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Research report

Elevated anxiety-like and depressive behavior in Desert hedgehog knockout male mice

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Abstract

To investigate the functional role of Desert hedgehog (Dhh) gene in the nervous system, we examined motor, sensory, learning and memory functions as well as mood in Dhh knockout (KO) mice. Dhh KO male mice exhibited prolonged immobility time compared with wild-type male mice in the forced swimming test, and showed enhanced inhibition in the Vogel's conflict model. These findings suggest that Dhh KO male mice exhibited enhanced anxiety and depressive behavior compared with wild-type male mice. In contrast, Dhh KO female mice did not show any significant difference compared to wild-type female mice. These behavioral abnormalities of Dhh KO male mice may be due to lower testosterone levels with abnormal development of the testes caused by Dhh-null mutation.

Keywords: Desert hedgehog; Knockout mice; Gonadal dysgenesis; Testosterone; Depression; Anxiety

1. Introduction

Hedgehogs (Hhs) are intercellular signaling proteins that are best known for controlling tissue patterning during development. In mammals, the Hh family consists of three members, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) [9]. They bind to the transmembrane receptors patched to activate complex intracellular signaling cascades involving smoothened (smo) and 3 gli transcription factors [11,13]. Dhh knockout (KO) mice have abnormal perineurial sheath formation, which results in minifascicles formation associated with abnormal testicular development [5,21]. These findings suggest that Dhh secreted by Schwann cells controls the correct formation of the connective tissue sheaths around peripheral nerves [21]. In humans, a null mutation in DHH also leads to peripheral neuropathy with minifascicle formation and 46XY gonadal dysgenesis [25,26]. Clinical features of Dhh KO mice were quite similar to those of the patient with DHH gene mutation. Although Dhh is not known to be expressed in brain, the patient with DHH gene mutation had impaired intellectual function [25,26]. Thus, the present studies were initiated to determine whether Dhh gene mutation induces behavioral and physiological abnormalities in mice.

2. Materials and methods

2.1. Animals

Dhh KO mice were obtained by homologous recombination as described previously [5]. Animals were genotyped as *Dhh*+/+, +/− or −/− by PCR on genomic DNA [21]. The Dhh KO mice and wild-type mice at 2–6 months of age with a mixed CD1/C57Bl6 genetic background were used for all experiments. Two independent experiments; experiment 1 (wild-type male mice versus Dhh KO male mice) and experiment 2 (wild-type female mice versus Dhh KO female mice), were carried out. They were tested repeatedly according to the experimental schedule (Fig. 1). They were kept under a constant light–dark cycle (lights on 07:00–19:00 h) in a temperature-controlled (23 ± 2 °C) room. Experiments were conducted during the light phase between 09:00 and 17:00 h. The animals had free access to food (CE-2; Clea Japan, Tokyo, Japan) and water in their home cages. All procedures regarding animal care and use were performed in

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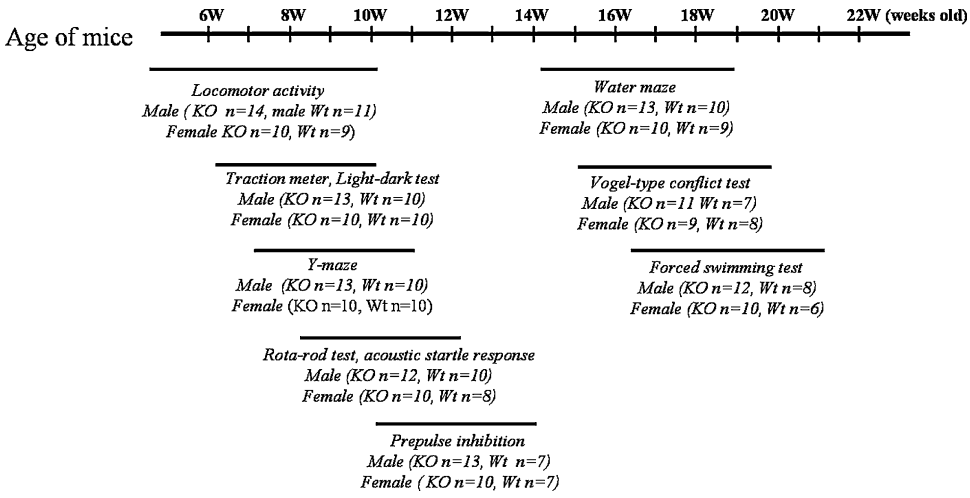

