2.5.8 Statistical analysis

For statistical analysis ofr behavioral analyses Phase I, II, and III, the Student's *t* test, one-way ANOVA, and repeated measures ANOVA (RMANOVA) were used. When a significant effect was found by one-way ANOVA, Tukey *post-hoc* comparisons were applied. When sphericity was rejected by the Mauchly test before the application of RMANOVA, the Greenhouse-Geisser estimate was used. Paired *t* test and two-sample *t* test were also used for *post-hoc* analysis when necessary. These statistical analyses were performed using SPSS 11.0 for Windows (SPSS Japan, Tokyo, Japan). Significance levels were set at 0.05 (two-tailed); df, degree of freedom. Average and standard error of mean (SEM) were presented for each experimental parameter in one group.

2.6 Immunohistochemistry

Because several computer programs predicted that mouse Wfs1 protein would be cleaved around position 36, we used the following amino acid sequence, Glu^{39}–Gly^{53} as an antigen. A hexadecapeptide (CEPPRAPRPQADPSAG) was synthesized, purified using high-performance liquid chromatography, and conjugated to keyhole limpet hemocyanin (KLH). Five Balb/c mice were injected intraperitoneally with the KLH-conjugated peptide emulsified in complete Freund's adjuvant. Antiserum was obtained 1 week after boosting with the same antigen. We performed Western blot analysis to selected sensitive antiserum specific to Wfs1 protein.

For immunohistochemical analysis using WFS1 antibody, wild-type B6 mice aged 20–22 weeks were used. The mouse brain was fixed by perfusion of

paraformaldehyde and embedded with paraffin. Coronal or sagittal sections with the thickness of 8 μm were sliced from paraffin-embedded mouse brain. After deparaffinization and hydration, the slices were incubated for 10 min at 95 °C in sodium citrate buffer. Endogenous peroxidase activity was quenched by H_2O_2/methanol treatment. For blocking, 0.8% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) in phosphate-buffered saline (PBS) was used. Anti-WFS1 antiserum was used by ×2500 dilution. For second antibody, biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) was used. Peroxidase/DAB staining was performed by Vectastain Elite ABC kit (Vector Laboratories).

2.7 DNA microarray analysis

DNA microarray analysis was performed in two developmental stages, 12 and 30 weeks old. Eight homozygous *Wfs1* KO mice and 8 WT littermates were sacrificed at the age of 12 weeks. Seven homozygous *Wfs1* KO mice and 5 WT littermates were also analyzed at the age of 30 weeks.

The hippocampus was rapidly dissected, and total RNA samples were extracted from the hippocampi using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Microarray analysis was performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Briefly, 5 μg total RNA of each sample was reverse-transcribed into cDNA, and biotinylated cRNA was synthesized from the cDNA by in vitro transcription. DNA microarray experiments were performed using Mouse Genome 430 2.0 GeneChips (Affymetrix). The hybridization signal on the chip was scanned by a GeneArray scanner and

processed by GeneSuite software (Affymetrix). The probe sets labeled as "present" in 8 of 16 samples at 12 weeks old (24703/45101 probe sets) or in 5 of 12 samples at 30 weeks old (24455/45101 probe sets) were selected. The raw data were analyzed using MAS5 (Affymetrix) and then imported into GeneSpring 7.3 software (Silicon Genetics, Redwood, CA). The signal intensity of each probe set on the microarray was divided by its median value using GeneSpring 7.3 software.

For statistical analysis, the Mann-Whitney *U* test was performed between the KO mice and their WT littermates, and $P < 0.05$ was considered statistically significant.

The probe sets were classified based on the information from GeneOntology (http://www.geneontology.org/) using GeneSpring software. For the GeneOntology analysis, the differentially expressed probes were selected. The categories showing overrepresentation at the level of $P < 0.05$ and containing 10 or more probe sets were selected.

2.8 Real-time quantitative polymerase chain reaction (RT-PCR) analysis

The representative probe sets that showed altered expression in the DNA microarray analysis of mouse brains were verified by RT-PCR. The cDNA used for the DNA microarray analysis was used. Primers and probes for Gapdh, cdc42ep5, Rnd1, Wnt2, and Garnl1 were commercially available by the Assay-on-Demand service (Applied Biosystems, Foster City, CA). The assays were carried out according to the protocols supplied by the manufacturer using 7900HT real-time PCR systems (Applied Biosystems). The relative values were

calculated by measuring ΔCt = Ct (each gene) − Ct (Gapdh) for each sample in quadruplicate. For statistical analysis, one-tailed Mann-Whitney *U*-test was applied, and $P < 0.05$ was considered statistically significant.

