Review

Tokai High Avoider rat (THA rat) that maintains an inborn high learning ability : Its application to screening of environmental factors with neurobehavioral effects

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Abstract

Learning behavioral tests are regarded as less sensitive tests than other psycomoter tests, since these animal experiments have large variance in their results partly due to the variance among the individual animals and its limited test schedules after their trainings.

We developed a strain of rat, which has a high learning behavioral ability innately by repeating breeding and selection by learning test. The learning behavior test was a Sidman Avoidance Test, and the strain has a very high avoidance rate (>98%) innately and the individual variance was very small (CV<1%). (The strain named as THA: Tokai High Avoider rat strain). The strain showed similar good results with little variance among animals not only in Sidman Avoidance Test, but also in Shuttle Box Avoidance Test and Water E-maze Test.

Acetylcholine release in the striatum and cerebral cortex of the rats showed high in concentration, which is reported to be highly correlated to learning ability.

This THA strain enabled us to evaluate effects of exposure to low dose of chemical agents on successive generations such as dams and pups, during perinatal and infantile periods.

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《**Key words**》 THA rat, new strain, learning behavior, animal experiment

Ⅰ. Introduction

We developed rats that innately exhibit high performance with small individual variation on learning behavior tests, and have maintained them to the 69th generation to date. Learning behavior tests are generally performed by raising animals after purchase until maturation, confirming that they have no abnormality in the development or health, training them in learning behavior, and then testing them. Because of such an experimental procedure, (1) the time of experiment (exposure) is restricted, and (2) commercially available animals show a large variation in the results of learning behavior so that the effects of chemicals and drugs on learning behavior are masked, possibly leading to underestimation of the effects. In addition, the use of animals in experiments involves

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a number of problems including (1) the difficulty of care and management of experimental animals, (2) a large number of animals necessary for evaluation, (3) differences in the species and strain of animals, and (4) individual variation. For these reasons, we prepared special experimental animals (rats) that have an inborn ability to perform well with a small individual variation on learning behavior tests. The use of these animals (THA) allows sensitive evaluation of the effects of chemicals at any exposure time including the next generation and perinatal period, which used to be impossible to evaluate, by learning behavior tests using a small number of animals.

Characteristics of these THA rats are described in comparison with the Wistar rat, which is the original strain of THA rats on the mother's side.

Ⅱ. Course of the establishment of THA rats

SD rats or Wistar rats have been used in learning behavior tests since the 1970's[1]. We also used Wistar rats, which were reported to perform relatively well on learning tests and had been used at many research institutions, for avoidance behavior learning tests.

We first purchased 5-week-old Jcl-Wistar rats (CLEA Japan, Inc., Tokyo), raised them until the age of 10 weeks in an animal room adjusted to a room temperature of 23±2℃ and a humidity of 50±10%, ventilated for 24 hours, and illuminated by a 12-hour light (8:00–20:00) and dark (20:00–8:00) cycles, and examined their general state of health. The animals were given a solid animal food (CE-2, CLEA Japan, Tokyo) and water ad libitum. We performed learning behavior tests using animals that were in good health and selected animals that showed an avoidance rate of 85% or higher by learning behavior tests. The selected male and female rats were paired one to one at random and were allowed to mate spontaneously by being placed in the same cage night and day. The pups delivered naturally were culled to 4 males and 4 females per mother on day 4 after birth. The pups were weaned on day 28 after birth, separated into males and females, and raised as groups of 4 in bracket cages until the end of the study.

From the second generation, pups with an avoidance rate of 95% or above were selected by learning behavior tests, and when less than 8 pups from a parent achieved an avoidance rate of more than 95%, all pups from this parent were excluded. Mating for breeding was made among siblings.

After inter-sibling mating was continued over 20 or more generations, the animals were named THA rats and registered as an inbred strain[2]. The animals have been bred further by the same procedure to the 65th generation to date.

