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New Rat Model for Attention Deficit Hyperactive Disorder (ADHD)

Eikichi Kamimura, PhD[1], Yoshikazu Ueno, PhD[1], Shinya Tanaka, MD[2], Hirofumi Sawa, MD[2], Mitsuhiro Yoshioka, MD[3], Ken-ichi Ueno, MD[3], Takeshi Inoue, MD[4], Xiaobai Li, PhD[4], Tsukasa Koyama, MD[4], Ryoichi Ishikawa, MD[2], and Kazuo Nagashima, MD[2,5*]

Purpose: **In a strain of the Long-Evans Cinnamon (LEC) rats, we found spontaneously hyperactive animals designated as "wiggling," and established a congenic wiggling (Wig) rat by transferring the gene from the LEC to the Wistar King-Aptekman/Hokkaido (WKAH) strain. We evaluated the feasibility of the Wig rat for an animal model of human attention deficit hyperactive disorder (ADHD).**

Methods: **Mode of inheritance was examined by use of linkage analyses. Motor activity, behavior, and working memory were assessed by use of electric digital counters, open field test, and Y-maze and water-maze tests.**

Results: **The abnormal behavior, including hyperactivity, was transmitted in autosomal recessive mode. Diurnal and nocturnal motor activity of 12- to 14-week-old Wig rats was markedly higher than that of controls, and this hyperactivity was more prominent during nighttime than daytime. Ambulation in the open-field test was significantly increased in Wig rats, but rearing was decreased in Wig rats, compared with controls. Results of the Y-maze tests indicated that spontaneous alternation behavior was significantly impaired in Wig rats, although there was no difference in the total arm entries. The water-maze test could not be performed because, when exposed to water, Wig rats panicked and almost drowned.**

Conclusions: **Wig rats are hyperactive and have impaired working memory and impulsive behavior, as assessed by the motor activity and open-field tests and the Y-maze test, and these abnormalities are transmitted by a single gene with Mendelian pattern. Wig rats represent an excellent animal model of human ADHD.**

Attention deficit hyperactive disorder (ADHD) is extremely common, affecting approximately 4% of all children in the United States (1) and one in 200 children in the United Kingdom (2), and is one of the causes of problems at school. The major symptoms of ADHD are inattention, excess impulsivity, and uncontrolled hyperactivity. The cause of ADHD remains unknown, but genetic factors have been implicated (2). To examine the pathophysiology of this disorder, animal models are required. Moreover, to determine the therapeutic efficacy of drugs, animal models are indispensable. Hyperactive mouse models have been produced in transgenic mice carrying multiple copies of the human S-100 beta gene (3). Various mice with motor abnormalities have been produced in knockout models lacking genes for α-calcium calmodulin kinase II (4), receptors of 5-hydroxytryptamine 1A or 1B (5-HTIA or 5-HTIB) (5), and a brain-derived neurotrophic factor (6). A similar mouse model was also produced by immunization with antiphospholipid antibody (7). The murine coloboma strain, which has a spontaneously hyperactive phenotype (8-11), and the acallosal mouse strain with resembling behavioral features (12) have been proposed as genetic models for ADHD.

Use of rats is more advantageous than that of mice because of a larger brain size for experimental analysis. Rats treated with various drugs (13-15) or irradiation (16) have been used as animal models for ADHD, while as a genetic model for ADHD, the spontaneous hypertensive rat (SHR) has been frequently used (17, 18) and various genetic, pharmacologic, and behavioral studies have been reported (19-26). However, recent studies have indicated that acquisition and performance of SHR are not impaired, compared with that of control animals (27). Moreover, to the authors' knowledge, the genes responsible for hyperactivity of SHR have not been identified.