3. Results

3.1 Wheel-running activity

To assess whether or not the *Wfs1* KO mice show bipolar disorder-like behavioral phenotypes, wheel-running activity of the *Wfs1* KO mice and WT littermates was recorded for a period up to 2 months. The levels of wheel-running activity and the circadian rhythm were assessed using male mice that were 34 weeks old at the initiation of this analysis (KO, $n = 11$; WT, $n = 9$). Average wheel-running activity per day of *Wfs1* KO mice during 28 days under the LD condition did not differ from that of WT littermates (Fig. 1a; WT, 221.3 ± 64.7 [mean ± SEM] counts; KO, 142.2 ± 57.6 counts, df = 18, U = 29, $P = 0.21$ by Mann-Whitney *U* test). Delayed activity index (WT, 7.70 ± 2.50; KO, 5.29 ± 1.16, df = 18, U = 42, $P = 0.84$) and anticipatory activity index (WT, 0.14 ± 0.05; KO, 0.34 ± 0.21, df = 18, U = 41, $P = 0.78$) did not differ between the KO mice and WT littermates (Fig. 1a-c).

There was no abnormality of free running period measured at the constant dark condition in *Wfs1* KO mice (average 23.7 h, $n = 6$). None of female KO mice showed significant periodicity in wheel-running activity with the duration of 4–5 d (data not shown). These results show that the behavioral phenotypes of *Wfs1* KO mice are different from the mPolg Tg mice that exhibit altered circadian rhythm of wheel-running activity (Kasahara et al., 2006).

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

To screen the behavioral abnormality of *Wfs1* KO mice, we performed a conventional behavioral test battery using 14 homozygous KO mice, 14 heterozygous KO mice, and 13 WT littermates. The results of behavioral tests were summarized in Table 1.

3.2.1 Open-field test

Although a significant effect of time was found for both locomotor activity (df = 8.6, F = 3.0, P = 0.002) and rearing (df = 11.0, F = 9.7, P = 0.000), no significant effect of genotype was found for locomotor activity (df = 2, F = 0.70, P = 0.49) and rearing (df = 2, F = 0.57, P = 0.56). There was no significant interaction between time and genotype (locomotor, df = 17.3, F = 21.1, P = 0.57; rearing, df = 22.0, F = 0.80, P = 0.71) (Fig. 2a,b).

3.2.2 Startle response and prepulse inhibition

When RMANOVA was applied for the data of startle response, significant effect of blocks was found (df = 5.12, F = 7.80, P < 0.001). However, no significant effect of genotype (df = 2,. F = 0.664, P = 0.52) or genotype × block interaction (df = 10.2, F = 1.30, P = 0.22) was found (Fig. 2c). No significant effect of genotype was found for the PPI ratio regardless of the interval of prepulse (50 ms, df = 2, F = 0.38, P = 0.68, 100 ms, df = 2, F = 0.65, P = 0.52, 200 ms, df = 2, F = 0.41, P = 0.66, one-way ANOVA) (Fig. 2d).

3.2.3 Elevated plus maze

The number of entry into the open arms ($F = 0.31$, df = 2, $P = 0.72$, one-way ANOVA) (Fig. 2e) and the time spent in the open arms ($F = 2.05$, df = 2, $P = 0.14$) (Fig. 2f) were not significantly different among the genotypes. A significant effect of genotype was found for the total number of boluses ($F = 7.16$, df = 2, $P = 0.002$) (Fig. 2g). The Tukey honest significant difference (HSD) test showed that homozygous KO mice had a significantly lower number of fecal boluses (2.7 ± 0.3 [mean ±SEM]) compared with heterozygous KO mice (4.8 ± 0.5, $P = 0.01$) and WT mice (5.2 ± 0.5, $P = 0.004$).