Learning behavior test used for animal selection

Learning of the lever-pressing manipulation was selected for the learning behavior test, because (1) it requires no preceding manipulation, (2) diverse learning behavior tests can be scheduled by computer control, and (3) data processing is easy. However, learning of lever pressing involves "positive reinforcement" of giving a reward (food or water) when the animal has responded correctly (pressing the lever) to the test situation and requires fasting the animals before the test (preceding manipulation). This preceding manipulation (1) may affect the learning behavior (e.g. nutritional impairment of the central nervous system), and (2) once the animal has been satiated, it stops pushing the lever regardless of whether it has learned the behavior or not. Therefore, we used the Sidman electric avoidance test[3,4].

The electric shock avoidance test was

performed using an operant behavior measurement apparatus (BRS/LVE, USA) consisting of a Skinner box, a control part with a mini-computer, and a recording part. Each Skinner box was placed in a soundproof box with a fluorescent lamp and a ventilator to eliminate the effects of the external environment (Fig. 1).

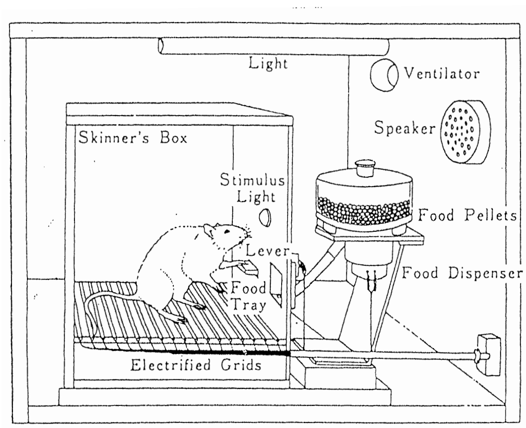

Fig. 1 Examination equipment of the learning behavior test.

The conditions of Sidman avoidance learning were: The interval between the lever pressing response (R) and electric shock (S) (R-S interval) was 30 seconds, the interval between electric shocks (S-S interval) was 5 seconds, the intensity of the electric shock was 100 V and 3 mA, and its duration was 0.5 seconds. One trial of this test, which was completed in 60 minutes, was performed each day on 10 consecutive days.

The results of each 60-minute trial were recorded every 10 minutes and evaluated according to the avoidance rate calculated by the formula below or according to the number of shocks suffered.

Avoidance rate = {(Number of shocks suffered when the lever was pressed 0 times) − (Number of shocks suffered)}/(Number of shocks suffered when the level was pressed 0 times) ×100

Ⅲ. Methods for Neurobehavioral Studies in THA rats Development

The two strains of rats were compared with regard to the development, spontaneous activity, learning behavior, sensitivity, and neurotransmitter levels.

Concerning the growth and development, the pups were weighed in the morning once a week from week 1 to week 10 after birth and examined twice a day at 10:00 and 18:00 for signs of differentiation (Pinna detachment, Incisol eruption, Eye opening) until they were observed in all pups.

Spontaneous activity was examined by the open-field method, which allows easy quantification of the results and analysis of behavior according to patterns, and by the Animex method, which allows prolonged observation. As for the open-field test, behavior was recorded for 3 minutes at the age of 5 weeks using an apparatus prepared by partial modification of the open-field apparatus of Petit et al[5].

The quantity of spontaneous activities during a 24-hour period was measured after the age of 5 weeks using an MK-Animex apparatus (SE-type: Muromachi Kagaku, Tokyo).

The learning behavior was examined by Shuttle box avoidance test, E-shaped water maze test, and electric shock avoidance test.

Shuttle box avoidance test was performed 50 trials per day on 5 consecutive days from the age of 35 days using a 2-way shuttle box (MK-RSC-001; Muromachi Kagaku, Tokyo). In the test, an alarm was given for 14 seconds, electric shocks were given at intervals of 30 seconds, and the intensity and duration of the electric shock were 100 V/3 mA and 5 seconds, respectively.

