down-regulated and nine up-regulated genes, six other genes in addition to *Wfs1* itself were on the chromosome 5. This is possibly caused by residual genomic region derived from the 129Sv mice. Thus, only a part of these changes can be attributable to the absense of Wfs1 itself. The present result is in accordance with a previous report that there were only small differences in expression profiles seen in fibroblasts obtained from patients with Wolfram disease (Philbrook et al., 2005).

Among the three down-regulated genes outside chromosome 5, two (Cdc42ep5 and Rnd1) were related to Rho GTPase. Down-regulation of Rnd1 was validated at the age of 12 weeks but not at 32 weeks.

Cdc42ep5 encodes CDC42 effector protein. CDC42 plays a role in dendrite development (Threadgill et al., 1997). Cdc42ep5 is one of the targets of CDC42 (Joberty et al., 1999), but its function in neurons is not known yet. Rnd1 also plays a role in activity-dependent dendrite development (Ishikawa et al., 2006). A recent fine mapping analysis of 13q33 in bipolar disorder revealed the linkage with DOCK9, an activator of Cdc42 (Detera-Wadleigh et al., 2007). This finding also suggested the possible role of Rho GTPase in mood disorder. Together with the GO analysis showing altered neural development related genes at age 30 weeks, these findings may suggest that dendrite development may be impaired in *Wfs1* KO mice. Although we did not observe morphologic difference between *Wfs1* KO mice and WT littermates using hematoxylin-eosin staining and Kluver-Barrera staining, dendrite morphology cannot be assessed using these methods. Further analysis by Golgi staining or other methods might be promising.

Up-regulation of two genes were validated at 32 weeks but not at 12 weeks. Up-regulation of Wnt2 is potentially interesting because Wnt signaling plays a role in neural plasticity and is implicated in the molecular pathology of bipolar disorder (Gould and Manji, 2002, Matigian et al., 2007).

Up-regulation of ribosome-related genes at both 12 and 30 weeks revealed by gene ontology analysis might be in accordance with the putative role of WFS1 in ER stress response (Fonseca et al., 2005, Yamada et al., 2006).

4.4 Phenotypic discordance between Wfs1 KO mice and patients with Wolfram disease

In this study, *Wfs1* KO mice did not show marked sensorimotor and general health problems that are seen in patients with Wolfram disease. This is in accordance with the lack of spontaneous diabetes mellitus in *Wfs1* KO mice on the B6 background (Ishihara et al., 2004). Although we detected some behavioral phenotypes in KO mice, it cannot be ruled out that some of detected behavioral alterations in *Wfs1* KO mice could be explained by the residual genomic region derived from 129Sv mice (Mouse Phenome Database, http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home).

It is possible that the symptoms in patients with Wolfram disease are the combination of the loss of function of WFS1 and the dominant-negative effect of the mutations. Meta-analysis of genotype-phenotype correlation in Wolfram disease suggested that nonsense or frame-shift mutations caused more severe phenotypes compared with missense mutations (Cano et al., 2007). The *Wfs1* KO mice we analyzed in this study are *Wfs1*-null mice. On the other hand,

another line of *Wfs1* KO mice, in which the exon 8 of *Wfs1* is deficient, was reported to show striking behavioral phenotypes (European Patent EP1353549). These findings suggest the possibility that the symptoms of Wolfram disease are accelerated by the aberrant proteins truncated around exon 8. Because function of WFS1 has not been well established yet, it is difficult to conclude which mechanism, loss of function or dominant-negative effect, is more influential. Further studies will be necessary to make draw a conclusion.

In summary, we studied the behavior and gene expression patterns in *Wfs1*-null mice. The *Wfs1* KO mice showed several behavioral features, such as retardation in emotionally triggered motion, decreased social interaction, and enhanced or attenuated behavioral despair depending on experimental conditions. These findings might be relevant to the neuropsychiatric phenotypes reported in patients with Wolfram disease.

