

—Review—

# Hatano rats selectively bred for high- and low-avoidance learning: an overview

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**Abstract:** This review compiles the results of a series of studies on Hatano high- and low-avoidance animals (HAA and LAA, respectively) established at the Hatano Research Institute, Food and Drug Safety Center, Japan. The HAA and LAA lines were selected and bred from Sprague Dawley rats for high and low avoidance learning, respectively, in a shuttlebox task since 1985. Although Hatano rats were selected only based on their behavioral traits in the active avoidance task, strain differences between the HAA and LAA lines were also observed in their stress responses and reproductive functions. However, the most noticeable finding of Hatano rats is a matched result in both active and passive avoidance tasks. The HAA and LAA lines are useful for next-generation toxicological studies, because the hereditary characters of behaviors or endocrine functions are well controlled.

**Key words:** avoidance learning, rat, reproduction, selective breeding, stress response

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## Two-way Shuttlebox Avoidance

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Avoidance learning is the process by which an animal learns a behavioral response to avoid aversive stimuli. In this task, the animal learns to predict the occurrence of an aversive event based on the presentation of a specific stimulus by moving to a different compartment. In the two-way shuttlebox avoidance procedure, an animal is placed in one of two compartments (Fig. 1). After a pause, a conditioned stimulus (e.g., tone and light) occurs. After the tone and light stimulus, an aversive event (e.g., shock) occurs, and the animal moves in response to the shock. These trials are repeated; a shuttling response after shock onset is an “escape” response, and a response prior to the shock is an “avoidance” response. If the animal does not respond, this is counted as a “freezing” response. The more quickly animals acquire an avoidance response, the better the learning in this task.

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## Selective Breeding for Avoidance Learning

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There are large individual variations in the rate of avoidance response among animals of different strains and from different commercial suppliers. Individual variations often make evaluation of behavioral tests difficult. To make animal models with small individual variations, two strains of rats were established from Sprague Dawley rats on the basis of active avoidance learning in a shuttlebox task [30]. High-avoidance animals (HAA) had a high rate of avoidance response, and low-avoidance animals (LAA) had a low rate of avoidance response. The HAA and LAA lines are the result of more than 20 consecutive generations of sister and brother mating (Fig. 2). All individuals in these two inbred strains are homozygous and genetically identical. These two strains, named Hatano rats, have been maintained by sib mating for over 25 years at the Hatano Research Institute, Food and Drug Safety Center. The Hatano rats (HAA/FDSC, LAA/FDSC) are also available