Members of the Long Evans Cinnamon (LEC) strain of rats spontaneously develop acute hepatitis and hepatocellular carcinoma and have excess accumulation of copper in the liver (28) and a remarkable decrease in serum ceruloplasmin activity, similar to findings in humans with Wilson's disease (29). A new rat model originated from the LEC strain presented here has visible hyperactivity; the abnormal behavior is transmitted by a single genetic locus as an autosomal recessive trait. This animal may provide a useful model for research of genetic, pharmacologic, and morphologic studies of disorders of the abnormal behavior of ADHD.

Materials and Methods

Origin of Wig and model establishment. The original line of the LEC rat strain has been maintained by sib-mating at the Center for Experimental Plants and Animals, Hokkaido University. In 1995, two 2-week-old females with distinctive motor abnormalities were found in the 66th generation of this LEC strain. These rats moved circularly with head shaking (wiggling) induced by stimulation, such as sound and oscillation, which were different from normal movement patterns. In addition, when the abnormal rats were lifted by the tip of their tail, they immediately twisted their body, whereas normal rats kept their body stretched. These abnormal females were mated with normal siblings, but did not produce off-

[1]Center for Experimental Plants & Animals, Hokkaido University, [2]Laboratory of Molecular & Cellular Pathology, Departments of [3]Pharmacology and [4]Psychiatry, Hokkaido University School of Medicine, [5]CREST, Science & Technology Corporation, Japan.

**Corresponding author: Kazuo Nagashima, MD, Laboratory of Molecular & Cellular Pathology, Hokkaido University School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan.*

spring. Thereafter, in the 68th generation, four rats (two of each sex), with similar motor abnormalities, were observed. These abnormal rats were separated and maintained; consequently, it has been able to constantly obtain offspring with motor abnormality.

Animals. The abnormal (wiggling) LEC rats were designated as LEC-Wig, and wiggling WKAH rats were designated as Wig. The normal and abnormal (wiggling) LEC and WKAH strain rats (12 to 14 weeks old) studied were maintained under specific-pathogen-free conditions. Serologic monitoring was carried out every four months for the following agents: *Corynebacterium kutscheri*, *Mycoplasma pulmonis*, ectromelia virus, mouse adenovirus, mouse hepatitis virus, *Salmonella typhimurium*, Sendai virus, and Tyzzer's organism. In addition, these animals were examined on April 20, 1999 for various other infective agents, which included *Bordetella bronchiseptica*, *Corynebacterium kutscheri* (again), *Pasteurella pneumotropica*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Streptococcus pneumoniae*, *Mycoplasma pulmonis* (again), dermatophytes, and *Staphylococcus aureus* by use of a culturing method; sialodacryoadenitis virus, Kilham rat virus, lymphocytic choriomeningitis virus, hantavirus, and cilia-associated respiratory bacillus by use of serologic testing; and *Giardia muris*, *Spironucleus muris*, and *Syphacia* spp. by use of microscopic examination. Rats had negative results of tests for all the aforementioned infective agents. Moreover, the conditions under which the rats are kept involve strict health monitoring of all incoming animals to avoid entry of infections; restricting exposure of animals to animal care personnel and adherence to rigorous animal health practices to avoid transmission of infection between cages; provision of sterilized bedding and autoclaved feed at the Center for Experimental Plants and Animals, Hokkaido University. The normal LEC and WKAH strains were maintained by sib-mating, whereas the LEC-Wig strain was maintained by outbreeding. The rats were housed in an environmentally controlled animal room at 23 ± 2°C with relative humidity of 50 ± 20%. Rats were fed a laboratory diet (CMF, Oriental Yeast, Tokyo, Japan) ad libitum. The humane care and use of animals was approved by the Laboratory Animal Use and Care Committee of the Center for Experimental Plants & Animals, Hokkaido University.

Mode of inheritance. To clarify the mode of inheritance of these motor abnormalities, we performed the following crosses between LEC and LEC-Wig rats: abnormal (LEC-Wig) females and males; LEC-Wig females and normal males; normal females and LEC-Wig males; and (LEC × LEC-Wig) F1 × (LEC × LEC-Wig) F1. All of the offspring in these test crosses were determined to be either normal or LEC-Wig according to motor abnormalities at three weeks of age.

