ATF4, the primary inducer of *Eif4ebp1* under ER stress, is activated by translational suppression by eIF2α phosphorylation during the acute phase. We found that 4E-BP1 protein is stable with a half-life of approximately 20 hr (Figure S6). Thus, 4E-BP1 protein seems to continue to be expressed abundantly during the later stages of the UPR. This is consistent with the recent observation that several prosurvival proteins involved in the UPR are stable, while proapoptotic proteins are not (Rutkowski et al., 2006). We found that global protein synthesis was higher in 4E-BP1-deficient β cells than in wild-type cells under ER stress conditions. In particular, expression of CHOP was augmented in 4E-BP1 deficiency. Enhanced CHOP expression in 4E-BP1-deficient cells suggests that a reduction in eIF4E availability due to 4E-BP1 induction suppresses CHOP translation during ER stress in wild-type cells, possibly accounting for one of the mechanisms by which 4E-BP1 plays a role in adaptation to ER stress. Important roles of translational control via eIF4E availability have also been suggested in prolonged hypoxia (Koritzinsky et al., 2006). However, the signaling mechanisms for translational control are different: ER stress increases 4E-BP1 protein levels via ATF4 in β cells, while hypoxia enhances 4E-BP1 activity via dephosphorylation and also causes eIF4E nuclear localization in HeLa cells.

The present results also suggest that variations in genes regulating eIF4E availability and/or eIF4F formation may have an impact on susceptibility to diabetes. In this context, a recent report demonstrating that a gene encoding eIF4A2, a component of eIF4F, is possibly linked to type 2 diabetes in French families (Cheyssac et al., 2006) is of great interest. Furthermore, our findings raise the possibility that 4E-BP1 may be a potential target for diabetes mellitus treatment.

EXPERIMENTAL PROCEDURES

Animal Experiments

All animal experiments were approved by the Tohoku University Institutional Animal Care and Use Committee. *Wfs1* $^{-/-}$ mice were backcrossed to a 129S6 (Taconic) background for six generations. *Ins2*$^{WT/C96Y}$ mice (Charles River Laboratories) were backcrossed to a 129S6 background for five generations. *Eif4ebp1*$^{-/-}$ mice were maintained on a 129S6 background. Only male mice were used. For the in vivo studies shown in Figures 4A, 4C, and 4D, littermates from crosses of male *Ins2*$^{WT/C96Y}$ *Eif4ebp1*$^{+/-}$ and female *Ins2*$^{WT/WT}$ *Eif4ebp1*$^{+/-}$ mice were used. For Figures 4B, 4E, and 4F, littermates from intercrosses of *Eif4ebp1*$^{+/-}$ *Wfs1*$^{+/+}$ mice and littermates from intercrosses of *Eif4ebp1*$^{+/-}$ *Wfs1*$^{-/-}$ mice were used. For isolated islet experiments (Figures 4G and 4H), age-matched nonlittermate mice were used. To induce ER stress in vivo, mice were given a 0.5 μg/g body weight intraperitoneal injection of tunicamycin. After 96 hr, kidneys and livers were removed. Tissue sample processing, immunostaining of pancreatic sections, and determination of β cell area and pancreatic insulin content were performed as described previously (Ishihara et al., 2004).

Cell Culture and Cell Viability Assay

Pancreatic tumors in *Eif4ebp1* $^{-/-}$:SV40Tag mice on a mixed background were excised, yielding MIN6*Eif4ebp1* $^{-/-}$ cells, which were used at 5–10 passages in this study. MIN6 cells were cultured in DMEM supplemented with 15% FCS. *Atf4* $^{-/-}$ MEFs were cultured in DMEM supplemented with a nonessential amino acid mixture and 10% FCS. Cells seeded in 24-well plates 2 days previously were treated with thapsigargin or tunicamycin and used for western blotting or cell viability assay. Cell viability was determined with a cell proliferation assay kit (Promega). Construction of adenoviruses and infection of MIN6 cells were performed as described previously (Ishihara et al., 2004).

Northern and Western Blotting and Cap-Binding Affinity Assay

Total RNA extracted using ISOGEN (Nippon Gene) was probed with ^{32}P-labeled cDNAs. Tissue homogenates and cell lysates were subjected to SDS-PAGE and probed with primary antibodies against 4E-BP1, 4E-BP2, eIF4E, eIF4G, cleaved caspase-3 (Cell Signaling), ATF4, CHOP (Santa Cruz), and actin (Sigma). Cell lysates were incubated with 7-methyl-GTP (^{7}mGTP)-Sepharose (Amersham) overnight at 4°C. The ^{7}mGTP-Sepharose was then pelleted and boiled. Experiments were performed at least three times. Band intensity was quantified using Scion Image software.