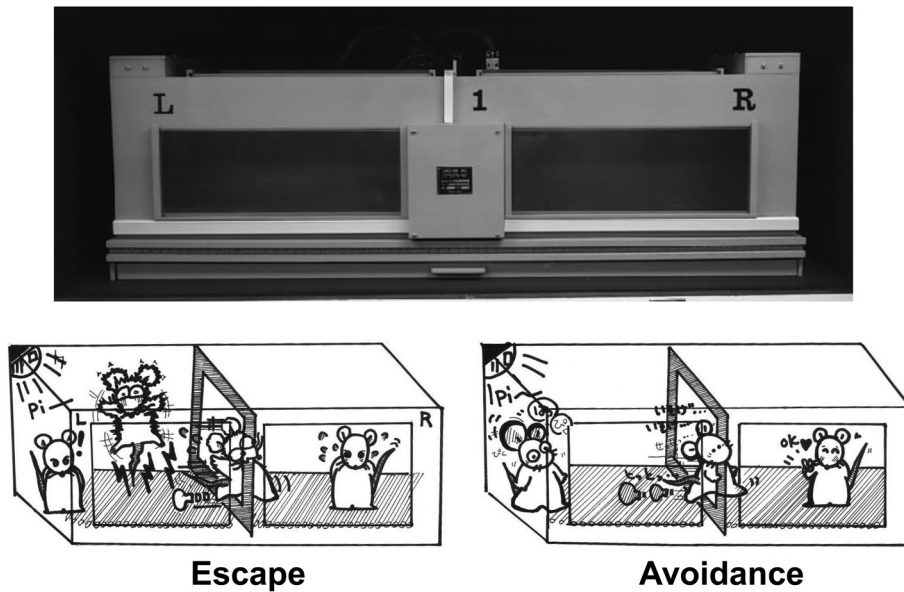
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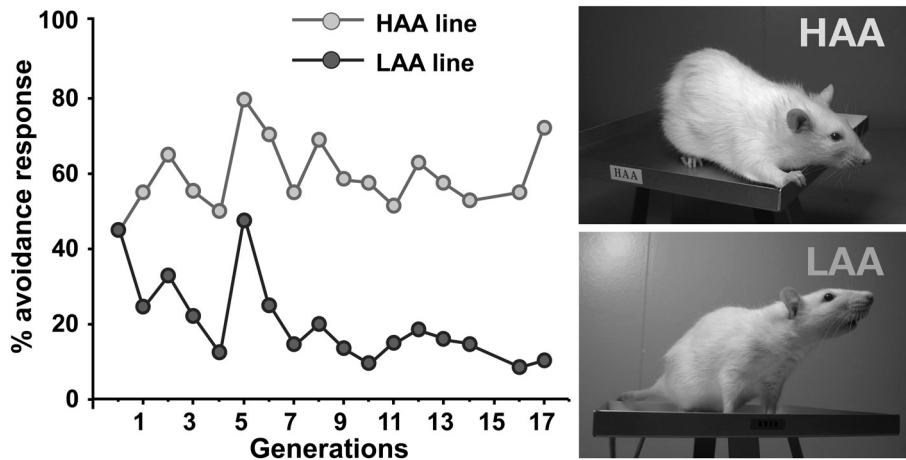
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**Fig. 1.** The shuttlebox (T401L, Unicm Inc. Japan) used for the studies consisted of two 50 L × 20 W × 25 H cm compartments separated by a central hurdle (height, 1.8 cm). The electric shock was delivered through the floor grid. The conditioned stimulus, consisting of light and a tone, was delivered through a light bulb and a loudspeaker located on the ceiling at the rear of each compartment. A shuttling response after shock onset was counted as an escape response, and a response prior to the shock was counted as an avoidance response.



**Fig. 2.** High avoidance animals (HAA) were selected for a high rate of avoidance responses, and low avoidance animals (LAA) were selected for a low rate of avoidance. Animals that showed freezing behavior were removed from the LAA line. By sister-brother mating within the HAA and LAA stocks for over 20 generations, two inbred strains were established that differed in terms of rate of avoidance.

from the National BioResource Project - Rat (<http://www.anim.med.kyoto-u.ac.jp/NBR/>).

### Roman and Syracuse Strains of Rats

Roman rats [7] and Syracuse rats [11] are famous

strains selectively bred for differential avoidance acquisition. Roman high-avoidance (RHA) and Syracuse high-avoidance (SHA) rats quickly acquire an avoidance response, whereas Roman low-avoidance (RLA) and Syracuse low-avoidance (SLA) rats fail to acquire a response. Other behavioral differences between high-

and low-avoidance rats include differences in emotionality [10, 16]. For example, RHA rats show more ambulation and less defecation in a novel environment than do their RLA counterparts [13, 14]. Furthermore, a higher neuroendocrine reactivity to stressors is observed in RLA rats than in RHA rats [6, 14, 17, 18, 43, 44]. Similar results have been obtained in Syracuse rats [9, 12].

The Hatano selection program was initiated with Sprague Dawley rats, but the Roman selection program used Wistar rats [7], and the Syracuse selection program used Long-Evans rats [11]. Furthermore, fewer freezing responses occur in the shuttlebox task in LAA rats than in RLA or SLA rats. Because freezing behavior is a defensive reaction rather than an indication of poor learning capacity [8], the breeding program for LAA rats has consistently discarded animals showing freezing behavior [35].