The E-shaped water maze was prepared by immersing an E-shaped maze in a pool (depth 25 cm, water temperature 22±2℃). A rat aged 5 weeks or above was placed at the center of the E-shaped maze, and the number of times that the animal advanced in the wrong direction (number of errors) were recorded. Ten trials of this test were performed per day on 3 consecutive days. The goal board was placed on the same side on days 1 and 2 but on the opposite side on day 3.

The electric shock avoidance test was performed in 7-week-old rats by the Sidman avoidance test same as that used at the establishment and maintenance of THA rats.

The sensitivity test was performed by using a heat stimulus in addition to the electric shock on the basis of the hypothesis that differences in the sensitivity to the electric shock used in the test affect the test results. The sensitivity to the electric shock was examined using a Skinner box by gradually increasing the voltage from 0 to 50 V at a fixed stimulation current of 3 mA and recording the voltage at which the rat first jumped. The sensitivity to heat stimulus was measured by placing a rat on a hot plate adjusted to 55℃ and recording the time until the rat first jumped or licked any one of the limbs.

The brain levels of neurotransmitters were measured by the microdialysis method, which (1) is free of the effects of animal handling, (2) allows continuous measurements of the brain levels of neurotransmitters in the same animal, and (3) allows measurements of rapidly metabolized materials such as acetylcholine (ACh). Analysis was performed by the use of a W-probe[6] that we developed independently for simultaneous measurements of neurotransmitters that require different analytical conditions. A W-probe with a 3-mm dialysis membrane was inserted 24 hours before the measurement into the right striatum (0 mm anteriorly from the bregma, 2.7 mm to the right and laterally from the median line, and 6.5 mm vertically from the dura mater toward the base of the brain) or the cerebral cortex of the right frontal cortex (2.5 mm anteriorly from the bregma, 2.5 mm to the right and laterally from the median line, and 4.0 mm laterally and 30° from the vertical line from the dura mater toward the base of the brain) according to Paxinos & Watson's sterotaxic coordinates[7] and fixed using dental resin.

A rat that had undergone surgery for insertion of the probe 24 hours before was placed in a free moving box, and perfusion was started after acclimating the animal for 1 hour. Recording was started 1 hour after the beginning of perfusion, when the animal, dialysis, and apparatus were stabilized. Monoamine metabolites were measured for 4 hours, and the mean value during this period was regarded as the value of the animal. ACh was measured every 20 minutes for 2 hours and, after an intraperitoneal administration of scopolamine at 1 mg/kg body weight, it was measured again every 20 minutes for 2 hours to measure its extracellular release induced by scopolamine, which is an ACh antagonist.

After completion of the above procedures, the rat blood were washed out, and brain was fixated by perfusion. Tissue sections were prepared from the brain, stained by the method of Karnovsky-Roots[8], and cells positive for acetylcholine esterase (ACh-E) were counted.

ACh-E-positive cells were counted at 5 sites (center, medial side, lateral side, parietal side, basal side) in the cross-section at the bregma, which passed near the center of the striatum.

Ⅳ. Newly established THA rats

In the course of the establishment and maintenance of THA rats, the results of learning tests (mean avoidance rate) improved progressively with repetition of trials in each of the 1st (F0), 10th (F10), 20th (F20), 30th (F30), 40th (F40), and 50th (F50) generations. The avoidance rate at the "establishment of the baseline behavior" (a stage in which the improvement in the avoidance rate is arrested and the results reach a plateau after a certain number of 10th trials) was 49.1±29.1% in F0, 96.6±1.3% in F10, 91.5±11.7 % in F20, 98.9±1.3% in F30, 99.1±1.3% in F40, and 98.5±1.0 in F50, showing a major improvement in the 10th generation. Also, the variation of the avoidance rate decreased with advances in the generation, and the coefficient of variation was small in and after F30 (1.3% in F30, 1.3% in F40, 1.0% in F50). No "establishment of baseline behavior" was observed in F0, but it was observed in the 5th trial in F10, 8th trial in F20, and 3rd trial in F30-F50 (Fig. 2).