Figure Legends

Figure 1. Long-term wheel-running activity analysis. (a) Wheel-running activity. (b) Delayed activity index. Delayed activity index is defined as a percent of the wheel-running activity during the first 3 h of the light period with the total activity during the previous dark period (12 h). (c) Anticipatory activity index, the wheel-running activity in the last 3 h of light phase in comparison with the activity during dark phase. +/+, WT mice; −/−, *Wfs1* homozygous KO mice. Bars indicate averages. Each circle represents the datum of a mouse.

Figure 2. Behavioral screening (1). (a–b) Open field test. Locomotion scores (a), and rearing scores (b). Bars indicate the standard errors. (c) Startle response. (d) Prepulse inhibition test. N50 means the prepulse 50 ms before the startle pulse. (e–g) Elevated plus maze test. (h–i) Morris water maze test. Time course of escape latency during 5-d training (h), and time spent in the target quadrant during the 60-sec session. Error bars represent standard error of mean. The dotted line represents the chance level. +/+, WT mice; +/−, *Wfs1* heterozygous KO mice; −/−, *Wfs1* homozygous KO mice. *, $P < 0.05$.

Figure 3. Behavioral screening (2). (a) Passive avoidance test. (b) Forced swimming test performed on two sequential days. (c–d) Active avoidance test. +/+, WT mice; +/−, *Wfs1* heterozygous KO mice; −/−, *Wfs1* homozygous KO mice. Error bars represent standard error of mean. *$P < 0.05$.

Figure 4. Further characterization of behavioral phenotypes of *Wfs1* KO

mice. (a) Morris water maze test. (b) Home cage activity. (c,d) Fear conditioning test. One time period is 30 sec. (c) Conditioning phase. Conditional stimuli (tone) and unconditional stimuli (foot shock) were applied during periods 5 and 8. (d) Cue test. Cue was applied between the time periods 4 and 5. (e) Context test. (f) Rotarod test. (g) Forced swimming test. (h) Social interaction test. Note that high number of particles indicates lower levels of social interaction.

Figure 5. Localization of Wfs1-like immunoreactivity in mouse brain. Immunohistochemistry analysis of mouse brain using anti-Wfs1 antiserum. Coronal sections are shown except for panel a. (a) Sagittal section of the whole brain. (b) Hippocampus. CA1 (corpus ammon 1) region is selectively stained. (c) Cerebral cortex. Layer II pyramidal neurons are stained. (d) Coronal section at the level of bed nucleus of striata terminalis (BNST). At this level, the regions with intense Wfs1-IR looked as if they are surrounding the internal capsule (arrowhead). (e,f) Hypothalamus. Suprachiasmatic nucleus and sub-paraventricular zone are indicated by an arrow and an arrowhead, respectively (e). Arcuate nucleus and ventromedial nucleus are shown by an arrow and an arrowhead, respectively (f). (g) Immunohistochemistry analysis of the brain of a *Wfs1* KO mouse using anti-Wfs1 antiserum. No staining is detected.

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Supplementary methods

2.3.6 Additional note for active avoidance learning

The illumination intensity was 110 lux in the light room, and 27 lux in the dark room. The mice was thrown into the dark box. The mice was remained in the box throughout the experiment. Twenty-five seconds after the initiation of the experiment, the first conditioned stimuli was given. Average interval between the trials was 25 seconds (range: 10 - 40 seconds). The strength of the stimulation was optimized to maximize the efficiency of leaning and minimize the number and the duration of the electric stimuli. The maximum length of the unconditioned stimuli was 13.5 seconds in the first session, 3.5 seconds in the second session, and 2.4 seconds in the third session.

2.4 Behavioral analysis. Phase II

Supplementary note on the housing conditions.
The mice were usually housed in a case together with several litter mates. Although the maximum number of litters of 5 was allowed, it was usually 2 or 3 after the weaning. The mice were transferred to the experimental room at 11:00 am. During the movement, the cage was covered by a canvas bag to minimize the excessive stimuli.