Linkage analysis between a causative gene for hepatitis and motor abnormality. Fifty-seven offspring were obtained by mating (WKAH × LEC-Wig with hepatitis) F1 with LEC-Wig. These rats were observed for 60 weeks after birth to determine whether clinical signs of jaundice appeared. Rats that did not appear jaundiced were further assessed by measuring serum ceruloplasmin concentrations, using the method described by Schosinsky and co-workers (30). Rats that developed jaundice and/or had low serum ceruloplasmin concentration (< 50 U/L) were identified as hepatitis type. Although rats that did not develop jaundice and/or had high serum ceruloplasmin concentration (> 50 U/L) were recognized as WKAH type.

Linkage analysis between motor abnormality and coat color. For the 57 backcrossed offspring, the segregation ratio of motor abnormality to coat color also was evaluated. The WKAH rats were albino, but their coat color was hooded agouti (AABBcchh), and that of LEC rats was hooded cinnamon (AABBCChhpp).

Diurnal and nocturnal motor activity. Male and female rats of wiggling WKAH (Wig) and WKAH, 12 to 14 weeks old and five or six rats in each group, were individually housed in home cages (30 × 35 × 17 cm) and were maintained on a 12-h light/dark cycle (light phase: 6:30 a.m. to 6:30 p.m.). After a 30-day acclimatization period, diurnal and nocturnal motor activity was measured for three days. Activity was recorded automatically every 12 h at 6:30 a.m. and 6:30 p.m. by use of electronic digital counters with infrared cell sensors, as described by Ohmori and co-workers (31). Horizontal movement was digitized and stored by computer. Locomotion contributed predominantly to the count, but other body movements, such as neck movements, also contributed when those movements contained substantial horizontal components.

Open-field test. In the open-field test, exploratory behavior and emotional reactivity of rats were assessed in the novel environment. Open-field activities of male and female rats of Wig and WKAH, 12 to 14 weeks old and five or six rats in each group, were measured by use of an apparatus consisting of a circular floor (diameter = 75 cm) divided by lines into 25 segments with a 35-cm-high wall (32). The test was conducted one week after evaluating diurnal and nocturnal motor activity and undertaken between 8 a.m. and midnight. Each rat was carefully placed in the central circle of the open-field and left undisturbed for 5 min, then its behavior was recorded on videotape. At a later time, one person, unaware of the experimental groups, reviewed the video and measured the number of sections entered (ambulation), rearings, and fecal pellets.

Y-Maze and water-maze tests. Spatial working memory was assessed by spontaneous alternation behavior in a black-painted Y-maze made of plywood. Each arm of the Y-maze was 45 cm long, 35 cm high, and 10 cm wide, and both arms were positioned at the same angle. The testing procedure was based on that described by Sarter and co-workers (33). Briefly, five rats in each group (Wig and WKAH) were placed at the end of one arm and allowed to move freely through the maze for an eight-minute test session without reinforcers, such as food, water, or electric shock. An arm entry was defined as the entire body of a rat except its tail completely entering into an arm. The sequence of arm entries was recorded manually. Spontaneous alternation behavior was defined as the entry into all three arms on consecutive choices in triplet sets with overlapping. The maximal number of alternations was then the total number of arms entered minus two, and the percentage of spontaneous alternation behavior was calculated as (actual alternations/maximal alternations) × 100. For example, if the three arms were called A, B and C, and a rat consecutively entered arms in the sequence ACBABACBAB, its performance (actual alternations) would comprise five alternations (ACB, CBA, BAC, ACB and CBA) out of eight (10 - 2) possible alternations, resulting in a percentage of alternation behavior of 62.5. When the water maze test was performed, Wig rats moved vigorously and almost drowned. Thus, further examination was not continued.