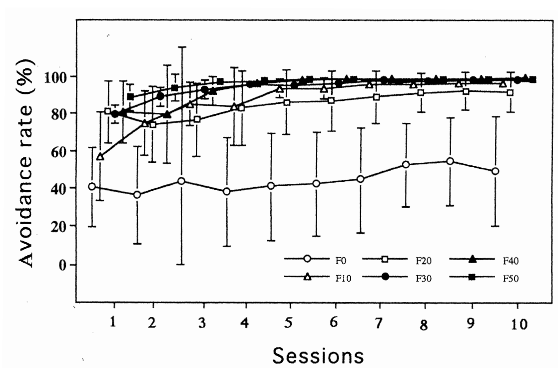

Fig. 2 The variation of the avoidance rate decreased with advances in the generation.

However, the mean test results were reversed between some generations, because comparisons were made according to the simple means of the avoidance rates of all pups obtained. When, therefore, the percentage of the pups that achieved the avoidance rate used as the selection criterion (95%) in all pups was compared among generations, it became 100% after the 45th generation while it was less than 100% until the 40th generation (Fig. 3). Thus, not all pups could acquire the avoidance behavior before the 45th generation, and the results remained unstable with some reversions of the results with progression of the generation up to this point. Therefore, THA rats that consistently show good learning of

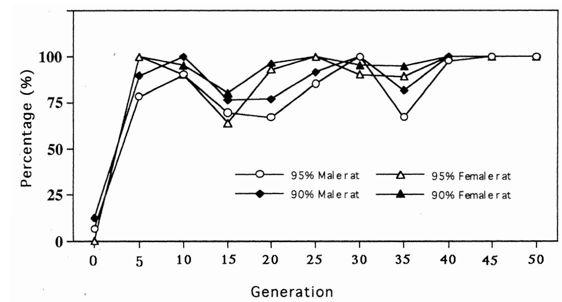

Fig. 3 The percentage of the pups that achieved the avoidance rate used as the selection criterion in all pups.

avoidance response are considered to have been established at the 45th generation.

We further evaluated the effect of the time of the beginning of learning tests on their results, i.e. effect of the speed of the brain development on the ability to learn the avoidance response. The Sidman-type avoidance test was performed in Wistar rats and THA rats raised to the age of 7 or 30 weeks under the same care conditions. The avoidance rate was higher in the THA rats than in the Wistar rats at both ages. Also, the results of the test were not different according to the time of the beginning of the training in the THA rats, but they were better at the age of 30 weeks than 7 weeks in the Wistar rats (Fig. 4).

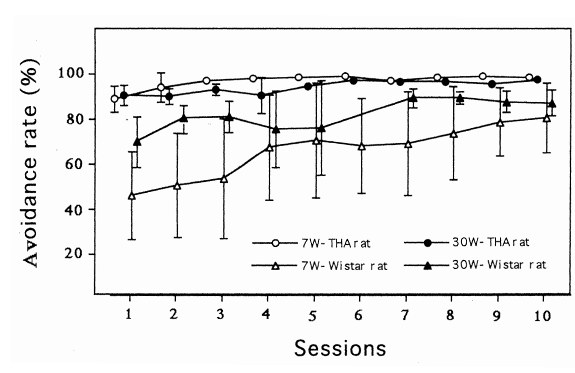

Fig. 4 Comparision of avoidance rate in the THA rat and Wistar rat on the time of the beginning of learning test.

Ⅴ. Neurobehavioral Comparisons between THA rats and Wistar rats

There was no difference between the two groups in the body weight gains or development. Also, the development has been reported to differ according to the number of impregnated embryos, number of littermates, and food type[9], but no difference was observed in the number of implant marks or number of pups delivered between the two strains. These results indicate that the development from during pregnancy to the age of 10 weeks is not different between THA rats and their mother strain, Wistar rats.