### Behavioral Characteristics of Hatano Rats

During the course of selection, additional behavioral differences between the HAA and LAA lines were found in running-wheel activity, with HAA rats showing higher activity than LAA rats, and in water maze performance, with HAA rats swimming faster than LAA rats [30]. Furthermore, strain differences were found in physical and behavioral development; eye opening, pivoting, and negative geotaxis occur at a younger age in HAA rats than in LAA rats [32].

A cross-fostering study indicated that the strain differences observed in shuttlebox avoidance, running-wheel activity, swimming speed, eye opening, pivoting, and negative geotaxis are independent of maternal care [32].

### Adrenal Gland and Stress Response

During the course of selection, it was found that HAA rats have bigger adrenal glands than their LAA counterparts [35]. These results suggested that these strains may differ in aspects of endocrine response to stress. Plasma levels of adrenocorticotrophic hormone (ACTH) were higher in HAA rats than in LAA rats during early avoidance acquisition in the shuttlebox [35]. Before shuttlebox avoidance tasks, hypothalamic concentrations of corticotropin-releasing hormone (CRH) were higher in LAA rats than in HAA rats. After shuttlebox tasks, the CRH levels were higher in HAA rats than in LAA rats. These

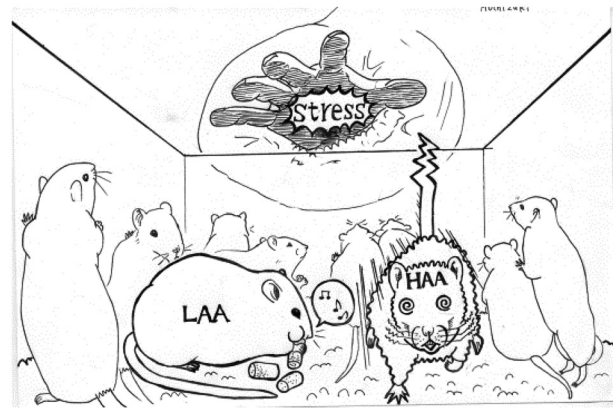


Fig. 3. Illustration of the difference in stress sensitivity between HAA and LAA lines (provided by Prof. Kazuyoshi Taya).

result suggest that the response of CRH-ACTH is higher in HAA rats than in LAA rats [1].

Strain differences between HAA and LAA rats have also been reported in hormonal responses to restraint stress [5]. The peak levels of plasma ACTH during stressful conditions are higher in HAA rats than in LAA rats, and the peak levels of prolactin (PRL) are lower in HAA rats than in LAA rats. These differences in endocrine responses to stress may be involved in the regulation of avoidance responses in the shuttlebox task.

When comparing stress-induced gastric erosion between HAA and LAA rats after restraint stress, the gastric erosion index was higher in HAA rats than in LAA rats [2]. These results indicate that HAA rats were more sensitive to stress than LAA rats. The peak levels of PRL were higher in LAA rats than in HAA rats under stressful conditions [2]. The number of PRL-receptor-positive cells determined by immunohistochemistry in the paraventricular nucleus was also increased in LAA rats compared with HAA rats after restraint stress [2]. Intracerebroventricular injection of PRL inhibits gastric ulceration under stress [15]. These results suggest that LAA rats are better protected against stress than HAA rats (Fig. 3).

### Puberty and Sexual Behavior

There were differences between the strains after postnatal development [32] including differences relating to male puberty, the timing of preputial separation, and plasma hormone levels [39]. Preputial separation occurred about 7 days earlier in HAA rats than in LAA rats.

Plasma testosterone levels began to rise at a younger age in HAA rats than in LAA rats. HAA rats exhibited more active eating, higher blood pressure, higher percentage fat, and higher plasma triglyceride levels than LAA rats—these features correspond to some of the risk factors associated with metabolic syndrome [21].

For female puberty, the timing of vaginal opening and the first ovulation were examined in HAA and LAA rats [42]. HAA rats exhibited a vaginal opening at a younger age and ovulated with more oocytes than LAA rats. Thus, there were phenotypic differences between HAA and LAA rats in male and female puberty.