Fig. 1. Experimental schedule. KO: Desert hedgehog knockout mouse, Wt: wild-type mouse.

compliance with the regulations established by the Experimental Animal Care and Use Committee of Fukuoka University.

2.2. Locomotor activity

Locomotor activity was measured for 24 h using an automated activity counter (NS-AS01; Neuroscience Inc., Tokyo, Japan) placed 15 cm above the activity cage (30 cm × 36 cm × 17 cm).

2.3. Rota-rod test

The rota-rod test for motor coordination was performed as described previously [10]. Mice were placed on a rotating rod (3 cm diameter; Neuroscience Inc.) with a non-skid surface, and the latency to falling was measured for up to 2 min. The test was performed three times, and the rotating speed was increased in the order of 5, 10 and 15 rpm each day.

2.4. Traction meter for muscle tone

The traction meter (Neuroscience Inc.) consisted of a detector (48.5 cm × 26 cm × 10 cm) for muscle tone with stainless grids (2 mm in diameter, 29 cm × 16 cm) connected to a spring and a printer as described previously [18]. The stainless grids can freely rotate. The interval of the grids is 2 cm. Each mouse was placed on the stainless grid of the apparatus and the tail was slowly pulled caudally in parallel to the long axis of the body at a constant speed by an experimenter. The forepaw resistance was measured by the detector as muscle tone.

2.5. Light/dark test

The light/dark test apparatus consisted of two acrylic resin compartments connected by a small opening (7.5 cm × 7.5 cm). One compartment (18 cm × 27 cm × 27 cm) was illuminated with white light, and the other (18 cm × 18 cm × 27 cm) remained dark. The animals were placed in the middle of the light compartment facing away from the opening, and the times spent in the light and dark compartments were recorded over a 10 min observation period.

2.6. Forced swimming test

The forced swimming procedure was a modification of that described by Porsolt et al. [22]. Animals were placed in individual plastic cylinders (diameter 11 cm; height 18 cm) filled with 10 cm of water (23 ± 1 °C) for 15 min and then removed and dried. Twenty-four hours later, a magnet was attached to one of their forelimbs, and they were replaced in the cylinders. The immobility time was recorded using the MicroAct Scratching Test (version 1.03; Neuroscience Inc.) during a 6 min observation period.

2.7. Vogel-type conflict test

The apparatus was a Plexiglas box (20 cm × 20 cm × 30 cm) with a stainless steel grid floor. A stainless steel spout extended 2.5 cm into the box at a height of 6.5 cm above the floor. The number of licks, drops of water (about 0.06 mL/drop) and shocks was recorded with a drinkometer system (O'hara, Tokyo, Japan). An electric shock (0.848 mA, 117 ms) was delivered by a shock generator (O'hara) through the spout and grid floor every 20 licks. On the first day, individual animals that had been deprived of water for 24 h were allowed to freely drink water from the spout for 3 min in the box without the shocks (shock free test). Three days later, the same mice, which were again deprived of water for 24 h prior to the test, were allowed to drink water freely for 10 min in the box with the shocks (shock 50 V test). The number of licks from the spout was recorded for 10 min in each test.

2.8. Y-maze apparatus for attention

A non-painted Y-maze made of plywood was used. Each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom and 10 cm wide at the top with the arms positioned at the same angle. Each mouse was placed at the end of one fixed arm (start arm) and was allowed to move freely through the maze for an 8 min test session. The apparatus was placed on the floor of the experimental room and was illuminated with a 100-W bulb suspended 200 cm above the maze. The sequence of arm entries was recorded manually. An alternation was defined as the entry into all three arms on consecutive choices. The number of maximum alternations was taken as the total number of arms entered minus 2, and the percent alternation was calculated as (actual alternations/maximum alternations) × 100. The arms were cleaned between testing periods for different animals.