Statistical analysis. Four-way analysis of variance (4-way ANOVA, Wig versus WKAH × Sex × Day × Night versus Daytime) was used for statistical analysis of the diurnal and nocturnal activity. Two-way ANOVA (Wig versus WKAH × Sex) was applied for

ambulation, rearing, and defecation measured in the open-field test. When an interaction effect was significant, the Duncan's multiple comparison test was used for further analysis. When values of $P < 0.05$ were obtained, they were considered to be significantly different. Results of the Y-maze test were expressed as the mean ± SEM, and were analyzed by use of a Student *t* test. The criterion for statistical significance was $P < 0.05$.

Neuropathologic analysis. When the LEC animals developed abnormal behavior, we examined the basal ganglia, including subthalamic nuclei by use of semi-serial sectioning, because the animals were considered to be a model of Wilson's disease (29). Two abnormal LEC-Wig rats and two normal Long Evans Agouti (LEA) rats, which are the normal counterpart of LEC rats, and two Wig and two normal WKAH rats were examined. After anesthesia with sodium pentobarbital (40 mg/kg of body weight, i.p.) animals were sacrificed by perfusion fixation via the left ventricle of the heart with physiologic saline followed by 4% paraformaldehyde. Brain slices were embedded in paraffin, and sectioned at 3-μm thickness. They were stained with hematoxylin and eosin, and by use of the Klüver Barrera method for myelin sheaths and the Bodian method for axons. For immunohistochemical staining, they were reacted with antibodies of neurofilament (NF, Dako, Kyoto, Japan), glial fibrillary acidic protein (GFAP, Dako), and microglia/macrophage (ED-1, Serotec, Oxford, England), and the reaction products were visualized with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.).

Results

Mode of inheritance. The results of the mating experiments for mode of inheritance are shown in Table 1. Crosses between the abnormal rats produced 29 offspring, and all of them exhibited the wiggling character. Reciprocal crosses between abnormal and normal rats produced 34 offspring and all of these were normal. Crosses between the F1 rats produced 69 offspring, and the number of normal and wiggling rats was 53 and 16, respectively. This segregation ratio of normal to abnormal was in good agreement with the theoretical segregation ratio of an autosomal recessive pattern (3:1). These results documented that the motor abnormalities are controlled by a single autosomal recessive gene. We now propose to designate the recessive gene *wig* (gene symbol for wiggling), and named the mutant wiggling rat or rats (Wig or Wigs).

Results of linkage analysis between the causative gene for hepatitis and motor abnormality are shown in Table 2. Fifty-seven backcrosses ([WKAH × wiggling hepatitis {LEC-Wig}] F1 with LEC-Wig) segregated as follows: wiggling with hepatitis phenotype, 15; wiggling with WKAH phenotype, 17; normal with hepatitis phenotype, 9; and normal with WKAH phenotype, 16. The segregation ratio of these backcrosses is in agreement with the 1:1:1:1 expected, and indicated that the causative gene for hepatitis is not linked to that of motor abnormalities.

Results of linkage analysis between wiggling and coat color are shown in Table 3. Wiggling with cinnamon (dilute-agouti) was 29, wiggling with agouti was 3, normal with cinnamon was 1, and normal with agouti was 24, producing a crossing-over value calculated as 7.02%. This value suggests a possibility of linkage between motor abnormalities (*wig* loci) and dilution (coat color loci).

Diurnal and nocturnal motor activity (Fig. 1). Four-way ANOVA revealed significant difference between the Wig and WKAH groups ($F[1,108] = 42.677$, $P < 0.0001$). Wigs were more active during daytime and nighttime, compared with WKAH rats,

Table 1. Results of mating experiments for mode of inheritance. Rats that did or did not have motor abnormality were identified as wiggling and normal rats, respectively

Mating			Wiggling		Normal			
F	M	N	F	M	F	M	χ^2	*P*
LEC-Wig	LEC-Wig	3	12	17	0	0	-	-
LEC-Wig	LEC	1	0	0	1	4	-	-
LEC	LEC-Wig	3	0	0	13	16	-	-
F1 x F1		12	7	9	28	25	0.121	< 0.7

LEC = Long Evans Cinnamon strain; Wig = "wiggling" strain of LEC rats.