Sperm motion, which was characterized by a computer-assisted motion analysis system, was more active in HAA rats than in LAA rats [40]. Sperm motion differences between HAA and LAA rats may be dependent on sperm adenosine triphosphate (ATP) content. There were also strain differences in the spontaneous incidence of sperm morphological abnormalities, and there were more abnormalities in LAA rats [41].

Male LAA rats have more rapid sexual behavior with lower testosterone than HAA or Sprague Dawley rats, and these differences increase with repeated sexual experiences [25], which suggests that active sexual behavior in LAA rats is not related to plasma testosterone levels. The strain difference in sexual behavior between male HAA and LAA rats may be caused by different emotional responses to novelty.

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### Endocrinology of Estrous Cycling

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The estrous cycling duration of Sprague Dawley rats is commonly between 4 days and 5 days. In Hatano rats, all HAA rats had a regular 4-day estrous cycle, but most LAA rats (70.8%) had a regular 5-day estrous cycle [3]. Therefore, differences in female reproductive endocrinology between the strains were investigated by measuring the plasma concentration of reproductive hormones during the estrous cycle [3, 22]. A prolonged elevation in plasma progesterone and a higher elevation in plasma PRL surge were characteristics of the LAA line with 5-day estrous cycles [3]. LAA rats also showed approximately threefold lower basal and surge levels of luteinizing hormone (LH), a more than fourfold lower level of follicle-stimulating hormone (FSH) surge levels, and higher levels of inhibin A and inhibin B during the estrous cycle compared with the levels in HAA rats. The concentration of estradiol-17 $\beta$  in the proestrous stage

was lower in LAA rats than in HAA rats. Lower LH secretion in LAA rats may be caused by reduced estrogenic responses of gonadotropin-releasing hormone (GnRH) neurons in the preoptic area and of kisspeptin neurons in the anteroventral periventricular nucleus [19].

Additionally, LH and FSH secretions from primary cultured anterior pituitary cells with or without GnRH stimulation were investigated [22]. LH and FSH secretions were lower in cells derived from LAA rats and, in terms of FSH secretion, were unresponsive to GnRH compared with cells derived from HAA rats. Although an increased number of preantral follicles in diestrus were observed in LAA rats, human chorionic gonadotropin (hCG)-induced ovulation was lower in LAA rats. LAA rats may exhibit more follicle growth during the early stage of folliculogenesis, but most follicles may not grow into mature follicles. These results strongly suggest that strain differences in ovarian function are the result of the difference in regulation of the hypothalamo-hypophyseal system for gonadotropin secretion.

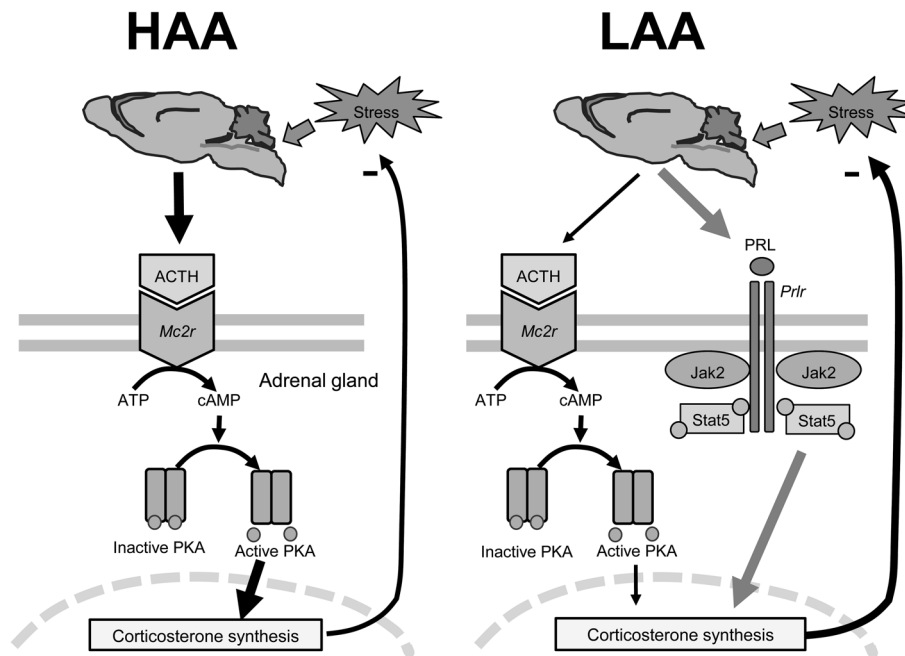
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### Maternal Behavior and Milk Ejection