2.4.3 Open field test

Four days after the termination of home cage activity measurement (Day 12), an open field test was conducted. A four channel of open field system was equipped in a small sound-proof room (185 × 185 × 225 cm (H)). Each field was made of white plastic (50 × 50 × 40 (H) cm), and illuminated by LEDs (70Lux at the center of the field). The behavior of a mouse was monitored by a CCD camera equipped on the ceiling of the rack for the open fields. During the measurements, lights of the sound-proof room were put off and an electronic fan was running as both for the ventilation and the source of background noise (35dB). In the open field test, mice were individually introduced at one corner of the field, and then were allowed to move freely for 15 min. Distance traveled (cm) and % duration of staying at the center area of the field (30% of the field) were adopted as the indices, and they were collected every 1 min.

2.4.5 Elevated plus maze test

The next day (Day 14) of the light-dark box test, an elevated plus maze test was conducted. A single channel of elevated plus maze (closed arms: 25 × 5 × 15 cm (H); open arms 25 × 5 × 0.3 cm (H)) was equipped in the same sound-proof room as the open field and the light-dark box. The floor of each arm was made of white plastic and the wall of closed arms and ridge of open arms were made of clear plastic. Closed arms and open arms were arranged orthogonally 60 cm above the floor. Light condition was 70Lux at the center platform of the maze (5 × 5 cm). In the elevated plus maze test, mice were individually put on the center platform facing to an open arm, and then mice were allowed to move freely in the maze for 5 min. Total distance traveled, % time stayed in the open arms, % number of the open arm entry were measured as indices.

2.4.6 Auditory startle response

The next day of the light-dark box test, an auditory startle response test was conducted for two days. One half of the mice were examined on Day 15, and the other half of the mice were examined on Day 16 because of the limitation of the capacity of the laboratory. The auditory startle response test was conducted in another sound-proof room exactly the same as the one in which the open field test, the light-dark box test and the elevated plus maze test were conducted. In this test, each mouse was put into the small cage for startle response (30 or 35 mm diameter, 12 cm long) and set on the sensor block equipped in a sound-proof chamber (60 × 50 × 67 cm (H)). A dim light was equipped on the ceiling of the sound-proof chamber (10 Lux at the center of the sensor block), and a 65 dB white noise was presented as background noise. In the auditory startle response test, mice were acclimatized to the experimental condition for 5 min, then the experimental session began. In the first session, only 120 dB startle stimuli (40 msec) was presented to the mice ten times in random inter-trial intervals (10-20 sec). In the second session, startle response to stimuli at various intensities were assessed. Five times of 70 to 120 dB (70, 75, 80, 85, 90, 95, 100, 110, 120 dB) white noise stimuli (40 msec) were presented in quasi-random order and random inter-trial intervals (10-20 sec). In the prepulse inhibition (PPI) session, mice experienced five types of trials; no stimulus, startle stimulus (120 dB, 40 msec) only, prepulse 70 dB (20 msec, lead time 100 msec) and pulse 120 dB, prepulse 75 dB (20 msec, lead time 100 msec) and pulse 120 dB, prepulse 80 dB (20 msec, lead time 100 msec) and pulse 120 dB. Each trial repeated ten times in quasi-random order and random inter-trial interval (10-20sec). In the final session, again only a 120 dB startle stimuli (40msec) was presented to the mice ten times in random inter-trial intervals (10-20sec). The total duration of an auditory startle response test was about 35 to 40 min. After each trial, holding chambers were washed by tap water, wiped by paper towel and dried. Apparatuses and software for data analysis used were commercially available ones (Mouse Startle; O'Hara, Tokyo, Japan).