Metabolic Labeling

Due to the low islet yields from *Ins2*$^{WT/C96Y}$, *Ins2*$^{WT/C96Y}$ *Eif4ebp1*$^{-/-}$, *Wfs1*$^{-/-}$, and *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ mice, islets with these genotypes were pooled from two or three mice. Fifty to eighty islets were cultured for 3 days in RPMI supplemented with 10% FCS. Islets washed with methionine/cysteine-free RPMI containing 10% dialyzed FCS were labeled with a protein labeling mix (PerkinElmer) (1.0 MBq/tube) for 15 min and then resolved in sample buffer (1.0 μl/islet for wild-type and *Eif4ebp1*$^{-/-}$ islets and 0.75 μl/islet for other genotypes). The level of protein synthesis was quantified from autoradiograms. For measurement of *Chop* translation, 4 × 10^6 cells treated with thapsigargin for 12 hr were washed with methionine/cysteine-free DMEM containing 15% dialyzed FCS and labeled with [^{35}S]methionine/cysteine (20 MBq/bottle) for 2 hr. Cells were then resolved in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, and protease inhibitors [Roche]). Lysates were precleared with Protein A Sepharose Fast Flow (Amersham) and incubated with anti-CHOP antibody (R-20, Santa Cruz) overnight.

Firefly Luciferase Reporter Assay

Oligonucleotides containing ATF4 binding sites were annealed and subcloned into the pGL3-Promoter vector (BamHI-SalI, Promega). MIN6 cells were transfected with luciferase reporters using Lipofectamine (Invitrogen). Luciferase activity was assayed with a dual-luciferase system (Promega) using a luminometer (Berthold).

Chromatin Immunoprecipitation Assay

Proteins bound to DNA were crosslinked with 1% formaldehyde at 4°C for 20 min. After sonication, the protein-DNA complexes were immunoprecipitated using an anti-ATF4 antibody (C-20, Santa Cruz). After reversal of the crosslinks at 65°C for 6 hr, DNA was purified on a DNA purification column (QIAGEN). PCR was performed with the primers 5′-GATGAGGAAGAGGAAGCTGAGT TG-3′ and 5′-AGTTGTAAGAGGAGTAGTTGGGGG-3′.

Statistical Analysis

Data are presented as means ± SEM. Differences between groups were assessed by Student's t test. $p < 0.05$ was considered significant.

SUPPLEMENTAL DATA

Supplemental Data include six figures and Supplemental References and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/7/3/269/DC1/.

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(H) [^{35}S]methionine/cysteine labeling as in (G) in islets of the indicated genotypes at 6–8 weeks of age. Lane 1, wild-type; lane 2, *Eif4ebp1* $^{-/-}$; lane 3, *Wfs1* $^{-/-}$; lane 4, *Eif4ebp1* $^{-/-}$ *Wfs1* $^{-/-}$. Data from three experiments are summarized in the right panel. *$p < 0.05$.
Error bars represent SEM.

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REFERENCES

Butler, A.E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R.A., and Butler, P.C. (2003). β-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes *52*, 102–110.

Cheyssac, C., Dina, C., Lepretre, F., Vasseur-Delannoy, V., Dechaume, A., Lobbens, S., Balkau, B., Ruiz, J., Charpentier, G., Pattou, F., et al. (2006). EIF4A2 is a positional candidate gene at the 3q27 locus linked to type 2 diabetes in French families. Diabetes *55*, 1171–1176.

Clemens, M.J. (2001). Translational regulation in cell stress and apoptosis. Roles of the eIF4E binding proteins. J. Cell. Mol. Med. *5*, 221–239.

Delepine, M., Nicolino, M., Barrett, T., Golamaully, M., Lathrop, G.M., and Julier, C. (2000). EIF2AK3, encoding translation initiation factor 2-alpha kinase 3, is mutated in patients with Wolcott-Rallison syndrome. Nat. Genet. *25*, 406–409.

Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell *6*, 1099–1108.

Harding, H.P., Zeng, H., Zhang, Y., Jungries, R., Chung, P., Plesken, H., Sabatini, D.D., and Ron, D. (2001). Diabetes mellitus and exocrine pancreatic dysfunction in perk−/− mice reveals a role for translational control in secretory cell survival. Mol. Cell *7*, 1153–1163.

Harding, H.P., Zhang, Y., Zeng, H., Novoa, I., Lu, P.D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., et al. (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol. Cell *11*, 619–633.

He, C.H., Gong, P., Hu, B., Stewart, D., Choi, M.E., Choi, A.M., and Alam, J. (2001). Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation. J. Biol. Chem. *276*, 20858–20865.

Holcik, M., and Sonenberg, N. (2005). Translational control in stress and apoptosis. Nat. Rev. Mol. Cell Biol. *6*, 318–327.

Ibrahimo, S., Holmes, L.E., and Ashe, M.P. (2006). Regulation of translation initiation by the yeast eIF4E binding proteins is required for the pseudohyphal response. Yeast *23*, 1075–1088.