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Although a cross-fostering study indicated that strain differences in physical and behavioral development were independent of maternal care, pup growth in both strains is strongly dependent on the maternal line [32]. A battery of tests was performed including nest building, home cage behavior, pup retrieval, and milk ejection tests [37]. Dams of both strains built good nests and spent an identical amount of time with their offspring. However, LAA dams had a prolonged latency time for pup retrieval and a decreased amount of milk ejection. These results suggest that maternal motivation and mechanisms responsible for maternal hormones related to suckling are involved in pup growth.

The endocrinological profile in lactating rats was studied to determine the effect of suckling during lactation in HAA and LAA rats [4]. Plasma concentrations of oxytocin, PRL, and ACTH were significantly higher in HAA dams than in LAA dams. There were, however, no strain differences in circulating corticosterone, indicating that the response of the hypothalamic-pituitary axis to the suckling stimulus was greater in HAA rats than in LAA rats, whereas the ACTH-induced adrenal response of corticosterone release was higher in LAA rats than in HAA rats. Because dopamine from the median eminence inhibits PRL secretion from the lactotrophs of the ante-



**Fig. 4.** Molecular biological features in the adrenal gland of Hatano rats. In HAA rats, corticosterone synthesis is mediated by the cAMP/protein kinase A (PKA) signaling pathway. In LAA rats, corticosterone synthesis is also mediated by the JAK2/STAT5 signaling pathway. Under stress, the pituitary gland secretes prolactin, which binds to *Prlr*. PRL/*Prlr* activates JAK2. The activated JAK2 mediates phosphorylation of STAT5, which induces corticosterone synthesis.

rior pituitary, the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis, was evaluated [4]. TH was significantly higher in HAA rats than in LAA rats before the suckling stimulus. After the suckling stimulus, TH activity in HAA rats was significantly lower than before suckling, whereas TH activity in LAA rats was not changed. The strain differences in endocrinological responses during suckling probably originate from neurotransmitter changes such as changes in dopamine.

#### Adrenocortical Responses *in Vitro*

An *in vitro* study was performed to identify differences in the regulation of the adrenal cortex of HAA and LAA rats [23]. Although incubation with PRL or ACTH resulted in a dose-dependent increase of corticosterone and progesterone release by adrenal cells from both HAA and LAA male rats, the responses were markedly increased for adrenal cells from LAA rats compared with those from HAA rats. This finding suggested that the adrenal glands of HAA rats are less sensitive to PRL and ACTH than the adrenal glands of LAA rats.

The adrenal glands of both strains were collected at 0, 15, and 30 min after stress. The protein levels of phosphorylated STAT5 and the mRNA levels of melanocortin receptor 2 (*Mc2r*) and PRL receptor (*Prlr*) were analyzed [24]. Furthermore, the effects of bromocriptine-induced hypoprolactinemia on adrenocortical responses to stress were investigated [24]. ACTH concentrations in HAA rats were greater than in LAA rats, but differences in PRL concentrations were found only at 120 min after stress induction. The stress-induced increase in *Mc2r* mRNA expression was higher in HAA rats, but there was lower *Prlr* mRNA expression. STAT5 became highly phosphorylated in response to stress in both strains, but bromocriptine led to a reduction in STAT5 phosphorylation. Exposure to bromocriptine was associated with a reduction in plasma PRL in response to stress in both strains, but ACTH levels were not altered. However, the decrease in corticosterone in response to stress was observed only in bromocriptine-treated LAA rats. These data show that PRL plays an important role in the regulation of corticosterone release in LAA rats but not in HAA rats under stress (Fig. 4).