3.2.4 Morris water maze

The time to reach the platform during the 5-d learning phase became shorter than the first day, shown by a significant effect of day by RMANOVA (df = 4, $F = 19.1$, $P < 0.001$) (Fig. 2h). However, there was neither significant effect of genotype (df = 2, $F = 0.56$, $P = 0.57$) nor significant interaction of day and genotype (df = 2, $F = 0.53$, $P = 0.94$). The time spent in the target quadrant (df = 2, $F = 0.10$, $P = 0.90$, one-way ANOVA) and immobility time (df = 2, $F = 0.58$, $P = 0.56$) at the probe test performed on day 6 did not show a significant difference among the genotypes (Fig. 2i).

3.2.5 Passive avoidance test

The latency to escape at the conditioning phase was significantly different among the genotypes (df = 2, $F = 4.70$, $P = 0.015$, one-way ANOVA). Multiple comparison showed that the latency in homozygous KO mice was

significantly longer than that in WT mice (P = 0.02) (Fig. 3a). There was no significant difference in escape latency at the test session (df = 2, F = 0.81, P = 0.92, one-way ANOVA).

3.2.6 Active avoidance test

The time course of mean escape latency was examined during 3-days' training, consisting of 5 blocks in each day (Fig. 3c). In the three-way RMANOVA with the within-group factors of day and block and the between-group factor of genotype, although no significant effect of genotype was found (df = 2, F = 0.75, P = 0.47), there was a significant interaction between genotype and block (df = 1.75, F = 2.94, P = 0.007). No other two-way or three-way interactions were statistically significant. This significant interaction may be caused by longer escape latency of KO mice only at the first block. A post-hoc analysis showed that the escape latency of the homozygous KO mice at the first block on day 3 was significantly longer than that in WT mice (df = 25, t = 2.34, P = 0.027, with no correction for multiple comparison).

Similar interaction between genotype and block was also seen for the numbers of avoidance (df = 6.3, F = 3.25, P = 0.004) (Fig. 3d). Both homozygous and heterozygous KO mice showed significantly lower numbers of avoidance at the first block on day 3 (homozygotes, df = 25, t = −2.82, P = 0.009; heterozygotes, df = 25, t = −2.15, P = 0.04).

3.2.7 Forced swimming test

When the immobility time was analyzed by RMANOVA, a significant

effect of day (df = 1, F = 5.5, P = 0.024) and a significant interaction of day and genotype (df = 2, F = 3.8, P = 0.031) were found, whereas no significant effect of genotype was found (df = 2, F = 0.48, P = 0.61) (Fig. 3b).

In the WT mice, immobility time was significantly longer on the second day (144.6 ± 53.0 sec) compared with the first day (103.0 ± 48.3 sec, df = 12, t = −3.45, P = 0.005, paired t test), possibly reflecting the learned despair (Parra et al., 1999). On the other hand, such a significant increase of immobility time on the second day was not observed for heterozygous (day 1, 128.5 ± 39.4 sec; day 2, 117.5 ± 39.4 sec; df = 12, t = 1.11, P = 0.28) and homozygous (day 1, 97.4 ± 50.6 sec; day 2, 122.0 ± 57.0 sec; df = 13, t = −1.44, P = 0.17) KO mice (Fig. 3b).

3.2.8 Summary of the phase I behavioral analysis

The results of the phase I behavioral analysis are summarized as follows.

1) There was no abnormality in open field, elevated plus maze, PPI, and Morris water maze. However, it cannot be ruled out that the mice develop behavioral phenotypes at later age because depression is an adult-onset disease.
2) The passive avoidance test showed the longer latency to enter the other chamber in *Wfs1* KO mice. This could be explained either by low anxiety or retardation, that is slow movement or delayed onset of motion. However, it is also possible that mice could have been just busy exploring the first box, or they had some kind of place neophobia.
3) The active avoidance test showed longer escape latency and lower numbers of avoidance at the first block on day 3 in *Wfs1* KO mice. This might suggest that the emotional memory is impaired in the *Wfs1* KO mice. It cannot be

excluded, however, that *Wfs1* KO mice have impairment of pain sensitivity.

4) Altered response to serial forced swimming test. This may suggest that the *Wfs1* KO mice tend to be resistant to behavioral despair.

3.3 Behavioral analysis: Phase II

To further characterize the behavioral phenotypes of *Wfs1* KO mice, additional behavioral analysis was performed.

3.3.1 Open-field, elevated plus maze, PPI tests, and Morris Water Maze at 31 weeks

At first, four of behavioral tests were repeated in the mice aged 31 weeks to assess the effect of age. There were no significant difference between WT mice and *Wfs1* KO mice for three of these behavioral tests: open-field, elevated plus maze, and PPI tests (data not shown).