2.9. Acoustic startle response and prepulse inhibition

The startle responses were measured with an illuminated startle chamber (39 cm × 38 cm × 58 cm, SR-LAB system, San Diego Instruments, San Diego, CA). It consisted of a Plexiglas cylinder (8 cm diameter, 16 cm long) mounted on a removable frame with a base unit. Movement of the mouse within the cylinder was detected by a piezoelectric accelerometer attached below the frame. A loudspeaker mounted 25 cm above the cylinder, provided the background white noise and acoustic stimuli. Presentation of the acoustic stimuli and the piezoelectric responses from the accelerometer were controlled and digitized by SR-LAB software and an interface system. The startle amplitude was defined as the average of 100 readings taken at 1ms intervals, starting from the beginning

of the startle stimulus onset. During the session the background noise was kept constant at 65 dB. Animals were placed into cylinders 10 min prior to the initial startle stimuli and only background noise was offered during this acclimation period. In the first experiment for measuring acoustic startle response, eight trial types of startles stimuli (70, 75, 80, 85, 90, 100, 110 and 120 dB, 20 ms broad band burst) were used. Each was repeated five times in a constant order. The trials were separated by a constant interval of 30 s. In the second experiment to measure acoustic prepulse inhibition, the five trial types were: no stimulus, startle stimulus only (100 and 120 dB, 20 ms broad band burst) and two types of startle stimulus proceeded by a prepulse (a 20 ms broad band burst). The prepulse onset was separated from the startle onset by 100 ms prepulse-startle interval (PSI), and the prepulse intensities used were 70 and 80 dB. Each was repeated 10 times in a random order. The trials were separated by an average interval of 30 s (15–30 s). Both acoustic startle response and prepulse inhibition were measured.

2.10. Water maze task for spatial learning

The swimming pool (Neuroscience Inc.) used was a circular water tank, 150 cm in diameter and 45 cm deep, which was modified according to that described by Morris [19]. It was filled to a depth of 30.5 cm with clear water at a temperature of 23 ± 2 °C. The test was performed using a 100-W bulb for illumination. A platform, 12 cm in diameter and 30 cm in height, was placed inside the tank, its surface being 0.5 cm below the surface of the water. The pool was located in a large test room, and surrounded by many cues external to the maze (e.g. the experimenter, ceiling lights, rack, etc.), which were visible from within the pool and could be used by the mouse for spatial location. Positions of the cues were kept unchanged throughout the test period. A CCD camera equipped with a personal computer was used for the behavioral analysis in the water maze task. Each mouse received three trials daily for 3 consecutive days. A trial consisted of placing the mouse by hand into the water facing the wall of the pool at one of three starting positions except in the quadrant where the platform was located. The pool was divided into sections (north: N, south: S, east: E or west: W). The platform was located in a constant position in the middle of one quadrant and the platform location was the same for all mice. During each block of three trials, each mouse started once at each of the three starting positions, with the sequence of the positions selected randomly. In each trial, the swimming time to escape onto the hidden platform was recorded and the cut-off time was 120 s. If a mouse found the platform, it was permitted to remain there for 30 s. If a mouse failed to find the platform within 120 s, the mouse was forced to remain on the platform for 30 s. At the end of a trial, the mouse was returned to its house cage. The inter-trial interval time was approximately 30 min. The performance of the test animal in each trial was assessed by three parameters; swimming time, swimming length and swimming speed via a personal computer for behavioral analysis (AXIS-30, Neuroscience Inc.).