2.4.7 Morris' water maze test

Three days after the termination of the auditory startle response test (Day 18), a series of Morris' water maze test began. A circular maze made of white plastic (1m diameter, 30cm depth) was filled with water to about 20 cm in depth (22 to 23 °C). Water was colored by white painting in order that mice could not see the platform (20 cm high, 10 cm diameter; 1cm below the surface of water) or other cues under the water. There were some extra-maze landmark cues (i.e., calendar, figure, plastic box) that were visible to the mice in the maze. The movements of mice in the maze was recorded and analyzed with Image J WM (O'Hara, Tokyo, Japan). Mice received six trials (1 session) per day for four consecutive days. Each acquisition trial was initiated by placing an individual mouse into the water facing the outer edge of the maze at one of four designated starting point quasi-randomly, but the submerged platform remained constant for each mouse throughout testing. A trial was terminated when the mouse reached the platform, and the latency and distance swam were measured. Cut-off time of the trial was 60 sec, and mice that did not reach the platform within 60 sec were removed from the water and placed on the platform for 30s before being toweled off and placed back into their home cage. The inter-trial interval was about 6 min. After the 4 days' training, a probe test was conducted on Day 22. In the probe test, the platform was taken away, and each mouse was placed into the water at the point of the opposite position of the target platform, and allowed to

swim in the maze for 60s. The distance swam, the number of crossings the position of the target platform and other three platforms, time staying in the quadrants of the four platforms were measured.