On the other hand, the Morris water maze test showed longer escape latency. Two-way RMANOVA showed significant effects of genotype (df=1, F=9.04, P=0.008) and day (df=3, F=8.45, P<0.001) (Fig 4a). The *Wfs1* KO mice showed longer escape latency than controls. There was no significant interaction between genotype and day (df=3, F=0.60, P=0.61). On the other hand, there was no significant effect of genotype on the distance (df=1, F=0.38, P=0.54) (Supplementary Fig 1a). Effect of day was significant (df=3, F=25.2, P<0.001), but the interaction between genotype and day was not significant (df=3, F=0.71, P=0.54). To assess the speed of swimming, a new index, swimming speed index = (total distance)/ (latency to reach platform), was calculated. Two-way

RMANOVA showed no significant effects of day (df=1.77, F=2.57, P=0.09) and genotype (df=1, F=0.04, P=0.83). There was no significant day-genotype interaction (df=1.77, F=0.33, p=0.69) (Supplementary Fig 1b). Spatial memory cannot be assessed because no significant difference was found between the time spent in the target quadrant and that in the other three quadrants, suggesting that the probe test did not work properly even for wild type mice (data not shown).

3.3.2 Home cage activity

To assess the general activity level, home cage activity was recorded for 8 days. When RMANOVA was applied, a significant effect of day (df=5, F=5.95, $P<0.001$) was found. There was no significant effect of genotype (df=1, F=0.61, $P=0.44$) and genotype-day interaction (df=5, F=0.53, $P=0.75$) (Fig. 4b).

3.3.3 Anxiety-like behavior

Next, the level of anxiety-like behavior was further assessed by the L-D box. The marble burying test was also performed in the 9-week-old mice in the phase III behavioral analysis.

In the L-D box test, no significant difference was found in the time spent in the light box (WT, 39.2 ± 9.0%, KO, 37.1 ± 9.8%, df = 18, t = 0.50, $P = 0.61$). There was no significant difference in the number of marbles buried (WT 16.0 ± 0.8, KO 17.1 ± 0.7, t = 1.0, $P = 0.33$). These findings suggest that longer latency to escape at the passive avoidance test was not due to lower anxiety-like behavior.

3.3.4 Emotional memory

To test the hypothesis that emotional memory is impaired in the *Wfs1* KO mice, the fear conditioning test was performed.

During the conditioning phase, two-way RMANOVA revealed significant effect of genotype (df=1, F=4.47, P=0.049) and time (df=3.54, F=22.1, P<0.001). No significant period × genotype interaction was found (df=3.54, F=1.73, P=0.16). The *Wfs1* KO mice showed significantly longer time of freezing during the conditional stimuli (periods 5 and 7) and at the final period (Student's t test, P < 0.05) (Fig. 4c).

For the cue test, two-way RMANOVA was applied to the data set before and after the cue, separately. For the data of freezing before the cue, a slight tendency of the effect of genotype (df=1, F=2.9, P=0.10) was seen, whereas there was significant effect of time (df=3, F=5.93, P=0.001) and no interaction between genotype and time (df=3, F=0.30, P=0.82). The *Wfs1* KO mice spent a significantly longer time for freezing (t = 2.48, P < 0.01) (Fig.4d). However, no significant effect of genotype was found after the cue (effect of genotype, df=1, F=1.48, P=0.23, effect of time, df=3, F=1.60, P=0.19, genotype × time interaction, df=3, F=0.40, P=0.75). There was no significant effect of genotype at the context test (Fig. 4e).

These findings suggested that memory of emotion is not impaired in the *Wfs1* KO mice.

3.4 Behavioral analysis. Phase III

3.4.1 Pain sensation

As noted above, it cannot be excluded that *Wfs1* KO mice have impairment of pain sensitivity. To rule out such possibility, the hot plate test and tail flick test were performed. No difference in the latency to licking (WT, 10.3 ± 1.2 sec; KO, 9.9 ± 0.9 sec, t = 0.219, df = 12, P = 0.83, by Student's t test) was found between the *Wfs1* KO mice and WT mice by the hot plate test. There was no significant difference in the latency to flick the tail (WT, 3.5 ± 0.2 sec; KO, 3.4 ± 0.2 sec, df = 12, t = 0.29, P = 0.77).