Inoue, H., Tanizawa, Y., Wasson, J., Behn, P., Kalidas, K., Bernal-Mizrachi, E., Mueckler, M., Marshall, H., Donis-Keller, H., Crock, P., et al. (1998). A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome). Nat. Genet. *20*, 143–148.

Ishihara, H., Takeda, S., Tamura, A., Takahashi, R., Yamaguchi, S., Takei, D., Yamada, T., Inoue, H., Soga, H., Katagiri, H., et al. (2004). Disruption of the WFS1 gene in mice causes progressive beta-cell loss and impaired stimulus-secretion coupling in insulin secretion. Hum. Mol. Genet. *13*, 1159–1170.

Koritzinsky, M., Magagnin, M.G., van den Beucken, T., Seigneuric, R., Savelkouls, K., Dostie, J., Pyronnet, S., Kaufman, R.J., Weppler, S.A., Voncken, J.W., et al. (2006). Gene expression during acute and prolonged hypoxia is regulated by distinct mechanisms of translational control. EMBO J. *25*, 1114–1125.

Laybutt, D.R., Preston, A.M., Akerfeldt, M.C., Kench, J.G., Busch, A.K., Biankin, A.V., and Biden, T.J. (2007). Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. Diabetologia *50*, 752–763.

Miyazaki, J., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y., and Yamamura, K. (1990). Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. Endocrinology *127*, 126–132.

Novoa, I., and Carrasco, L. (1999). Cleavage of eukaryotic translation initiation factor 4G by exogenously added hybrid proteins containing poliovirus 2Apro in HeLa cells: effects on gene expression. Mol. Cell. Biol. *19*, 2445–2454.

Novoa, I., Zeng, H., Harding, H.P., and Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α. J. Cell Biol. *153*, 1011–1022.

Pirot, P., Naamane, N., Libert, F., Magnusson, N.E., Orntoft, T.F., Cardozo, A.K., and Eizirik, D.L. (2007). Global profiling of genes modified by endoplasmic reticulum stress in pancreatic beta cells reveals the early degradation of insulin mRNAs. Diabetologia *50*, 1006–1014.

Riggs, A.C., Bernal-Mizrachi, E., Ohsugi, M., Wasson, J., Fatrai, S., Welling, C., Murray, J., Schmidt, R.E., Herrera, P.L., and Permutt, M.A. (2005). Mice conditionally lacking the Wolfram gene in pancreatic islet beta cells exhibit diabetes as a result of enhanced endoplasmic reticulum stress and apoptosis. Diabetologia *48*, 2313–2321.

Rutkowski, D.T., Arnold, S.M., Miller, C.N., Wu, J., Li, J., Gunnison, K.M., Mori, K., Sadighi Akha, A.A., Raden, D., and Kaufman, R.J. (2006). Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. PLoS Biol. *4*, e374.

Scheuner, D., Mierde, D.V., Song, B., Flamez, D., Creemers, J.W., Tsukamoto, K., Ribick, M., Schuit, F.C., and Kaufman, R.J. (2005). Control of mRNA translation preserves endoplasmic reticulum function in β cells and maintains glucose homeostasis. Nat. Med. *11*, 757–764.

Schroder, M., and Kaufman, R.J. (2005). The mammalian unfolded protein response. Annu. Rev. Biochem. *74*, 739–789.

Strom, T.M., Hortnagel, K., Hofmann, S., Gekeler, F., Scharfe, C., Rabl, W., Gerbitz, K.D., and Meitinger, T. (1998). Diabetes insipidus, diabetes mellitus, optic atrophy and deafness (DIDMOAD) caused by mutations in a novel gene (wolframin) coding for a predicted transmembrane protein. Hum. Mol. Genet. *7*, 2021–2028.

Teleman, A.A., Chen, Y.W., and Cohen, S.M. (2005). 4E-BP functions as a metabolic brake used under stress conditions but not during normal growth. Genes Dev. *19*, 1844–1848.

Tettweiler, G., Miron, M., Jenkins, M., Sonenberg, N., and Lasko, P.F. (2005). Starvation and oxidative stress resistance in Drosophila are mediated through the eIF4E-binding protein, d4E-BP. Genes Dev. *19*, 1840–1843.

Tsukiyama-Kohara, K., Poulin, F., Kohara, M., DeMaria, C.T., Cheng, A., Wu, Z., Gingras, A.C., Katsume, A., Elchebly, M., Spiegelman, B.M., et al. (2001). Adipose tissue reduction in mice lacking the translational inhibitor 4E-BP1. Nat. Med. *7*, 1128–1132.

Wang, J., Takeuchi, T., Tanaka, S., Kubo, S.K., Kayo, T., Lu, D., Takata, K., Koizumi, A., and Izumi, T. (1999). A mutation in the insulin 2 gene induces diabetes with severe pancreatic β-cell dysfunction in the Mody mouse. J. Clin. Invest. *103*, 27–37.