Concerning spontaneous activity, no difference was observed between the two strains except that the distance of walking and number of rearing in males were greater in Wistar rats than in THA rats. There was also no difference in the 24-hour activity level between the two strains although the activity pattern with two peaks during the nighttime characteristic of rodents was observed[10, 11]. These results suggest that Wistar rats increased activities and the number of rearing in the explorative behavior after entry into a new environment as they usually do when they are emotionally stimulated[12]. However, no difference was observed in the frequency of defecation or urination, which is increased when Wistar rats are emotionally stimulated. Also, THA rats did not bite the breeders' hands or fingers or escape during handling. Therefore, it is more appropriate to consider that THA rats are relatively inactive and docile animals by nature rather than that the Wistar rats were emotionally stimulated. In the Shuttle box avoidance test, and the number of escapes was greater and less varied in THA rats than in Wistar rats (Fig. 5), similarly to the results of the Sidman avoidance test. In the water E-maze test, also, the number of errors was smaller (Fig. 6), with smaller variation, in THA rats than in Wistar rats.

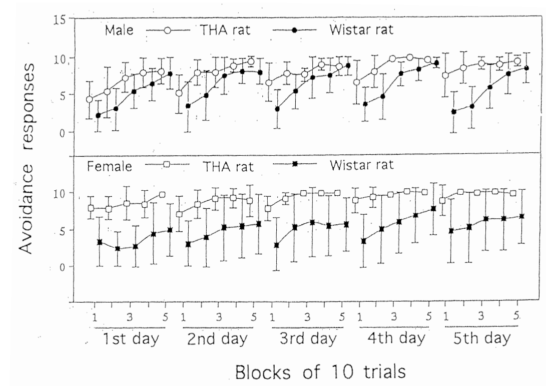

Fig. 5 The number of avoidance responses in THA rat and Wistar rat in a two-way shuttle box avoidance test on daily 50 trials for 5 days.

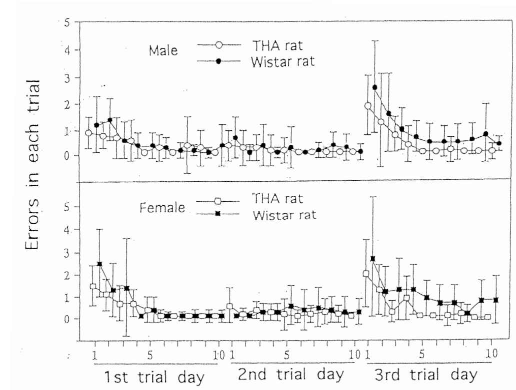

Fig. 6 The number of errors in THA rat and Wistar rat in a water E-maze test on daily 10 trials for 3 days.

The platform was placed at the same side on the 1st and 2nd trial days and the reverse side on the 3rd trial day.

From these results of learning behavior tests, THA rats established as a strain through selective mating according to the performance in the electric shock avoidance test are considered to have excellent ability not only in learning the positive lever pressing manipulation but also in acquiring spatial perception.

On the sensitivity tests, the voltage at which the animal first jumped was higher, and the time until the animal placed on a hot plate licked a limb was longer in THA rats than in Wistar rats.

These indicate that THA rats, which performed better in the electric shock avoidance test, were less sensitive to these stimuli. Therefore, the high learning ability of THA rats is not explained by high sensitivity to the electric shock.

Concerning brain levels of neurotransmitters, there was no difference in the release of ACh or 3 monoamine metabolites. However, the ACh release after scopolamine administration was greater in the cerebral cortex in THA rats than in Wistar rats (Fig.7). The result of striatum was same.