Table 2. Results of linkage analysis between causative gene for hepatitis and motor abnormality. Rats that developed jaundice and or low serum ceruloplasmin concentration were identified as LEC type

	Motor abnormality		Normal			
	LEC	WKAH	LEC	WKAH	χ^2	*P*
N	15	17	9	16	-	-
Expected N	14.25	14.25	14.25	14.25	2.72	$0.3 < P < 0.5$
Ratio	1	1	1	1	-	-

WKAH =Wistar King-Aptekman/Hokkaido rat strain.

Table 3. Results of linkage analysis between abnormal behavior and coat color

Motor abnormality		Normal		
Cinnamon	Agouti	Cinnamon	Agouti	Linkage ratio
29	3	1	24	7.02

regardless of sex (no interaction between Wig versus WKAH and sex, $F[1,108] = 3.759$, $P = 0.0551$). There was a significant difference between daytime and nighttime ($F[1,108] = 36.317$, $P < 0.0001$], and significant interaction effect between WKAH versus Wig and Nighttime versus Daytime ($F[1,108] = 15.516$, $P = 0.0001$). In general, animals were markedly more active during nighttime than during daytime. Moreover, high levels of motor activity of Wigs were more marked during nighttime. There was a significant effect of sex ($F[1,108] = 4.103$, $P = 0.0453$]; female rats were more active than male rats. Significant effect of experimental days was not observed ($F[2,108] = 0.67$, $P = 0.5138$).

Open-field test. Table 4 summarizes the results obtained in the open-field test. Two-way ANOVA indicated that ambulation of Wig rats was significantly greater than that of WKAH rats ($F[1,18] = 4.894$, $P = 0.0401$; Fig. 2). There was no significant sex effect and no interaction (Effect of Sex, $F[1,18] = 0.134$, $P = 0.7182$; Interaction, $F[1,18] = 0.091$, $P = 0.7658$).

There was a significant difference between WKAH and Wig rats, a significant sex effect, and a significant interaction effect on rearing (WKAH versus Wig, $F[1,18] = 37.425$, $P < 0.0001$; Effect of Sex, $F[1,18] = 10.294$, $P = 0.0049$, Interaction $F[1,18] = 9.273$, $P = 0.007$]. Post-hoc analysis indicated that female WKAH rats indicated significantly ($P < 0.01$) more rearing, compared with male WKAH, male Wig, and female Wig rats.

There was no significant difference between WKAH and Wig rats, no significant effect of sex, and no significant interaction on defecation (WKAH versus Wig, $F[1,18] = 0.929$, $P = 0.348$; Effect of Sex, $F[1,18] = 2.771$, $P = 0.1133$; Interaction, $F[1,18] = 2.771$, $P = 0.1133$].

Y-Maze test. Mean percentage of alternation behavior and total arm entries of WKAH and Wig rats are shown in Fig. 3. The WKAH rats had percentage of alternation behavior of 85 ± 7% and total arm entries of 14 ± 1%. In Wigs, spontaneous alternation behavior was significantly impaired (57 ± 6%, $P < 0.05$), although there was no change in the total arm entries, compared with those for WKAH rats. In addition, the water maze test was