Figure 1

Figure 1

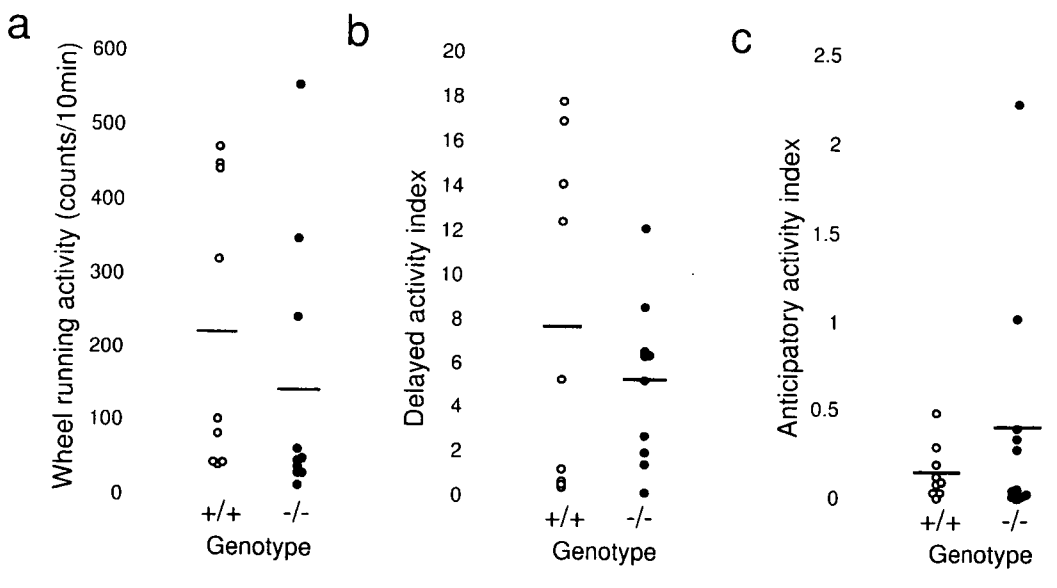

Figure 2

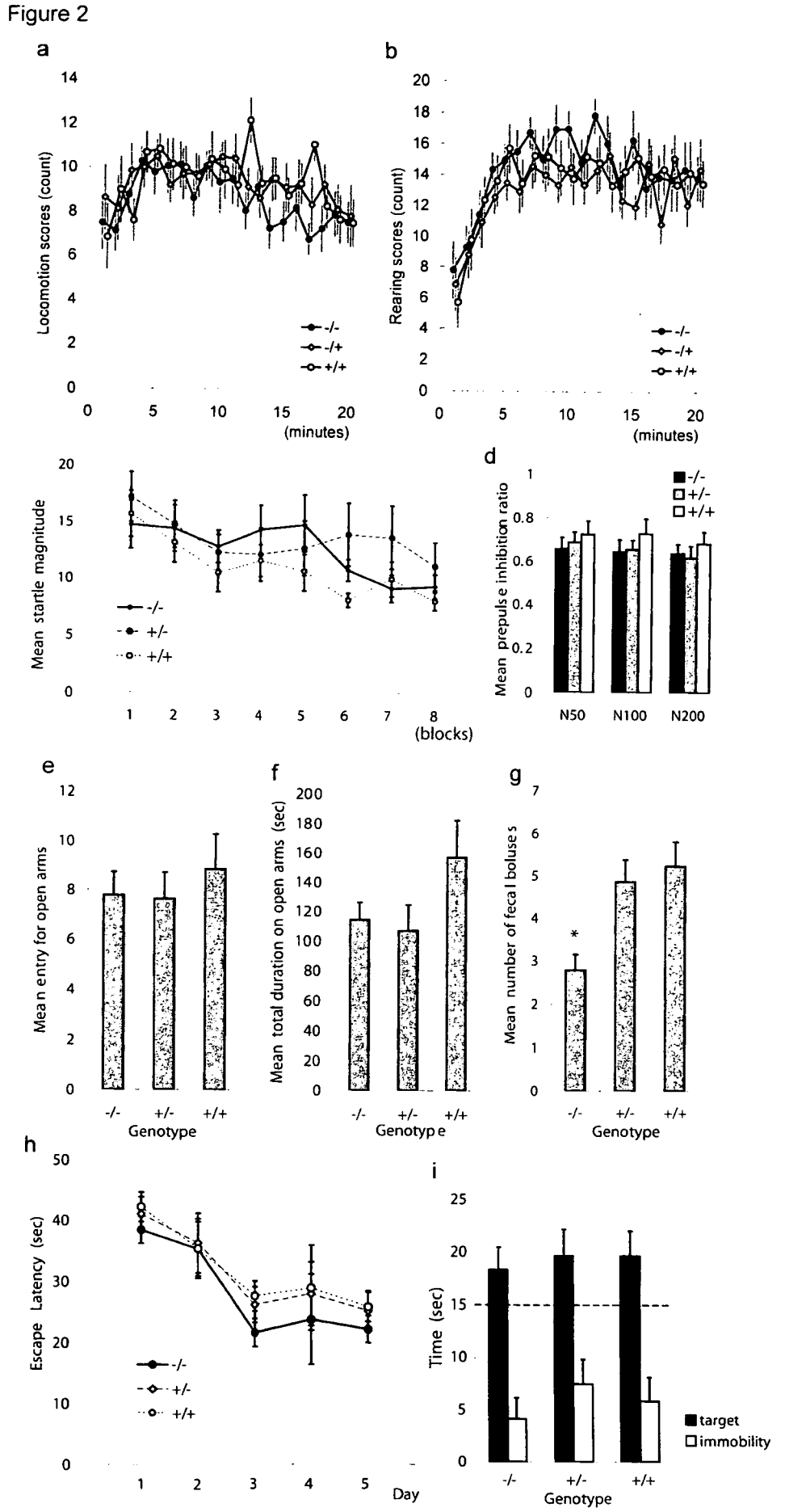
Figure 2
a
Locomotion scores (count)
(minutes)
-/-
-/+
+/+
b
Rearing scores (count)
(minutes)
-/-
-/+
+/+
Mean startle magnitude
(blocks)
-/-
+/-
+/+
d
Mean prepulse inhibition ratio
N50
N100
N200
-/-
+/-
+/+
e
Mean entry for open arms
Genotype
f
Mean total duration on open arms (sec)
Genotype
g
Mean number of fecal boluses
Genotype
h
Escape Latency (sec)
Day
-/-
+/-
+/+
i
Time (sec)
target
immobility
Genotype