3.4.2 Motor function

As noted above, many of the positive findings in behavioral tests can be interpreted as reflecting retardation. Such findings can be explained by altered motor functions, such as impairment in muscle contraction, voluntary movement, or motor coordination. To test this possibility, the rotarod test was performed. Three-way RMANOVA with the intraindividual factors of day and trial and interindividual factor of genotype showed no significant effect of genotype (df=1, F=1.02, P=0.33). Whereas significant effects of day (df=3, F=15.6, P<0.001) and trial (df=3, F=51.0, P<0.001) were found, no significant two-way or three-way interactions were detected except for a trend of interaction between day and trial (df=3, F=2.54, P=0.07) (Fig. 4f).

3.4.3 Behavioral despair

As noted above, *Wfs1* KO mice showed altered response to the serial forced

swimming test. To further confirm this finding, the forced swimming test was performed again.

RMANOVA revealed a tendency of effect of day (df=1.0, F=3.83, *P*=0.07). There was no significant effect of genotype (df=1, F=0.18, *P*=0.67) and day × genotype interaction (df=1.0, F=0.17, *P*=0.68).

Although no day × genotype interaction was found in this analysis, the paired *t* test was applied similarly to the first experiment (Fig. 4g). Immobility time tended to be longer on the second day (99.7 ± 17.4 sec) compared with the first day (71.0 ± 10.55 sec, r=0.68, *P* = 0.064, paired *t* test) in WT mice, whereas no significant difference was found in Wfs1 KO mice (day 1, 74.5 ± 17.1 sec, day 2, 96.2 ± 10.0 sec, r = 0.50, *P* = 0.24). This analysis showed a similar tendency to the first experiment.

The other test of behavioral despair, the tail suspension test, was also performed. There was no significant difference in the immobile time between the *Wfs1* KO mice and WT mice (WT, 9.0 ± 2.8%, KO, 9.4 ± 3.0%, t = 0.09, *P* = 0.92).

3.4.4 Other aspects of depression

Some of these noted findings in the *Wfs1* KO mice can be explained by the retardation in emotionally triggered motion. This could not be explained by abnormalities in instrumental motor functions. Such findings seem to be similar to "psychomotor retardation" seen in human depressive patients. Though the findings in the forced swimming test and tail suspension test are equivocal, behavioral despair is not always a valid depression model. Thus, we further examined the other aspects of depression.

The sucrose preference test is an established test for anhedonia, one of the core symptoms of depression. In the choice test for 3 days, there was no significant effect of genotype (df=1, F=0.95, *P*=0.34) by two-way RMANOVA (WT, day 1, 44.2±16.7 %, day 2, 94.1±5.2 %, day 3, 84.5±9.7 %; KO, day 1, 37.3±12.9 %, day 2, 97.3±1.7 %, day 3, 60.2±16.0 %). A significant effect of trial (df=2, F=10.7, *P*=0.001) and no significant interaction of genotype × trial was found (df=2, F=0.68, *P*=0.51). The 1-h choice test after 24-h water deprivation did not show a significant difference between genotypes (WT 90.2 ± 1.7%, KO 86.3 ± 5.3%, *U*=28, NS).

The social interaction test is an established test for anxiety-like behavior (File and Seth, 2003). However, its response to drugs is different from elevated plus maze, and it is more sensitive to serotonergic drugs. Recently, this test is also applied to animal models of schizophrenia (Miyakawa et al., 2003) and autism, and to genetic models of anxiety and depression (Overstreet et al., 2003). Thus, social behavior of the *Wfs1* KO mice was examined by this test. Two-way RMANOVA revealed no significant effect of genotype (df=1, F=2.0, p=0.17) and time (df=11, F=0.93, *P*=0.51). There was a trend of genotype × time interaction (df=11, F=1.67, P=0.08) (Fig. 4h). The *Wfs1* KO mice showed significant decrease of social interaction at the periods 3 and 10 (Student's t-test, *P*<0.05) shown by the higher number of particles observed.

3.5 Wfs1 Immunohistochemistry

To determine the molecular basis of behavioral abnormality in *Wfs1* KO mice, we verified whether the distribution of Wfs1 protein in the brains of WT B6

mice is similar to that in rats (Fig. 5a-f) (Takeda et al., 2001). We verified that no staining was observed in *Wfs1* KO mice, suggesting the specificity of the anti-Wfs1 antibody (Fig. 5g).