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### Behavioral Teratology

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It is difficult to predict avoidance learning in the next generation because there are large individual variations in data from a heterogeneous animal. Although the Sprague Dawley rats which are generally used in toxicology were made to retain or acquire characteristics of high lactation and rapid growth, behavioral traits had not been hereditarily controlled. Because Hatano rats have little interindividual variation in the shuttlebox avoidance task, they are a suitable model for the risk assessment of behavioral teratology.

In a study by Ohta *et al.* [31], pregnant rats from HAA and LAA lines and an unselected line of Sprague Dawley rats were given an intraperitoneal (IP) injection of methylnitrosourea (MNU) on gestational day 13, and offspring with microcephaly were subjected to the shuttlebox avoidance task. During the acquisition trials, there were no effects of MNU on avoidance learning in any of the lines. During the extinction trials, however, the MNU offspring of the HAA line showed more responses than the controls. The lack of treatment effects in LAA and Sprague Dawley rats during the extinction trials suggests that the effects induced by MNU on the shuttlebox extinction trials are dependent upon the baseline levels of acquisition.

In a subsequent study [33], pregnant rats of both lines were given a low dose IP injection of methylazoxymethanol (MAM) on gestational day 14, and their offspring were subjected to several behavioral tests. There were no significant effects of MAM on running-wheel activity or shuttlebox avoidance learning, whereas open-field activity of the MAM offspring was markedly decreased in the LAA line but not in the HAA line. These results suggest that the behavioral baseline was an important factor influencing behavioral effects and that behaviorally selected strains make neurobehavioral teratogens, such as MNU and MAM, detectable.

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### Immunological Differences in Hatano Rats

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Because there are close interconnections among nervous, endocrine, and immune systems, HAA and LAA lines may have differences in immunological activity. Therefore, the plasma levels of IgG, IgM, complement 3 (C3), classical pathway hemolytic complement (CH50), and beta-2-microglobulin were compared between males of the two lines at 5 and 24 weeks of age [28]. The

plasma levels of IgG and CH50 were lower in the LAA line than in the HAA line at 5 weeks of age, whereas the differences disappeared at 24 weeks of age. There were no differences between the two strains in the plasma levels of IgM, C3, and beta-2-microglobulin. Antibody production to sheep red blood cells (SRBC) and mitogen-induced lymphocyte proliferation were compared between 12-week-old males of the two lines [28]. Antibody responses in the PFC assay, plasma anti-SRBC-IgM levels, and spleen weights were higher in LAA lines than in HAA lines. LPS-induced lymphocyte proliferation was greater in the LAA line than in the HAA line. The HAA line had earlier immunological development, but antibody production and mitotic response of B lymphocytes was more pronounced in the adult LAA line than in the HAA line. The immunological differences between the strains suggest that Hatano rats are useful for analysis of behavioral-immunological relationships.

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### Endocrine Disrupting Effects on the Next Generation

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The results of animal studies on endocrine active compounds often vary, especially in the effects on the next generation. Because the HAA and LAA lines have well characterized endocrine and immune systems, the effects of an endocrine active compound were examined using these strains. Neonates from both lines were fostered by Sprague Dawley rats receiving genistein by gavage from day 17 of pregnancy through day 21 of lactation [36]. Genistein is a typical phytoestrogen that exhibits estrogenic activity in a rodent uterotrophic assay [38]. IgM antibody production was decreased in HAA offspring reared by genistein-treated dams. During restraint stress, the plasma concentration of corticosterone was lower in LAA offspring reared by genistein-treated dams. These results demonstrate that transmaternal exposure to genistein potentially affects immunological and stress responses and suggest that Hatano rats are useful for examining endocrine active chemicals in whole body systems.

To examine the effects of a low dose of diethylstilbestrol (DES), which is a synthetic non-steroidal estrogen, female neonates from HAA and LAA lines were administered DES daily by oral gavage for the first 5 days after birth [26]. Accelerated puberty and excessive body weight gains were observed only in LAA rats treated with a low dose of DES, which suggests that neonatal DES exposure varies by genetic background.