Figure 3

Figure 3

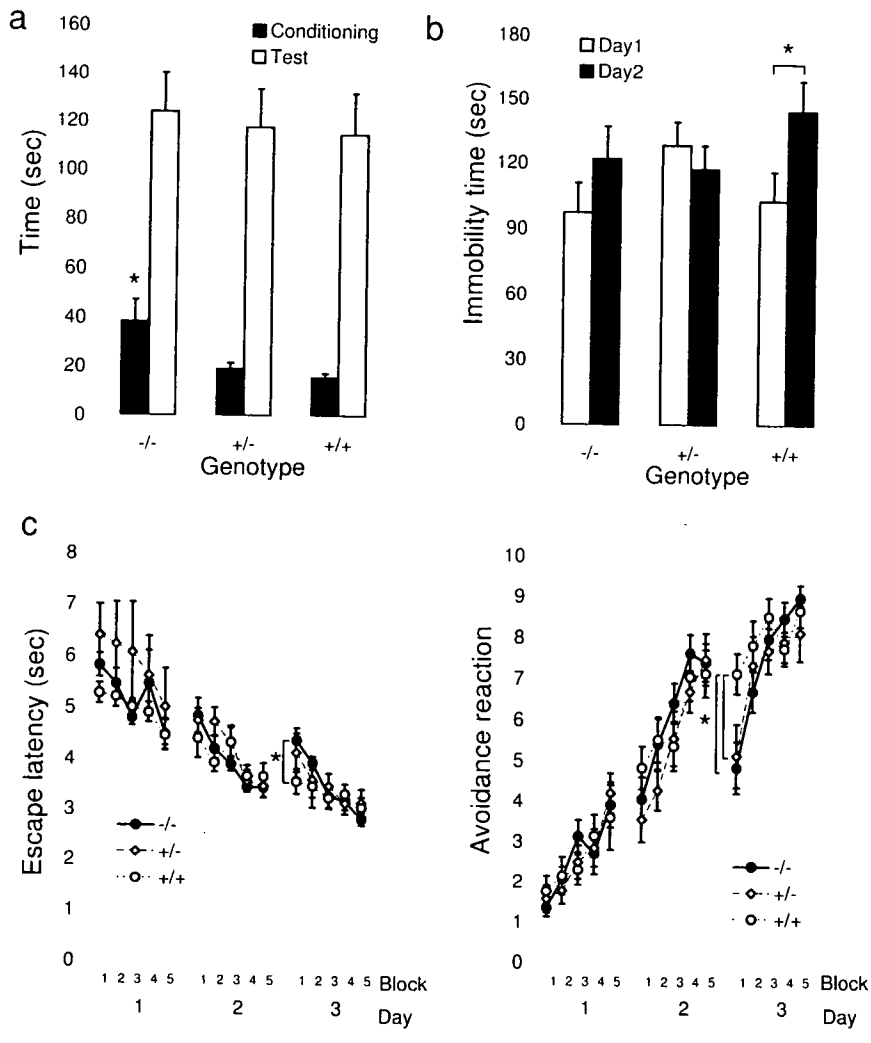

Figure 4

Figure 4

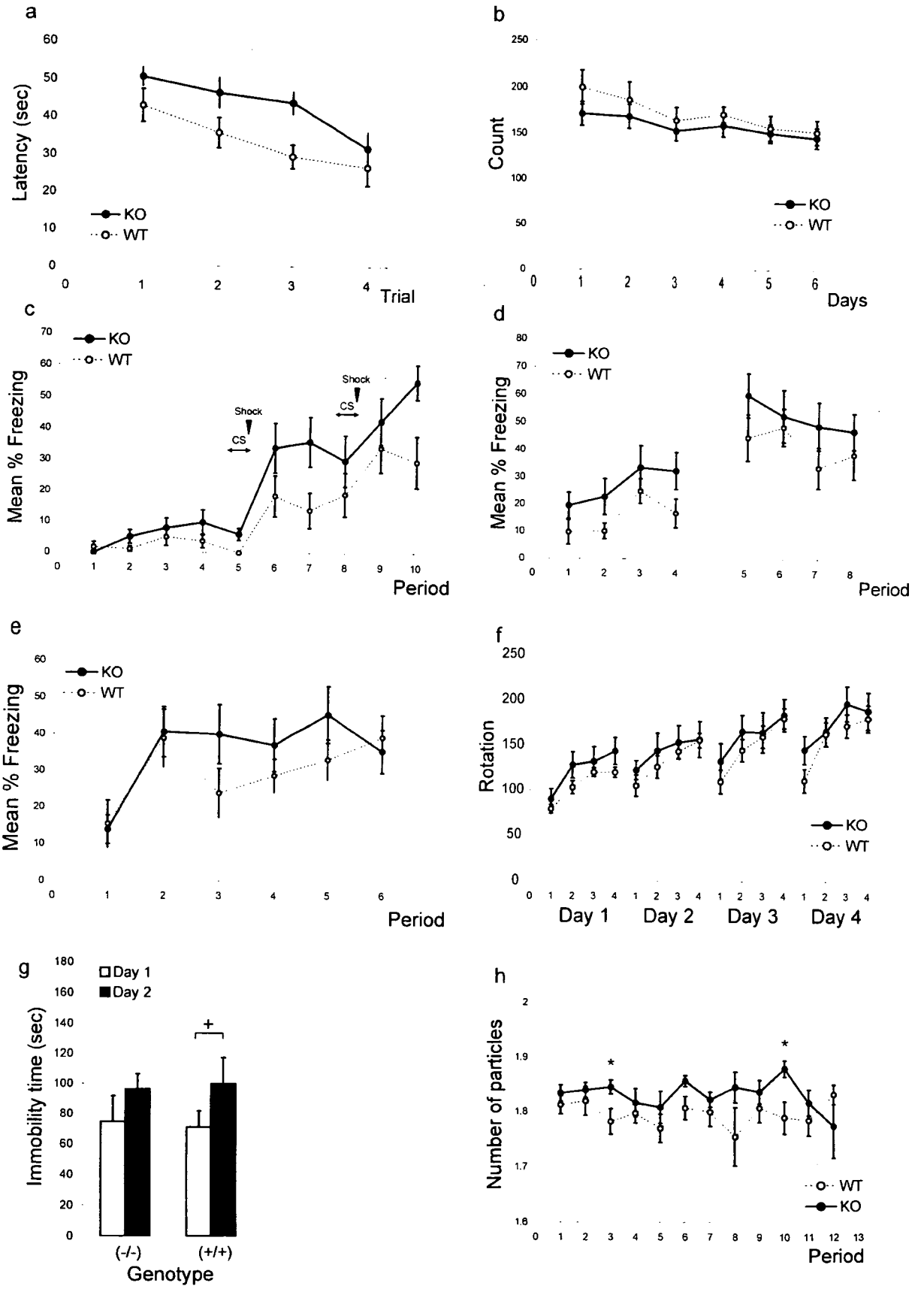

Figure 5

Figure 5

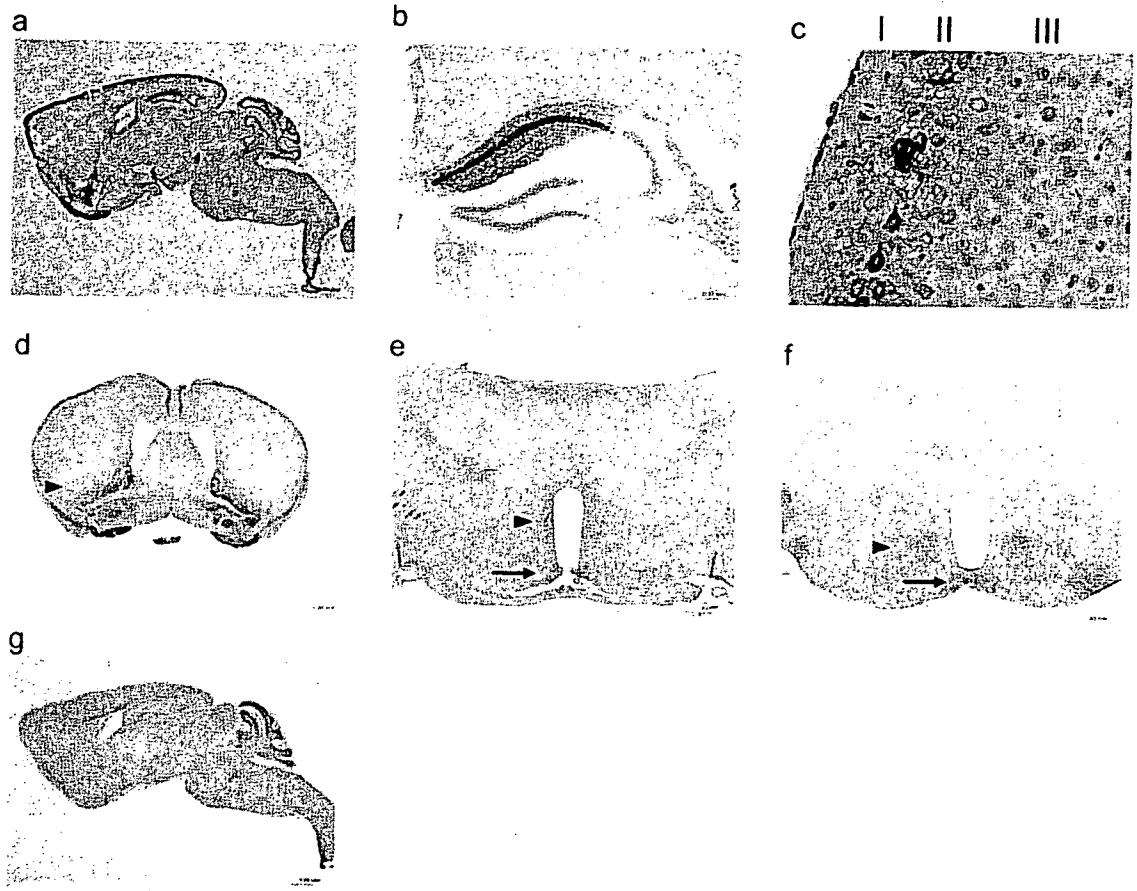

Table 1

Table 1. Summary of findings in behavioral tests

Test battery	Findings
Wheel running activity (34 weeks, 11 KO, 9 WT)	
periodicity	NS
diurnal activity rhythm	NS
Phase I (12 weeks, 13 KO, 14 Hetero, 13 WT)	
Open field	NS
Startle/PPI	NS
Elevated plus maze	NS
Morris Water Maze	NS
Passive avoidance test	Longer latency to move
Active avoidance test	Reduced number of escape at day 3
Forced swimming test	Reduced immobility on the second day
Phase II (31 weeks, 9 KO, 11 WT)	
Home cage activity	NS
Open field	NS
Light-dark box	NS
Elevated plus maze	NS
Startle/PPI	NS
Morris Water Maze	Increased escape latency without the change of distance traveled
Fear conditioning	Enhanced freezing during conditioning and before the cue at the cue test
Phase III (9 weeks, 7 KO, 8 WT)	
Social interaction	Decreased interaction
Rota-rod	NS
Sucrose preference	NS
Tail suspension test	NS
Forced swimming test	Reduced immobility on the second day
Marble burying test	NS
Hot plate test	NS
Tail flick test	NS

KO, Wfs1(-/-); Hetero, Wfs1(-/+); WT, Wfs1 (+/+)
PPI: prepulse inhibition test
NS, non-significant