2.11. Apparatus and behavioral procedures for the eight radial maze

Behavioral testing was conducted as previously reported [10,15], in an eight-arm radial maze (Neuroscience, Tokyo, Japan), which was a modification of the previous study [20]. The apparatus (Neuroscience Co., Tokyo, Japan) consisted of a central platform 18 cm in diameter, with eight arms extending radially. Each arm was 30 cm long, 6 cm wide and 2.5 cm high with transparent plastic sidewalls. It was elevated 50 cm from the floor. Food cups for the food pellets were placed near the end of each arm. A confinement procedure was used utilizing transparent guillotine doors at the entrance to each arm. The doors were lowered and kept closed for 2 s after the mice returned to the central platform. This procedure is known to disrupt chaining responses and kinesthetic strategies in rodents. The maze was located in a room containing many extra-maze visual cues. The animals were trained so that they would become habituated to the apparatus and food pellets for 3 days before each test. In each training session, the animal was placed on a central platform at the middle of the eight-arm radial maze. Then, after 1 min, the doors were lifted and the animal was allowed to move freely in the maze. The trial continued until either the animal had entered all eight arms or 5 min had elapsed. The performance of the animal in each trial was assessed using two parameters: (1) the number of CC in the initial eight chosen arms and (2) the number of EC which was defined as choosing arms which had already been visited.

2.12. Serum concentration of testosterone

The serum concentration of testosterone was estimated by using a testosterone EIA kit (Cayman chemical, Michigan, USA) according to the manufacturer's instruction.

2.13. Statistical analyses

Values are expressed as the mean ± S.E.M. Data analyses of the Rota-rod test, forced swimming test, water maze, acoustic response, PPI and eight-arm radial maze were evaluated for statistical significance using two-way (with repeated measures) analysis of variance (ANOVA) followed by the unpaired *t*-test to determine differences among the groups. The other data were analyzed by the unpaired *t*-test. A *p*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Rota-rod performance for motor coordination

The rota-rod performance for motor coordination was carried out with mice aged 9–13 weeks. At rotating speeds of 5, 10 and 15 rpm, there were no significant differences between the wild type and both male and female Dhh KO mice (Fig. 2). The repeated measures of two-way ANOVA showed a group difference ($F(1, 20) = 0.115$, $p = 0.734$), effect of speed ($F(2, 40) = 10.060$, $p < 0.001$) and a group × speed interaction ($F(2, 40) = 0.050$, $p = 0.951$).

3.2. Locomotor activity and light/dark test

The 24-h activities of Dhh KO male mice were not significantly different from those of the wild type and female Dhh KO mice (data not shown). Moreover, there was no significant difference between wild type and Dhh KO male mice for their time spent in the light compartment in the light/dark test (data not shown). There was no significant difference between wild-type female mice and Dhh KO female mice.

3.3. Traction meter for muscle tone

The traction of wild-type male mice was 0.165 ± 0.007 kgf ($n = 10$), and that of Dhh KO male mice was 0.151 ± 0.008 kgf

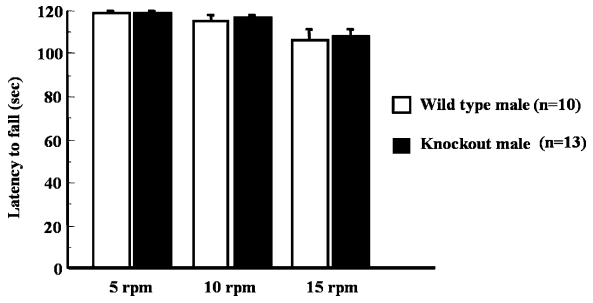

Fig. 2. Rota-rod performance in Dhh KO mice. Dhh KO male mice show no impairment on the rota-rod task. Data are represented as mean ± S.E.M.

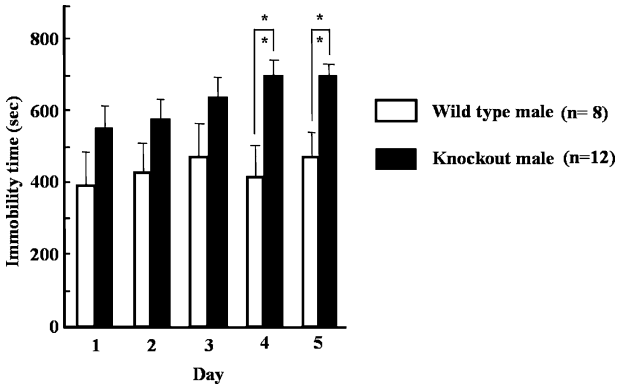

Fig. 3. Forced swimming test. Dhh knockout male mice show prolonged immobility time on day 4 and 5. Significant differences are indicated as $^{*}p < 0.05$, $^{**}p < 0.01$(Student's *t*-test). Data are represented as mean ± S.E.M.