Zhang, P., McGrath, B., Li, S., Frank, A., Zambito, F., Reinert, J., Gannon, M., Ma, K., McNaughton, K., and Cavener, D.R. (2002). The PERK eukaryotic initiation factor 2α kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. Mol. Cell. Biol. *22*, 3864–3874.

Behavioral and Gene Expression Analyses of *Wfs1* Knockout Mice as a Possible Animal Model of Mood Disorder

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Abstract

Wolfram disease is a rare genetic disorder frequently accompanying depression and psychosis. Nonsymptomatic mutation carriers also have higher rates of depression and suicide. Because *WFS1*, the causative gene of Wolfram disease, is located at 4p16, a linkage locus for bipolar disorder, mutations of *WFS1* were suggested to be involved in the pathophysiology of bipolar disorder. In this study, we performed behavioral and gene expression analyses of *Wfs1* knockout mice to assess the validity as an animal model of mood disorder. In addition, the distribution of Wfs1 protein was examined in mouse brain. *Wfs1* knockout mice did not show abnormalities in circadian rhythm and periodic fluctuation of wheel-running activity. Behavioral analysis showed that *Wfs1* knockout mice had retardation in emotionally triggered behavior, decreased social interaction, and altered behavioral despair depending on experimental conditions. Wfs1-like immunoreactivity in mouse brain showed a similar distribution pattern to that in rats, including several nuclei potentially relevant to the symptoms of mood disorders. Gene expression analysis showed down-regulation of *Cdc42ep5* and *Rnd1*, both of which are related to Rho GTPase, which plays a role in dendrite development. These findings may be relevant to the mood disorder observed in patients with Wolfram disease.

Key words: wolframin, Wolfram disease, depression, bipolar disorder, DNA microarray, forced swimming test

1. Introduction

Wolfram disease (Online Mendelian Inheritance in Man [OMIM] 222300) is a rare autosomal recessive neurodegenerative disorder characterized by early-onset diabetes mellitus, progressive optic atrophy, diabetes insipidus, and deafness (Domenech et al., 2006); *WFS1/wolframin* has been identified as the causative gene (Strom et al., 1998, Inoue et al., 1998). Approximately 60% of the patients with Wolfram disease have mental symptoms such as severe depression, psychosis, impulsivity, and aggression (Swift et al., 1990). More importantly, carriers of *WFS1* mutations, who are not affected with Wolfram disease, have a 26-fold higher likelihood of psychiatric hospitalization mainly due to depression (Swift and Swift, 2000). The *WFS1* gene locates at 4p16.1 (Strom et al., 1998, Inoue et al., 1998), a replicated linkage locus of bipolar disorder (Ewald et al., 1998, Detera-Wadleigh et al., 1999, Ewald et al., 2002). Some studies showed that bipolar disorder with psychosis (Als et al., 2004, Cheng et al., 2006) or suicidal behavior (Cheng et al., 2006) is linked with this locus. These lines of evidence suggested the possible role of *WFS1* mutations in the pathophysiology of bipolar disorder and related phenotypes.

To date, mutation screening of the *WFS1* gene has been reported in 84 patients with bipolar disorder, 54 with major depression, 119 with schizophrenia, 100 suicide victims, 3 with schizoaffective disorder, and several other patients with other psychiatric diagnoses (Ohtsuki et al., 2000, Martorell et al., 2003, Torres et al., 2001, Crawford et al., 2002, Evans et al., 2000). However, none of these patients had mutations causing Worfram disease.

Despite the fact that *WFS1* mutations may not be a frequent cause of

mental disorders, the mechanism underlying how *WFS1* mutations lead to mental symptoms in patients with Wolfram disease will shed light on the pathophysiology of mood disorders. Mice lacking the *Wfs1* gene might be useful as a genetic animal model of mood disorders.

The symptoms of Wolfram disease resemble those of mitochondrial diseases and, indeed, initial studies suggested mitochondrial dysfunction in Wolfram disease based on mitochondrial DNA (mtDNA) deletions found in patients (Rotig et al., 1993). However, the protein coded by *WFS1* was found to be localized in endoplasmic reticulum (ER) (Takeda et al., 2001, Philbrook et al., 2005). WFS1 expression was induced by ER stress (Fonseca et al., 2005) or *XBP1* overexpression (Kakiuchi et al., 2006), and disruption of *Wfs1* caused a dysfunctional ER stress response (Fonseca et al., 2005, Riggs et al., 2005, Yamada et al., 2006). Recent studies have provided insight into the function of WFS1 protein; WFS1 induces cation channel activity on ER membranes (Osman et al., 2003) and regulates calcium levels in ER (Takei et al., 2006). It also plays a role in stimulus-secretion coupling for insulin exocytosis in pancreatic β cells (Ishihara et al., 2004). Disruption of *Wfs1* increased vulnerability to cell death in the knockout (KO) mice (Ishihara et al., 2004, Philbrook et al., 2005, Riggs et al., 2005, Yamada et al., 2006). In the rat brain, WFS1 was distributed predominantly in neurons of the so-called limbic system (Takeda et al., 2001). WFS1 mutations could lead to loss of WFS1-expressing neurons in particular brain regions of patients with Wolfram disease, which may underlie progression of mental symptoms.