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### Genetic Analysis of Hatano Rats

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To determine whether co-selection observed with avoidance learning and some biological characteristics of the Hatano rats were genetically determined, the inheritance patterns of avoidance learning, locomotion activity, adrenal weight, and reproductive functions were examined [34]. In the first experiment, two F1 hybrids produced by a reciprocal cross between HAA and LAA lines were tested for shuttlebox avoidance rate, running wheel activity, and adrenal gland weight. Although the avoidance rate in F1 hybrids was similar to that in HAA rats, running wheel activity and adrenal gland weight in F1 hybrids were similar to the average of values obtained from the HAA and LAA rats.

In the second experiment, F1 hybrids produced by mating between female Sprague Dawley rats and male HAA or LAA rats were tested. The avoidance rate, running wheel activity, and adrenal gland weight in F1 hybrids were similar to the average of values obtained from the parental strains.

In the third experiment, two F1 hybrids produced by a reciprocal cross between HAA and LAA rats were examined for time of puberty, type of estrus cycle, ovulation number, and genital organ weight. The time of puberty, ovulation number, and ovarian weight were similar to the average of values obtained from HAA and LAA rats, but the type of estrus cycle in F1 hybrids was similar to that in LAA rats. There was a significant difference in testicular weight between reciprocal hybrids. These results indicate that the inheritance pattern of avoidance learning and estrus cycle may be complete dominance but that the inheritance pattern of the time of puberty, ovulation number, and ovarian weight may be incomplete dominance in Hatano rats. A sex chromosome effect may be involved in testicular weight.

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### Passive Avoidance and Anxiety

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There are two forms of avoidance learning: active and passive. In the active form, avoidance depends on moving from an aversive event. In the passive form, however, avoidance learning depends on not moving to an aversive event. In a step-through-type passive avoidance task, the apparatus consists of two chambers: a light box and a dark box. Once an animal is placed in the light box, it quickly moves to the dark box because rodents prefer a darker environment. In the dark box, the animal

is given an unavoidable foot shock in a learning session. After a few minutes, 24 h, several days, or more, the same procedure is repeated, and the latency in entering the dark box is recorded as memory retention. Because remaining in the light box allows the animal to avoid an aversive event, it is called a passive avoidance task.

Avoidance performance in the passive task was significantly greater in HAA rats than in LAA rats [20]. Furthermore, HAA rats had high anxiety-like behaviors compared with LAA rats in the open-field and elevated plus maze tests [20]. These results suggest that selection by shuttlebox task and breeding of Hatano HAA and LAA strain rats were properly performed with high and low avoidance performance, respectively, and that HAA rats were predisposed to high anxiety compared with LAA rats. Unlike Hatano rats, other rat strains selected through avoidance performance in the shuttlebox task do not exhibit the same result in the other avoidance task. For instance, SLA rats rapidly learn the passive avoidance task compared with SHA rats [10], and RHA and RLA rats are not significantly different in the passive avoidance task [16].

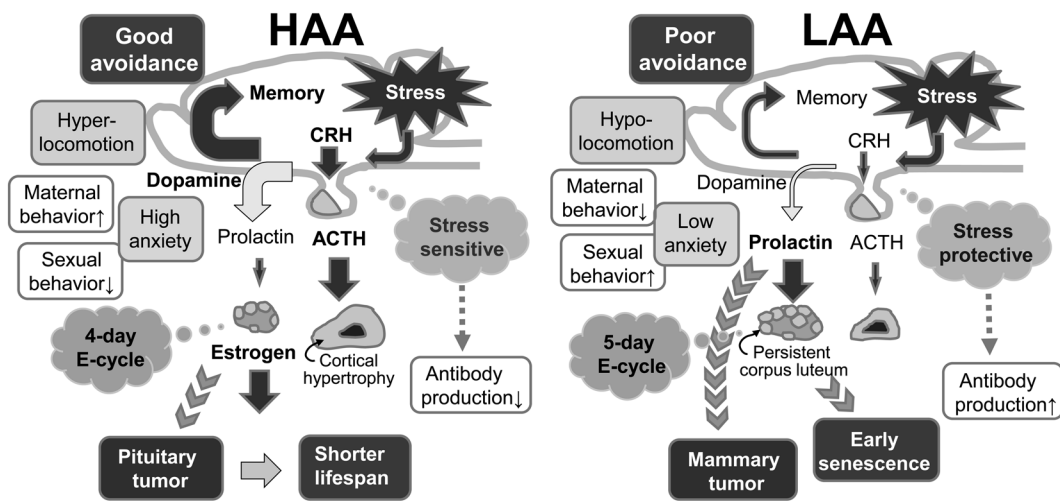
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### Fur Loss and Aggressive Behavior