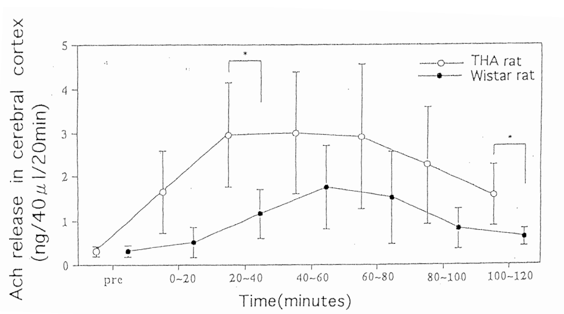

Fig. 7 Acetylcholine release in the microdialysate output from the cerebral cortex in THA rat and Wistar rat coused by scoporamine (1mg/kg, ip).

* :Significant difference between THA rat and Wistar rat ($p<0.05$).

There have been a large number of reports concerning the relationship between learning and brain levels of neurotransmitters[13~16]. Attention has been directed to the cerebral cortex, striatum, and hippocampus among areas of the brain and to ACh and dopamines among transmitters. No clear difference was observed in the dopamine level between the two strains. However, the ACh level in the cerebral cortex tended to be higher before scopolamine administration and clearly higher after scopolamine administration, in THA rats than in Wistar rats. These results suggest that ACh is released from a greater number of cells, or it is released in greater quantities from each cell, or both. At any rate, THA rats were shown to release more ACh than Wistar rats.

The number of ACh-E-positive cells in the striatum was not different among the site examined in the same strain but was greater in THA rats than in Wistar rats at all 5 sites examined (Fig.8). In consideration of the above results and the ACh release, the ACh level is considered to be higher in THA rats before scopolamine administration due to a greater number of ACh-releasing cells and after scopolamine administration due to a greater quantity of ACh release per cell.

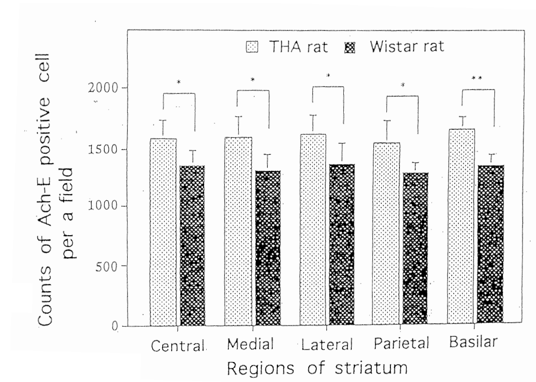

Fig. 8 Counts of ACh-E positive cell in the striatum in male rats of THA and Wistar strains.

* :Significant difference between THA rat and Wistar rat ($p<0.05$).

** :Significant difference between THA rat and Wistar rat ($p<0.01$).

We confirmed the establishment, through selection according to the results of the electric shock avoidance test and inter-sibling mating over more than 60 generations, of THA rats, a strain with an intended high inborn ability to perform well in the avoidance test with small individual variation despite no difference in the development compared with their mother strain. Moreover, THA rats were also confirmed to have an excellent ability in the Shuttle box avoidance test and water E-maze test.

Ⅵ. Usefulness of THA rats

THA rats have an excellent inborn ability to perform well with a small individual variation not only on the Sidman avoidance test but also on the Shuttle box avoidnce test and water E-maze test. Because of the inborn nature of this ability, THA rats have made it possible to evaluate the effect of exposure to the stimulus in not only the exposed animals itself but also the next generation, perinate and infancy[16~20]. Also, because of the high learning ability, effects of damages that may impair the learning ability appear more emphatically to facilitate their evaluation. In addition, in studies of methods for improving the learning ability, for example, effects of methods on the learning ability can also be evaluated after reducing the learning ability. When toxicological effects of lead and toluene were experimented either Wistar and THA strain rats, no significant difference was observed in Wistar rats, however, in THA rats, significance was observed, which showed THA strain is more sensitive to reveal effects of interventon[21, 22]. Moreover, the small individual variation leads to a decrease in the number of animals needed for studies.

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