In this study, we performed behavioral analysis of *Wfs1* KO mice to

characterize their behavioral abnormality. We previously developed neuron-specific mutant polymerase γ-transgenic mice (mPolg Tg mice) based on a mitochondrial dysfunction hypothesis of bipolar disorder (Kato and Kato, 2000) and demonstrated that these mice had bipolar disorder-like phenotypes such as altered circadian rhythm and periodic fluctuation of wheel-running activity (Kasahara et al., 2006). Whether or not the *Wfs1* KO mice show such wheel-running activity was examined. A behavioral test battery was also conducted to search for other behavioral phenotypes. Distribution of *Wfs1* in the brain was examined to search for the neural basis of behavioral alteration. In addition, gene expression analysis was performed to search for the molecular basis of behavioral phenotypes of *Wfs1* KO mice.

2. Experimental procedures

2.1 Generation of Wfs1 KO mice

The methods for the generation of *Wfs1* KO mice have been described elsewhere (Ishihara et al., 2004). In brief, a neomycin-resistance gene was inserted into exon 2 of the *Wfs1* gene in the targeting vector. The targeting vector was injected into 129Sv embryonic stem (ES) cells, and the ES cells with homologous recombination were obtained. By crossing the chimeric mice with C57BL/6J (B6) mice, *Wfs1* heterozygous KO mice were obtained. Genotyping was performed as previously described (Ishihara et al., 2004). The heterozygous KO mice were crossed with the B6 mice for at least 8 generations before the analysis. The mice were maintained in a 12-h light/12-h dark cycle, except for several specific experiments as indicated. Wild-type (WT) littermates were used

for the control whenever possible. All animal experiments were approved by the local animal experiment committees of RIKEN and Behavioral and Medical Sciences Research Consortium (BMSRC) (Akashi, Japan). Animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

2.2 Wheel-running activity

For this analysis, 11 homozygous KO mice (*Wfs1*$^{-/-}$) and 9 WT littermates (*Wfs1*$^{+/+}$) were used. All were males aged 34 weeks at the initiation of the analysis. The groups did not differ significantly in body weight.

The methods for the analysis of wheel-running activity were described in detail elsewhere (Kasahara et al., 2006). In brief, mice were individually housed in cages (width, 24 cm; depth, 11 cm; height, 14 cm) equipped with a steel wheel (width, 5 cm; diameter, 14 cm) (O'Hara & Co., Tokyo, Japan). Wheel-running activity was monitored by measuring the rotation of the wheel (3 counts/1 rotation). Food and water were available *ad libitum*. The data of initial 7–10 days were omitted from the analysis. Delayed and anticipatory activity indices, referring to the wheel-running activity during the initial 3 h of a light phase and that during the last 3 h of a light phase, were calculated. The periodicity of wheel-running activity was assessed by Lomb-Scargle periodogram (Kasahara et al., 2006).

The Mann-Whitney *U* test was used for statistical analyses. Significance levels were set at 0.05 (two-tailed; df, degree of freedom). The average and

standard error of mean (SEM) were presented for each experimental parameter in one group.

2.3 Behavioral analysis. Phase I: Screening by a test battery

This analysis was performed at BMSRC (Akashi, Japan). For this analysis, 14 homozygous KO mice (*Wfs1*$^{-/-}$), 14 heterozygous KO mice (*Wfs1*$^{-/+}$), and 13 WT littermates (*Wfs1*$^{+/+}$) were analyzed. All were males aged 12 weeks at the initiation of the behavioral analysis. The analyses were performed in the order of open-field test, startle response and prepulse inhibition test, elevated plus maze, Morris water maze, passive avoidance learning, active avoidance learning, and forced swimming test. After the behavioral test battery, the non-fasting blood glucose level was examined to rule out the possibility that elevated blood glucose levels might affect the results of behavioral analysis. There was no significant difference among the genotypes, consistent with a previous report that there was no apparent increase in blood glucose levels in *Wfs1* KO mice on the B6 background (Ishihara et al., 2004).

2.3.1 Open-field test

A transparent cubic box without a ceiling (30 × 30 × 30 cm) was placed in a ventilated sound-attenuating chamber. A 40-W white lamp provided room lighting, which was approximately 110 lux on the floor of the chamber. In addition, a fan attached on the upper part of the wall at one end of the chamber presented a masking noise of 45 dB. Two infrared beams were set on each wall 2 cm above the floor with an interval of 10 cm. The total number of successive interceptions of

two adjoining beams on each bank was scored as locomotion behavior. The other 12 infrared ray beams were attached 4.5 cm above the floor in 2.5-cm intervals, and the total number of vertical beam interceptions was scored as rearing behavior. Each mouse was allowed to explore freely in the open-field area for 20 min.