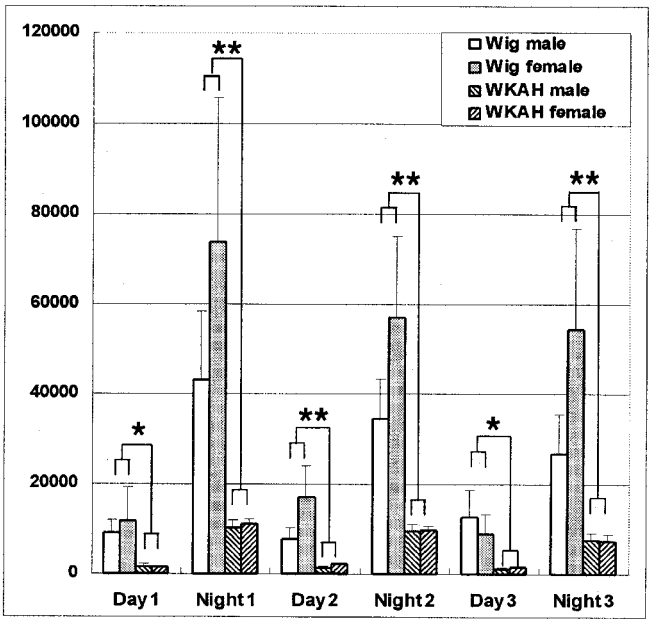

Figure 1. Diurnal and nocturnal motor activity of Wistar King-Aptekman/Hokkaido (WKAH) rats and "wiggling" Long Evans Cinnamon (Wig) strain of rats. Rats were individually placed in their home cages under infrared cell sensors. Motor activity (counts) of daytime and nighttime was measured every 12 h for three days. Represented are the mean and SEM counts of motor activity. N = 5 or 6, in each group. *$P < 0.05$, **$P < 0.01$ between WKAH and Wig groups.

Table 4. Behavioral parameters of WKAH and Wig rats in the open-field test

Group	Ambulation	Rearing	Defecation
Male WKAH	79.3±6.7	14.3±2.2	4.5±1.3
Female WKAH	125.2±7.1	29.7±2.2*	1.3±0.6
Male Wig	251.6±68.4†	6.8±3.3	2.0±0.9
Female Wig	256.0±135.0†	7.2±2.0	2.0±0.8

*$P < 0.01$ vs. male WKAH, male Wig, and female Wig groups; †$P < 0.05$ between WKAH and Wig groups; n = 5 or 6/group.
Data are expressed as mean ± SEM number of sections entered (ambulation) rearings, and defecation.

performed, but when Wig rats were placed on water, they vigorously rolled their body into deeper water, and almost drowned, because of panic.

Neuropathologic analysis. After anesthesia with sodium pentobarbital (40 mg/kg, i.p.) animals were sacrificed, and pathologic examination was performed. The cerebral cortex, white matter, basal ganglia, cerebellum, and brainstem had no abnormalities detected microscopically in LEC-Wigs and Wigs. A few slight vacuolar changes were found in LEC-Wig and Wig rats, but similar changes also were found in the control animals. The GFAP-positive astrocytes were frequently observed in the hippocampus of Wigs, but a similar profile also was found in the controls. There was no increased number of ED-1-positive cells in Wigs, compared with controls. Thus, on the basis of these microscopic immunohistochemical examinations, histologic or immunohistochemical differences were not observed between Wigs and normal controls.

Discussion

The aim of the study reported here was to establish an animal model for human ADHD. Initially, abnormal animals were considered to result from infectious diseases (34, 35), but linkage analyses clearly verified that the disorder is transmitted by

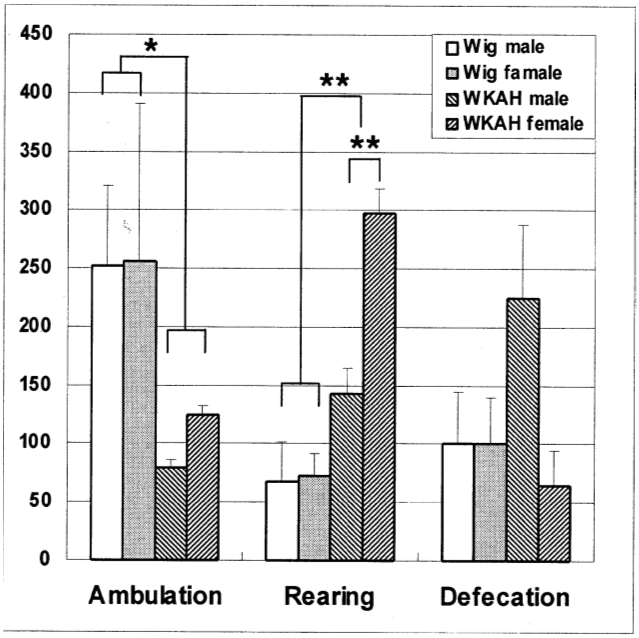

Figure 2. Results of the open-field tests described in Table 4.