($n = 13$). The traction/body weight × 100 of wild-type male mice was 0.535 ± 0.013 ($n = 10$), and that of Dhh KO male mice was 0.557 ± 0.021. There was no significant difference between wild-type female and Dhh KO female mice (data not shown).

3.4. Forced swimming test

The repeated measures of two-way ANOVA showed a group difference ($F(1, 19) = 8.226$, $p < 0.01$), effect of day ($F(4, 76) = 2.228$, $p = 0.738$) and a group × day interaction ($F(4, 76) = 0.804$, $p = 0.5263$) in experiments 1 (male). Post hoc analysis showed that Dhh KO male exhibited a significant increase in the immobility time on day 4 and 5, compared to wild-type male mice (Fig. 3). In contrast, there was no significant difference between Dhh KO female mice and wild-type female mice in experiments 2 (data not shown).

3.5. Anxiety behavior using conflict apparatus

In the Vogel's conflict test, the number of licks under shock free condition was not significantly different between wild type and Dhh KO male mice. However, the number of licks under shock condition was significantly decreased in Dhh KO male mice compared with wild-type mice (Fig. 4). (The number of licks under a 50 V shock test/the number of licks under a shock free test) × 100 was 50.7 ± 8.8% in wild-type male mice and 32.2 ± 9.4% in Dhh KO male mice ($p < 0.05$). In contrast, there was no significant difference between Dhh KO female mice and wild-type female mice in the same experiment (data not shown).

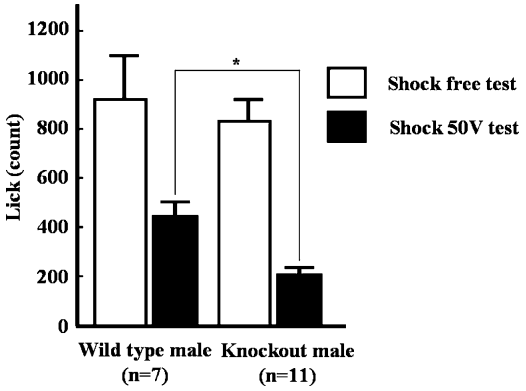

Fig. 4. Vogel's conflict test. Dhh knockout male mice show enhanced suppression of lick count under shock condition. Significant differences are indicated as $^{*}p < 0.05$ (Student's *t*-test). Data are represented as mean ± S.E.M.

3.6. Spontaneous alternation performance in Y-maze task

There was no difference in the total numbers of arm entries between the wild type and Dhh KO mice in both experiments 1(male) and experiments 2 (female) (Table 1).

3.7. Acoustic startle response and prepulse inhibition

In the first experiment to measure the acoustic startle responses, Dhh KO male mice tended to display higher levels of acoustic startle responses than the wild-type male mice, although this was not significant (Fig. 5A). The repeated measures of two-way ANOVA showed a group difference ($F(1, 21) = 1.718$, $p = 0.2041$), effect of pulse intensity ($F(7, 147) = 18.533$, $p < 0.001$) and a group × pulse intensity interaction ($F(7, 147) = 1.737$, $p = 0.1046$). In the second experiment for measuring acoustic prepulse inhibition by a startle stimulus of 100 and 120 dB, there was no significant difference between Dhh KO male mice and wild-type male mice (Fig. 5B). The repeated measures of two-way ANOVA showed a group difference ($F(1, 18) = 2.472$, $p = 0.1333$), effect of prepulse–pulse intensity ($F(3, 54) = 34.727$, $p < 0.001$) and a group × prepulse–pulse intensity interaction ($F(3, 54) = 0.583$, $p = 0.6286$). Similar results were obtained in the experiments using Dhh KO female mice and wild-type female mice (data not shown).