For statistical analysis, repeated measures analysis of variance (ANOVA) with the intrasubject factor of time (1–20 min) and the intersubject factor of genotype (−/−, +/−, and +/+) was applied.

2.3.2 Startle response and prepulse inhibition (PPI)

Each mouse was enclosed in a transparent acrylic box (7 × 7 × 10 cm). Startle response was detected as vibration of the box, using an accelerometer (GH-313A, Keyence, Osaka, Japan). The acoustic startle pulse of broadband burst (115 dB, 50 ms) and tone prepulse (85 dB, 30 ms) were presented via a speaker located in front of the box. Light prepulse (30 ms) was applied by LED. At the beginning of the session, 40 startle pulses were presented to test for basal startle responsiveness and its habituation. The average values of 8 blocks, consisting of 5 startle pulses each, were used for the statistical analysis. After that, three different types of trials were performed: startle pulse alone (n = 12), startle pulse preceded by a tone prepulse (n = 12), and startle pulse preceded by a light prepulse (n = 12). Prepulses were presented 50, 100, or 200 ms before the startle pulse. In total, 6 types of prepulse (n = 4, each) were applied. The mean interval averaged 25 sec (15–45 sec) throughout the session. The startle response was recorded for 200 ms with the sampling frequency of 1000 Hz. The PPI was

assessed by the ratio of the mean response of trials with one type of prepulse (n = 4) divided by the mean response of trials without prepulse (n = 12). Because light prepulse did not attenuate the startle response at all, only the data of tone prepulse were presented.

2.3.3 Elevated plus maze

The maze consisted of four arms, two open arms and two closed arms, 5 cm wide and 30 cm long with a gray acrylic floor, that met at a 10 × 10-cm center zone. Two closed arms had the transparent walls of 15 cm height on both sides, and the open arms had the low walls of 3 mm height on the both sides. The apparatus was mounted 75 cm above the floor of the room. The room lighting was approximately 20 lux on the maze. The video camera was placed 80 cm above the maze. A fan generated a masking noise of 45 dB. The animal was placed gently onto the center of the maze and was allowed to explore the maze freely for 10 min. Number of entries into each arm and time spent in each arm were recorded from videotapes.

2.3.4 Morris water maze

A round pool, with the diameter of 95 cm and the depth of 21.5 cm, was placed in the center of a 140 cm × 130 cm room. A platform with the diameter of 11 cm was set in one of quadrants and 5 mm beneath the surface of black water maintained at 21 ± 1 °C. On the first to fifth days, five trials per day were performed for learning phase. The mouse was released on one of three quadrants of the pool without the platform, and the time to reach the platform was

measured. When the mouse could not reach the platform within 60 s, the experimenter placed the mouse on the platform. On the sixth day, a probe test was performed to examine whether the mouse remembered the place of the platform. The mouse was released in the quadrant on the opposite side of the platform and its behavior for 60 s was videotaped. The time staying in the target quadrant, where the platform had been placed, and immobility time were measured.

2.3.5 Passive avoidance learning

A mouse was placed in a box, consisting of two rooms separated by a shutter, that is, light and dark compartments (10 × 10 cm each). In the acquisition trial, the mouse was kept in the light compartment. Five seconds later, the door to the dark compartment was opened. When the mouse moved into the dark compartment, the shutter was closed, and 10 s later, an electrical shock (160 V, 3 sec) was delivered through the grid floor. Twenty-four hours later, each mouse was placed again in the light compartment and the latency to enter the dark compartment was recorded up to a maximum of 180 s.

2.3.6 Active avoidance learning

The same apparatus with the one used for passive avoidance learning was used, but there was no shutter between the light and dark compartments. The box was set in a soundproof chamber, and illuminated by a 20W white light set on the champer. The ceiling of the dark room is made of a black acryl board, and the ceiling of the light room was a transparent acryl board.

The training was performed for 3 days. On each day, one session consisting of 50 trials was performed. In each trial, a condition stimulus (CS) of 1500 Hz sound (85 dB) was followed by an unconditioned stimulus (US) of 140-V electrical shock. The US was given 5 sec after the initiation of the CS and continued until the mouse escaped to the other compartment. If the mouse did not move to the other compartment, the US lasted 15 sec together with the CS. If the mouse moved within 5 sec after the CS, CS was stopped and no US was given. None of the mice experienced the maximum length of the CS. The time from the CS to the escape and the number of escapes were used for the performance of learning.