an autosomal recessive mode. The LEC-Wig strain was crossbred to WKAH to obtain a congenic rat that expresses the gene for the behavioral abnormality, because LEC rats develop hepatitis at around three to four months of age and around half of them die within one week after the onset of jaundice (28). Furthermore, liver cancer develops in surviving rats after recovery from jaundice as well as in a few rats without prior clinical signs of disease. To establish a congenic strain, however, it is generally agreed that more than 12 generations must be passed. Because the disorder is transmitted in autosomal recessive mode, it takes twice the time until the final establishment of the congenic strain. To date, we have examined the behavior and pathologic changes through the sixth generation; however, more than five or six years will be needed to obtain animals with a theoretically pure genetic background. Considering that preliminary information on the presence of the behaviorally abnormal rats will be useful to those who are interested in behavioral brain research, we report here the process of establishment of congenic rats.

According to the present results, Wig are more hyperactive than WKAH rats, especially during nighttime. Extremely high ambulatory activity of Wig might lower the occurrence of rearing since ambulation scores of about 250 (sections entered) are two or three times as high as scores of naive rats or rats treated with motor stimulants in the open-field test from other studies (32, 36). It is well known that ambulation and rearing are good indices for mainly spontaneous activities (36).

There was no difference in defecation between Wig and WKAH rats. Defecation has good correlation with emotional states of the animal (37). Our results do not suggest that emotional states of Wig was different from that of WKAH rats. Thus, the results from the open-field test indicate that the motor activity of Wig was increased not only in their home cages, but also in the novel environments.

We investigated the profiles of Wig rats on working memory processes by using spontaneous alternation behavior, because percentage of alternation behavior is considered to reflect cognitive