3.8. Water maze task for spatial learning

The water maze task was carried out at ages 9–13 weeks. The repeated measures of two-way ANOVA for swimming length showed a group difference ($F(1, 21) = 0.964$, $p = 0.3373$), effect of day ($F(4, 84) = 7.228$, $p < 0.001$) and a group × day interaction ($F(4, 84) = 3.564$, $p < 0.001$). Post hoc analysis showed that the Dhh KO male mice showed longer swimming time (data not shown) and length than wild-type male mice on day 1 (Fig. 6A). However, the repeated measures of two-way ANOVA for swimming speed showed a group difference ($F(1, 21) = 5.765$, $p < 0.05$), effect of day ($F(4, 84) = 6.277$, $p < 0.001$) and a group × day interaction ($F(4, 84) = 1.597$, $p = 0.1826$). Post hoc analysis showed that the swimming speed of Dhh KO male mice was significantly lower than that of wild-type mice on days 2, 4 and 5 (Fig. 6B). There was no significant difference between wild type and Dhh KO female mice (data not shown).

3.9. Eight-arm radial maze

In the eight-arm radial maze task, no significant changes in performance were observed between Dhh KO male mice and wild-type male mice. The repeated measures of two-way

Table 1
Spontaneous alternation performance in Y-maze task in male mice

	No. of mice	Total arm entries	No. of entries alternations	Alternation (%)
Wild type	10	21.8 ± 1.9	13.0 ± 1.8	64.0 ± 3.4
Knockout	13	22.0 ± 1.3	12.2 ± 0.6	62.2 ± 3.5

Experimental schedule and the number of animals tested in each experiment (KO: Dhh KO mice, Wt: wild-type mice).

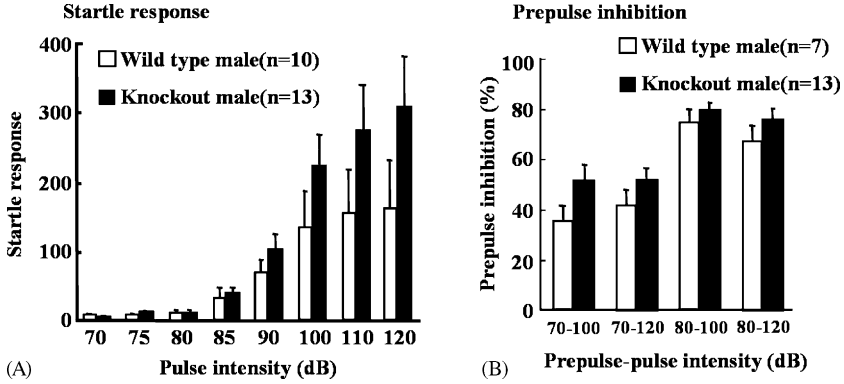

Fig. 5. Acoustic startle response and prepulse inhibition. (A) Startle response, (B) prepulse inhibition. Dhh knockout male mice did not show significant difference in either A or B. Data is represented as mean ± S.E.M.