2.3.7 Forced swimming test

Animals were thrown into a square pool 24 cm × 24 cm in size. The water was maintained constant at 21 °C. On the first day, the mice were left in the pool for 20 min, and the mobility during the first 5 min recorded by videocamera was assessed. On the second day, animals were thrown into the pool for 5 min and the immobility time during the 5 min was recorded. The immobility was defined by the two criteria. 1) No movement of all legs and the tail. 2) Completely stationary state in the pool, or the movement only by inertia by the adjacent movement. The immobility was assessed by three independent raters by scrutinizing the video and the median value of the three raters was used for the analysis.

2.4 Behavioral analysis. Phase II

This analysis was performed at the Support Unit for Animal Experiment, RIKEN BSI. For this analysis, 9 homozygous KO mice (*Wfs1*$^{-/-}$) and 11 WT littermates (*Wfs1*$^{+/+}$) were analyzed. All were males aged 31 weeks at the initiation of the behavioral analysis. There was no significant difference of body weight at the initiation of the behavioral tests.

The analyses were performed in the order of home cage activity, open-field test, light-dark (L-D) box test, elevated plus maze, startle response and PPI test, Morris water maze, and fear conditioning. After each trial (except the auditory startle response and the water maze), apparatuses were wiped and cleaned by 80% alcohol and damp towel. For data acquisition, the Image J program (http://rsb.info.Nih.gov/ij) was used after some modification.

2.4.1 Environment of behavioral laboratory and housing condition of mice

Mice were housed individually for several days before they were transferred to the behavioral laboratory. The laboratory was air-conditioned and maintained temperature and humidity within approximately 22–23 °C and 50–55%. Food and water were freely available except during experimentation. Large tweezers were used to handle mice to avoid individual differences in the handling procedure. All of the experiments were conducted in the light phase (9:00–18:00), and the starting times of the experiments were kept constant.

2.4.2 Home cage activity measurement

Spontaneous activity of mice in their home cage was measured using a 24-ch ABsystem 4.0 (Neuroscience, Tokyo, Japan). Cages were individually set

into the compartments made of stainless steel in the negative breeding rack (JCL, Tokyo, Japan). An infrared sensor was equipped on the ceiling of each compartment and it detected movements of the mice. Home cage activity was measured for 1 week from the afternoon of the day of transferring to the behavioral laboratory (day 1) until the next day of the week (day 8). After the termination of home cage activity measurement, cages and bedding materials were changed to fresh ones and then mice were maintained in the micro-isolation rack (Allentown Inc., Allentown, PA, USA), the same as those used in breeding rooms throughout the behavioral screening.

2.4.3 Open-field test

Four days after the termination of home cage activity measurement (day 12), an open-field test was conducted. The detailed protocol is shown in the supplementary information.

2.4.4 L-D box test

The next day (day 13) after the open-field test, an L-D box test was conducted. A four-channel of the L-D box system was equipped in the same sound-proof room as the open field. Each light box was made of white plastic (20 × 20 × 20 [H] cm) and illuminated by LEDs (250 lux at the center of the box); a CCD camera was equipped on the ceiling. Each dark box was made of black plastic (20 × 20 × 20 [H] cm) and an infrared camera was equipped on the ceiling. There was a tunnel for transition on the center panel between the light box and dark box (3 × 5 cm) with an automatic sliding door. In the L-D box test, mice were

individually introduced into the light box, and the door of the tunnel automatically opened 3 sec after the introduction of a mouse. Then mouse was allowed to move freely in the LD box for 10 min. Total distance traveled, percent distance traveled in the light box, percent duration staying in the light box, numbers of the transition between light and dark box, and the first latency to enter the dark box were measured as indices.

2.4.5 Elevated plus maze test

The next day (day 14) after the L-D box test, an elevated plus maze test was conducted. The detailed protocol is shown in the supplementary information.

2.4.6 Auditory startle response

The next day after the L-D box test, an auditory startle response test was conducted for 2 days. The detailed protocol is shown in the supplementary information.

2.4.7 The Morris water maze test

Three days after the termination of the auditory startle response test (day 18), a series of the Morris water maze test began. The detailed protocol is shown in the supplementary information.

2.4.8 Classical fear conditioning

Three days after the termination of the Morris water maze test (day 25), a classical fear-conditioning test was conducted. This test consisted of three parts:

a conditioning trial (day 25), a context test trial (day 26), and a cued test trial (day 27). Fear conditioning was carried out on a clear plastic chamber equipped with a stainless steel grid floor (34 × 26 × 30 [H] cm). A CCD camera was equipped on the ceiling of the chamber and was connected to a video monitor and computer. The grid floor was wired to a shock generator. White noise (65 dB) was supplied from a loudspeaker as an auditory cue (CS). The conditioning trial consisted of a 2-min exploration period followed by two CS-US pairings separated by 1 min each. A US (foot-shock: 0.5 mA, 2 sec) was administered at the end of the 30-sec CS period. A context test was performed in the same conditioning chamber for 3 min in the absence of the white noise at 24 h after the conditioning trial. Further, a cued test was performed in an alternative context with distinct cues; the test chamber was different from the conditioning chamber in brightness (almost 0–1 Lux), color (white), floor structure (no grid), and shape (triangular). The cued test was conducted 24 h after the contextual test was finished and consisted of a 2-min exploration period (no CS) to evaluate the nonspecific contextual fear followed by a 2-min CS period (no foot shock) to evaluate the acquired cued fear. Rate of freezing response of mice was measured as an index of fear memory.