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Fur loss on the back is often observed in rodents and is probably caused by stress, hormonal abnormalities, or fighting. It is often observed in HAA rats but not in LAA rats. The frequency of fur loss on the back was investigated in one or two animals/cage after weaning [27]. In two animals/cage, fur loss was found in more than 90% of male HAA rats and 11% of female HAA rats. In one animal/cage, fur loss was found in about 30% of male HAA rats and 0% of female HAA rats. Fur loss was not found in LAA rats independent of housing conditions. Fur loss on the back observed in HAA rats occurred mainly between 8 and 10 weeks of age. These data suggest that increased play fighting is correlated with fur loss on the back of HAA rats.

To evaluate the relationship between fur loss and aggression, play behavior and an intruder test were examined in Hatano rats [27]. The frequency of play fighting was higher in HAA rats than in LAA rats. Although no strain difference in aggressiveness was observed in the male intruder test, more maternal aggression was found in HAA dams than in LAA dams. These data suggest that increased play fighting may underlie higher aggression in HAA rats.



**Fig. 5.** A summary of the characteristics of Hatano rats. Although Hatano rats were selected only based on avoidance learning, strain differences between the HAA and LAA lines were also observed in locomotion activity, stress reactions, anxiety, mating behavior, estrous cycling, antibody production, reproductive senescence, tumorigenesis, and lifespan.

### Reproductive Senescence and Lifespan

Reproductive aging was examined in female HAA and LAA rats by observing estrous cycles between 8 and 11 months of age [29]. Females were kept for their natural lifespans until 24 months of age to compare survival and clarify the relationship between reproductive aging and tumor development [29]. At 8 months of age, 2 of 35 HAA rats and 20 of 35 LAA rats had abnormal estrous cycles. The median lifespan of HAA rats (673 days) was shorter than that of the LAA rats (733 days). The incidence of pituitary neoplasia was higher in HAA rats than in LAA rats. However, mammary gland tumors were observed more frequently in LAA rats than in HAA rats. These results suggest that female HAA rats have a shorter lifespan than female LAA rats and develop pituitary neoplasia, which is a causal factor for accelerated mortality. In the kidney, all of the LAA rats had chronic progressive nephropathy (CPN), and all of the HAA rats had dilatation of the renal pelvis [29]. Because a spontaneous membranous glomerulonephropathy was found in the LAA line supplied by the National BioResource Project - Rat [45], attention should be paid to distinguish this lesion from CPN.

### Conclusions

The comparative characteristics of Hatano HAA and LAA rats are summarized in Fig. 5. Hatano rats have

identical results in both active and passive avoidance learning and have been well characterized by stress reactions and reproductive parameters. The strain differences between HAA and LAA lines probably result from differences in the hypothalamus-pituitary-adrenal (HPA) axis and the hypothalamus-pituitary-gonadal axis. Research on the neuroendocrine system related to the hypothalamic-pituitary axis is an active area of investigation. A mechanism-based molecular study would contribute to this rapidly advancing field. Unfortunately, we have not been successful in elucidating the biological mechanisms that underlie differences in the HPA axis of these rat strains. In the future, we would like to investigate these mechanisms by extending our current research to incorporate molecular biological factors affecting the HPA axis. These studies have the potential to impact neuroendocrinology and the biology of reproduction.

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