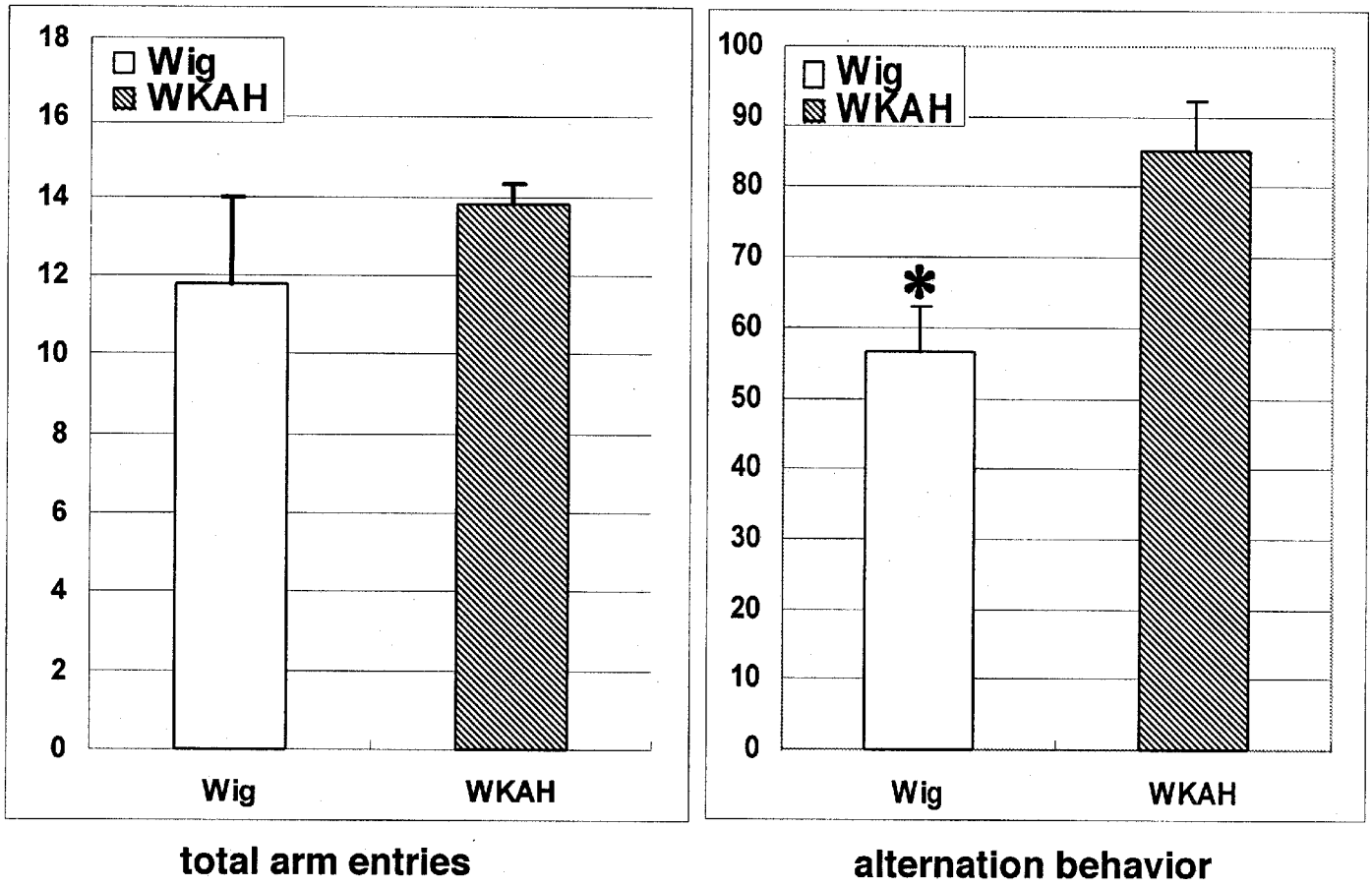

Figure 3. Results of Y-maze tests. N = 5, in each group. *$P < 0.05$.

functions (33, 38). In contrast to WKAH control rats, Wig rats had significant impairment of spontaneous alternation behavior without affecting total arm entries, a measure considered to reflect locomotor activity. Working memory, classified as short-term memory, is thought to underlie spontaneous alternation behavior (33, 38). Spontaneous alternation behavior has been used for assessing cognitive function because it can be impaired by administrating amnesic drugs, such as scopolamine or MK-801 (33, 38) and by transient cerebral ischemia (39, 40). It also is reported that spontaneous alternation behavior is affected by several psychological factors, such as spatial working memory (38, 41, 42), attention (43), preservation (44, 45), and decision making (46). Although we have considered that impairment of spontaneous alternation behavior observed in the Wig rats of this study is based on dysfunction of spatial working memory, these other factors may also play an important role. As described in the Material and Methods section, the water maze test was performed, but Wig rats almost drowned because of panic, suggesting that Wig's behavior is impulsive and that Wig is a rat that cannot swim.

Neuropathologic findings indicate neither malformation nor degenerative changes detected in LEC-Wig and Wig rats. This suggests that the disorder is not progressive and may be caused by functional abnormalities with minimal morphologic changes.

Thus, hyperactivity, impulsiveness, and impairment of spontaneous alternation behavior found in Wig rats may fulfil some of the symptoms observed in humans with ADHD. Moreover, hyperactivity seems less prominent in aged animals, a feature which also is observed in people with ADHD. Segregation analysis of the traits involved in ADHD suggested that the fittest model was a sex-influenced, single-gene, Mendelian pattern (9). Hyperactivity in female Wig rats is a dominant trait, which is the reverse to that seen in people with ADHD. However, Wig transmitted by a single gene with Mendelian pattern may be a good model of ADHD.

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