2.5 Behavioral analysis. Phase III

This analysis was performed at the Support Unit for Animal Experiment, RIKEN BSI. For this analysis, 7 homozygous KO mice (*Wfs1*$^{-/-}$) and 8 WT littermates (*Wfs1*$^{+/+}$) were analyzed. All were males aged 9 weeks at the initiation of the behavioral analysis. There was no significant difference of body weight at the initiation of the behavioral tests (WT, 25.2 ± 0.6 g; KO, 25.2 ± 0.5 g).

The analyses were performed in the order of social interaction, rotarod test, sucrose preference test, tail suspension test, forced swimming test, marble burying test, hot plate test, and tail flick test. Inter-test intervals were 1 day to a week. After each trial (except the auditory startle response and the water maze), apparatuses were wiped and cleaned by 80% alcohol and damp towel. For data acquisition, the Image J program was used after some modification.

2.5.1 Social interaction test (encounter method)

Subject mice were individually put into the center of a white-colored open field (40 × 40 × 30 [H] cm). Immediately after the introduction of the subject mouse, a target mouse was also introduced into the same open field. The duration of contact behavior was measured for 60 min to assess passive contact. Contact or separation of mice was expressed as "1" or "2" by computerized image analysis. If the two mice contacted, the software return value of "1," and if separated, return value "2." Thus, smaller number means higher contact. Data were collected and analyzed using a personal computer and commercially available software (Time HC; O'Hara, Tokyo, Japan).

2.5.2 Motor coordination and motor learning test (rotarod)

Mice were individually placed on a rotating rod (O'Hara, Tokyo, Japan) and the time each animal was able to maintain its balance walking on top of the rod was measured. The speed of the rotarod was 4 rpm (on the first day) or accelerated from 4 to 40 rpm over a 4-min period and 40 rpm another 1 min (day 2 to day 5). Mice were given a trial for 2 min (day 1) or four trials with a maximum

time of 300 sec (inter-trial intervals were 20–30 sec). Time between placement and falling or revolving around the rod was recorded manually.

2.5.3 Sucrose preference test

Mice were tested for a 3-days 24-h test and 1-day 1-h test with water deprivation. The 24-h tests were free choice between two bottles, one with sucrose (3% in filtered water) and another with filtered water. To eliminate the side preference, the position of bottles was switched every 24 h. The consumption of water and sucrose solution was assessed daily. After the choice test, mice were deprived water for 24 h and then a1-h choice test between water and sucrose was conducted.

2.5.4 Tail suspension test

Mice were individually hung by the tail using an adhesive tape placed approximately 1.5 cm from the tip of the tail attached to a wire and 30 cm above the floor. The duration of immobility was scored and analyzed using Image J TS (O'Hara, Tokyo, Japan) for 5 min.

2.5.5 Forced swimming test

Mice were individually placed for 15 min (day 1) or 5 min (day 2) in glass cylinders (30 cm high, 10 cm in diameter) containing 10 cm of water maintained at 23–25 °C. The duration of immobility was scored and analyzed using Image J software. The immobility time during the first 5 min was assessed.

2.5.6 *Marble burying test*

The test was performed in the test cage identical to their home cage with a 5-cm thick layer of bedding material (TEK-FRESH, Edstrom Industries, Waterford, WI, USA). Mice were habituated to fresh bedding for 30 min and then briefly returned to their home cage; 20 glass marbles (1.5 cm in diameter) were placed evenly on the bedding. Mice were then reintroduced into the test cage and the number of buried marbles (buried into the bedding over 2/3) was analyzed 30 min later.

2.5.7 *Hot plate and tail flick test*

In the hot plate test (Model MK-350C, Muromachi-kikai, Tokyo, Japan), mice were individually placed on the plate (52 °C ± 0.5 °C) enclosed in a translucent plastic wall, and the time between placement and licking of the paws and jumping was recorded manually as the response latency. A cut-off time was 90 sec. Because most of the mice did not jump, latency to licking was used for statistical analysis.

In the tail flick test (Model MK-330B, Muromachi-kikai, Tokyo, Japan), mice were individually restricted on the radiant heat meter and focused heat was applied to the surface of the tail at 2–3 cm from its tip; the latency to reflexive removal of the tail from the heat was recorded manually as the tail flick latency. A cut-off time was 10 sec.

In these tests, data were obtained by two observers, and the shorter scores were adopted as the response time.