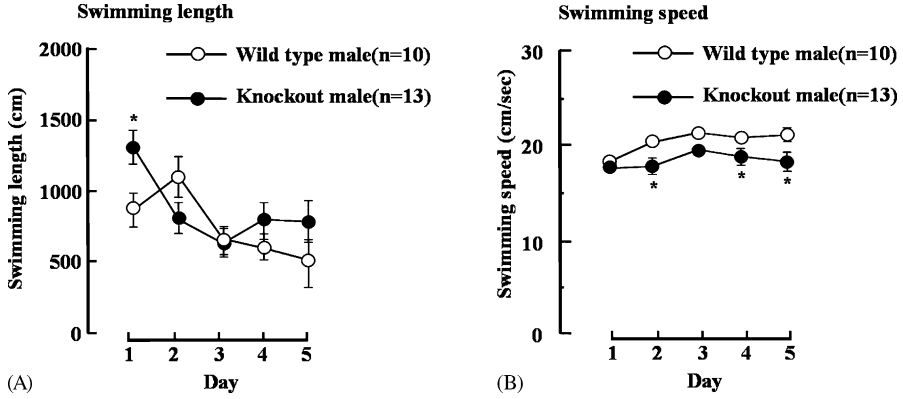

Fig. 6. Water maze task. Swimming length (A) and speed (B) of Dhh KO male mice was not significantly different from those of wild-type mice. Swimming length of Dhh KO male mice became lower than that of wild-type mice on day 1. Swimming speed of Dhh KO male mice became lower than that of wild-type mice on day 2, 4 and 5. $^{*}p<0.05$ (Student's *t*-test). The data are represented as mean ± S.E.M.

ANOVA showed a group difference ($F(1, 20)=0.248$, $p=0.6241$), effect of block ($F(4, 80)=13.509$, $p<0.001$) and a group × block interaction ($F(4, 80)=1.162$, $p=0.3338$) for correct choices, and a group difference ($F(1, 20)=2.828$, $p=0.1082$), effect of block ($F(4, 80)=10.599$, $p<0.001$) and a group × block interaction ($F(4, 80)=0{,}647$ $p=0.6308$) for errors. However, the number of error choice in Dhh KO male mice tend to show more than that in wild-type mice, although statistically not significant (Fig. 7).

3.10. Serum testosterone levels

Serum testosterone levels in Dhh null male mice ($n=5$, 61.6 ± 10.2 pg/mL) were significantly lower than those of wild-type male mice ($n=5$, 796.3 ± 23.5 pg/mL).

4. Discussion

In the present study, Dhh KO male mice exhibited a prolonged immobility time compared with wild-type male mice in the forced swimming test, a test with high predictivity for antidepressant efficacy in human depression [22]. In addition, Dhh KO male mice showed enhanced inhibition in the Vogel's conflict model, which reflect anxiety-like behaviors [27]. Taken together, Dhh KO male mice exhibited enhanced anxiety-like and depressive behavior compared with wild-type male mice. In contrast, there was no significant difference between Dhh KO female mice and wild-type female mice. Therefore, abnormal behavior of Dhh KO male mice is unlikely to have been caused by the absence of Dhh, but may be an associated sex-related dysfunction.

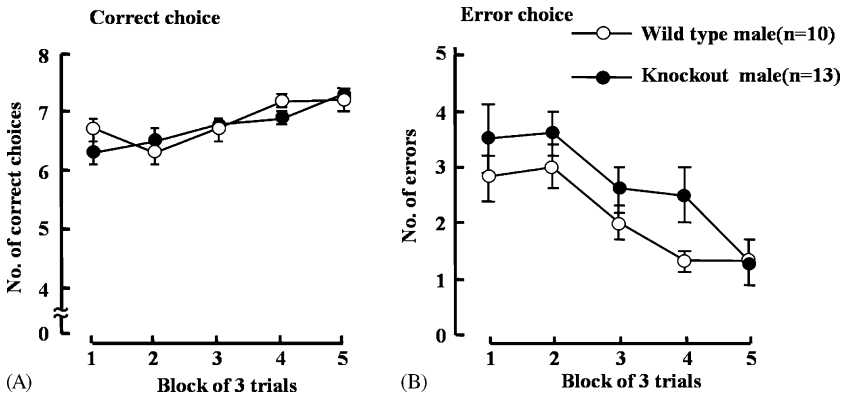

Fig. 7. Eight-arm radial maze. Although the number of correct choices were not significantly different between Dhh KO male mice and wild-type male mice, the number of error choice in Dhh KO male mice tend to be more than that in wild-type mice.