Wfs1-like immunoreactivity (Wfs1-IR) localized mostly to neurons and its regional distribution was mostly similar to that in rats (Fig. 5a). Wfs1-IR was most abundant in the hippocampal CA1 pyramidal neurons (Fig. 5b), and strong in the layer II pyramidal neurons of the cerebral cortex (Fig. 5c). Similar to rats, Wsf1-IR was also rich in the striatum, nucleus accumbens, thalamus, cerebellar Purkinje cells, amygdala, and bed nucleus of the stria terminalis (Fig. 5d). In addition, Wfs1-IR was observed in several hypothalamic nuclei such as the paraventricular nucleus and suprachiasmatic nucleus (SCN) in mice (Fig. 5e). In the adjacent region of SCN, sub-paraventricular zone, some cell bodies showed Wfs1-IR. The ventromedial nucleus and arcuate nucleus also showed Wfs1-IR (Fig. 5f). Wfs1-IR was also found in the locus coeruleus and cochlea nucleus (data not shown).

3.6 DNA microarray analysis

To examine what sort of functional impairment occurs in the neurons of *Wfs1* KO mice, we performed gene expression analysis using DNA microarray. Because Wfs1-IR was most abundant in hippocampus, we performed DNA microarray analysis in the hippocampus of the *Wfs1* KO mice. A total of 1012 probe sets were changed at the age of 12 weeks. To narrow down the gene list, we repeated the experiment at the age of 30 weeks. We assumed that the true gene expression difference observed at age 12 weeks should be replicated at

age 30 weeks. At the age of 30 weeks, 3508 probe sets showed significant differences. The genes altered in the same direction at both the ages of 12 and 30 weeks, and the fold change higher than 1.2 are shown in Table 2.

GeneOntology (GO) analysis showed that genes related to ribosome biogenesis (GO:3735: structural constituent of ribosome, GO:7046: ribosome biogenesis, GO:3723: RNA binding) or other basic cellular functions (GO:5622: intracellular, GO:44249: cellular biosynthesis, GO:15399: primary active transporter activity, GO:5623: cell, GO:7028: cytoplasm organization and biogenesis) were commonly up-regulated at the age of 12 and 30 weeks (Supplementary Tables 1 and 2). The major difference between the week 12 and week 30 is the inclusion of neurodevelopment-related genes at the age of 30 weeks (GO:48666: neuron development, GO:30182: neuron differentiation, GO:7409: axonogenesis, GO:48667: neuron morphogenesis during differentiation, and GO:31175: neurite morphogenesis) (Supplementary Table 2).

3.7 RT-PCR analysis

To test whether the findings by DNA microarray analysis are chance findings, RT-PCR analysis was performed. For this purpose, two down-regulated genes, *cdc42ep5* and *Rnd1*, as well as two up-regulated genes, *Wnt2* and *Garnl1*, were examined using Gapdh as a reference.

The level of cdc42ep5 and Rnd1 tended to be lower at 12 weeks but not at 32 weeks. On the other hand, Wnt2 and Garnl1 were significantly up-regulated at 32 weeks but not at 12 weeks (Table 3).

4. Discussion

4.1 Behavioral analyses

We recently reported that mPolg Tg mice show bipolar disorder-like behavioral phenotypes, such as altered circadian rhythm in both males and females and periodic fluctuation of wheel-running activity in females (Kasahara et al., 2006). Based on previous reports suggesting that patients with Wolfram disease are frequently affected with depression or bipolar disorder, we speculated that the *Wfs1* KO mice might also show these bipolar disorder-like phenotypes, which were seen in the mPolg Tg mice. However, *Wfs1* KO mice did not show similar phenotypes (Fig. 1).

