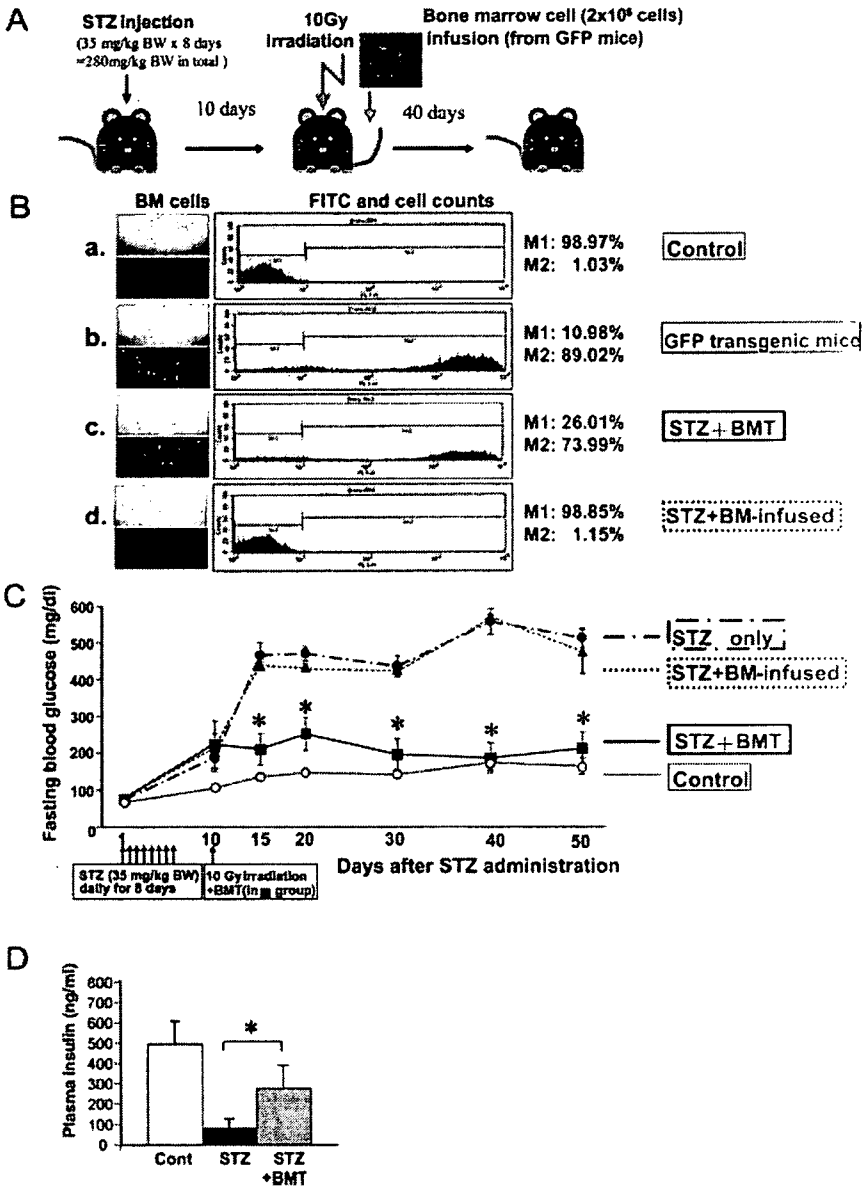

FIG. 1. BMT in STZ-treated mice. A, Experimental protocol. B, Bone marrow chimerism. Bright field (*left upper panels*), FITC (*left lower panels*), and representative FACS analyses of bone marrow chimerism from C57BL/6J mice (a), GFP transgenic mice (b), STZ-treated mice receiving lethal irradiation and BMT from GFP transgenic mice (c), and STZ-treated mice infused with BM cells of GFP transgenic mice, without preirradiation (d) (*right panels*) are shown. C, Fasting blood glucose of STZ-treated mice with or without BMT. ▲, STZ-treated mice without BMT (hyperglycemic control); ■, STZ-treated mice receiving BMT (lethal irradiation and subsequent BM cell infusion from GFP transgenic mice); ●, STZ-treated mice infused with BM cells of GFP transgenic mice, without preirradiation; ○, mice with neither STZ nor BMT (normoglycemic control). *, $P < 0.05$ for ■, compared with ▲ group (n = 5–6 in each group). D, Fasting plasma insulin on d 40. Cont, Mice with neither STZ nor BMT (normoglycemic control); STZ, STZ-treated mice without BMT (hyperglycemic control); STZ+BMT, STZ-treated mice receiving BMT. *, $P < 0.05$ for STZ+BMT, compared with STZ group (n = 5–6 in each group).

BMT, but not simple BM cell infusion without preirradiation, improved hyperglycemia in STZ-treated mice

As shown in Fig. 1C, STZ-treated mice receiving neither irradiation nor BM cell infusion (hyperglycemic controls) showed markedly higher fasting blood glucose than mice without STZ treatment (normoglycemic controls). Notably, blood glucose levels of STZ+BMT mice were significantly lower than those of hyperglycemic controls. Forty days after the first STZ administration, blood glucose levels of STZ+BMT mice were similar to those of normoglycemic controls. However, blood glucose levels of STZ+BM-infused mice did not decrease, instead remaining similar to those of hyperglycemic controls for 50 d after STZ administration. We additionally examined the effects of BMT, performed 30 d after STZ treatment. This late BMT did not significantly decrease blood glucose levels (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Together, these findings suggest that BMT improves hyperglycemia after acute injury of pancreatic β-cells with STZ treatment.

Next, we measured fasting plasma insulin levels on d 40 (Fig. 1D) in STZ+BMT mice. STZ administration markedly decreased plasma insulin levels, whereas BMT partially but significantly restored these levels by d 40.

STZ administration followed by BMT increased pancreatic islets in the vicinity of pancreatic ducts

We histologically analyzed pancreatic islets in the four groups. With hematoxylin-eosin staining on d 35, islet number and size were markedly decreased in hyperglycemic (Fig. 2A, b and f), as compared with normoglycemic (Fig. 2A, a and e), controls. Whereas simple BM infusion without preirradiation did not reverse the diminished number and size of