One of the most characteristic findings in Dhh KO mice is the presence of developmental abnormalities in the testes [5]. Dhh encodes a signaling molecule expressed in the testis, and plays a role in the regulation of spermatogenesis. Male mice homozygous for a Dhh-null mutation showed developmental abnormalities in the testes, which may result in lower testosterone levels. Because behavioral abnormalities in Dhh KO mice were restricted to male mice, we postulated that such phenomenon may relate to abnormal testicular development in Dhh KO male mice. Some evidences exist that testosterone and its metabolites are anxiolytic in male rodents [4,8]. Reduced testosterone signaling in rodents leads to behavior indicative of increased anxiety. Removal of testes (gonadectomy) increases anxiety behavior in several tasks [1]. Long-term castration of mice significantly increases the duration of immobility in the forced swimming test [3]. That testosterone exerts an anti-conflict effect in Vogel's conflict test is in line with a previous report showing an anxiolytic-like effect of anabolic-androgenic steroids in another model of anxiety [24]. These evidences support the idea that increased anxiety-like and depressive behavior in Dhh KO male mice may be associated with abnormal development of the testes and lower testosterone levels.

There is a strong link between testosterone and emotional disturbances in humans. Mood fluctuations, depression and anxiety have often been associated with low levels of testosterone in older men and hypogonadal young men [12]. Although the brain site at which testosterone exerts its anxiolytic effects is not known, several lines of research suggest possibilities. One possible non-genomic mode of testosterone action in the brain involves testosterone's conversion by oligodendrocytes into 3α, 5α-reduced metabolites [7] which are powerful $GABA_A$ agonists [4]. Since $GABA_A$ agonists are anxiolytic, testosterone reduces anxiety. Testosterone also increases dopamine levels in the nucleus accumbens [2]. Since depletion of dopamine in the nucleus accumbens impairs avoidance responding to an aversive stimulus (foot shock) [17], testosterone may act to modulate behavioral changes mediated by dopamine responsiveness [23].

In the present study, both Dhh KO male and female mice showed no impairment in the Rota-rod test for motor coordination, in locomotor activity and in the traction meter for muscle tone. The results suggest that the motor function of Dhh KO mice does not change in the absence of Dhh. On the other hand, Dhh KO male mice showed longer swimming length on day 1 and lower swimming speed on days 2, 4 and 5. This longer swimming length may come from following reasons: (1) lower swimming ability, (2) greater stress against water or (3) impairment of spatial learning. Firstly, the longer swimming length is associated with lower swimming ability because Dhh KO mice produced lower swimming speed on days 2, 4 and 5. Secondly, the impairment was transient on day 1 and did not continue in the water maze. Thirdly, Dhh KO mice did not have any impairment in spontaneous alternation in the Y-maze test for the evaluation of attention and short-term memory. Thus, these results suggest that this longer swimming length might be related to lower swimming ability or greater stress against water, as shown in the forced swimming test, rather than spatial learning impairment in Dhh KO male mice. However, this finding do not exclude the possibility that Dhh KO male mice have spatial learning impairment, because Dhh KO male mice tend to show more frequent error choice in the eight-arm radial maze. Previous studies demonstrated that perinatal gonadal steroid manipulations can alter hippocampal morphology, which correlates with spatial performance in adulthood [14,16]. Further studies will be required to confirm the spatial learning ability in Dhh KO male mice.

In the present study, Dhh KO male mice showed anxiety-like behavior in the Vogel's conflict test, but not in the light/dark test. In light/dark test based upon natural behaviors, rodents move between light (aversion) and dark (preference) compartments. In contrast to experimental models involving such spontaneous (untrained) behaviors, in Vogel's conflict test, subjects receive a punishment (mild electric shock) leading to suppression of a conditioned (learned) response for reinforcement (water), thereby producing a conflict between punishment and reinforcement. In addition, it is difficult to detect the effect of non-benzodiazepine on anxiety-like behavior in the Vogel's conflict test. The results suggest that Dhh gene may be related to anxiety-like behavior under strong conflict situation in male mice.

Patients with male gonadal dysgenesis often have intellectual or mental abnormalities. Women with Turner syndrome reported a higher rate of lifetime depression compared with rates observed in community-based studies but similar to those obtained from gynecologic clinic samples [6]. Although the mechanism of these phenomenon has not been fully understood, inappropriate sexual development may have effects on the development of the central nervous system. Therefore, Dhh KO male mice may be a good model to analyze the effect of testosterone in disorders of fear and in anxiety in patients with gonadal dysgenesis.

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