Thus, we next examined the possibility that *Wfs1* KO mice show other types of behavioral phenotypes. At first, a battery of established behavioral tests was applied. There was no marked difference found in most of conventional behavioral tests, such as the open-field test, startle response, prepulse inhibition test, and elevated plus maze. The lack of marked difference in these tests was replicated in the mice aged 31 weeks. On the other hand, several tests in the initial test battery showed equivocal findings. In the passive avoidance test, the mice showed longer latency to move into the dark compartment at the training phase (Fig. 3a). The active avoidance test showed subtle differences between the KO and WT mice. On the third day of training, WT mice kept the same level of escape latency and number of avoidance reactions as the final block of the second day. Although *Wfs1* KO mice seemed as if they forget the previous memory of escape training (Fig. 3c,d.), it is unlikely considering the fact that there were no differences in the day 2 of the active avoidance test and in the contextual

testing of the fear conditioning test. Otherwise, they may remember the events, but could not take the adequate action under the situation for some other reasons. For example, a possibility is that they showed retardation or increased behavioral despair without any problems in memory retention. In the forced swimming test performed for two sequential days, WT mice showed an increase of immobility time on the second day (Fig. 3d). This is in accordance with a previous study showing that mice became immobile on the second day of the sequential forced swimming test (Parra et al., 1999). This phenomenon was not observed in the homozygous and heterozygous KO mice (Fig. 3d). Although statistical analysis did not show the same difference, a similar tendency was observed in the second forced swimming test. In addition, the other test for behavioral despair, the tail suspension test, did not show any significant difference. On the other hand, the longer escape latency of the KO mice without the difference of the distance traveled in the Morris water maze might reflect the longer time for immobility during the session. Thus, the *Wfs1* KO mice might show enhanced or attenuated behavioral despair depending on experimental conditions.

As described above, it was speculated that longer latency to move at the passive avoidance test can be explained either by low anxiety or retardation of *Wfs1* KO mice. The former possibility was not supported by two established tests for anxiety-like behavior, the L-D box test and the marble burying test.

Wfs1 KO mice also showed longer escape latency and lower numbers of avoidance during the active avoidance test. This was not due to decreased pain sensitivity. This cannot be explained by the impairment of emotional memory, because there was no significant abnormality in fear conditioning test. This test

instead showed increased freezing during conditioning phase. Freezing was also increased during the cue test, not after the cue but before the cue.

The *Wfs1* KO mice did not show impairment in fundamental motor functions that can explain these findings.

In summary, the following findings were obtained.

1) Longer latency to move in passive avoidance test
2) Diminished avoidance reaction during active avoidance test
3) Longer escape latency in Morris water maze
4) Increased freezing during conditioning
5) Normal sensorimotor function and anxiety-like behavior

These findings together suggest that the *Wfs1* KO mice might show retardation in the emotionally triggered motion. We could not discriminate whether this feature of the Wfs1 KO mice reflects the slow movement, longer time to initiate movement, or mixture of both. Psychomotor retardation, that is, slow voluntary movement and thoughts and/or taking longer time to initiate movement, is one of the characteristic symptoms of melancholic depression. The observed retardation of the *Wfs1* KO mice resembled such a characteristic symptom of depression. Thus, other aspects of depression were also examined. Although the sucrose preference test did not show any difference, the social interaction test showed decreased social interaction in *Wfs1* KO mice.

Together these results suggest that *Wfs1* KO mice have some similarity to patients with depressive disorder. It should be noted, however, that the observed difference in the social interaction test might also reflect the retardation noted above.The *Wfs1* KO mice did not show marked abnormalities in the

conventional behavioral despair paradigm such as the forced swimming test and tail suspension test. These tests are established as screening tests for compounds having tricyclic antidepressant-like properties. However, its construct validity as a depression model is questioned (Crawley, 2007).

Taken together, the *Wfs1* KO mice show behavioral alterations at least partly mimicking the symptoms of depression. Further studies to examine the effects of antidepressive agents would be extremely interesting.

4.2 Morphologic analyses

Immunohistochemistry demonstrated that the distribution of Wfs1-IR was similar to that in rats (Fig. 5) (Takeda et al., 2001). In addition, we found that Wfs1-IR is also present in the hypothalamus. The presence of Wfs1-IR in the arcuate nucleus seems to be in accordance with diabetes insipidus, the major symptom of Wolfram disease. In a similar way, Wfs1-IR in the cochlea nucleus may be relevant to deafness in patients with Wolfram disease. It is also interesting that Wfs1-IR is found in the locus coeruleus and substantia nigra, from which noradrenergic and dopaminergic fibers originate. In *Wfs1* KO mice, however, we did not observe marked morphologic alterations in these regions using hematoxylin-eosin staining and Kluver-Barrera staining (data not shown).

4.3 Gene expression analysis

The fact that *Wfs1* itself is included in the list of altered genes (Table 2) supports the validity of our experiment and data analysis. Among the eight