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Behavioral Study on the Gracile Axonal Dystrophy (GAD) Mutant Mouse

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We investigated motor function and pain sensation in the gracile axonal dystrophy (GAD) mutant mouse, using the tail-flick test and the rotarod test. GAD (*gad/gad*) and normal sib mice (*gad/+* or *+/+*) were used between 5 and 11 weeks of age, during which time the behavioral signs of GAD mice shifted from sensory ataxia (about 4 to 8 weeks of age) to paresis (after about 9 weeks of age). In the tail-flick test, significant shortening of latency was observed at 6 and 8 weeks of age in female GAD mice, in comparison with normal female mice. This may be related to dysfunction or degeneration of axons in the fasciculus gracilis, whose collaterals are thought to control the transmission of nociceptive information. In the rotarod test, a cumulative χ^2 test showed significant reduction in the performance times of GAD mice beginning at 5 and 6 weeks of age in males and females, respectively, indicating that the rotarod test can detect the development of motor incoordination as early as these ages. The performance times of GAD mice dropped sharply from 9 weeks of age onwards, and this is believed to reflect the progression of paresis. The rotarod test therefore appears to be a good method of quantifying behavioral changes in GAD mice and to be applicable both to objective selection of GAD mice before 8 weeks of age and to evaluation of drugs to treat ataxia or paresis. — KEY WORDS: gracile axonal dystrophy mouse, motor function, rotarod test

Gracile axonal dystrophy (GAD) is a neurological autosomal recessive mutant mouse with the gene symbol, *gad* [10]. The mutants were first discovered because of the animals' ataxic gait and dragging of the hindlimbs beginning at about 11 weeks of age. Thereafter, the disease progressed gradually, causing muscular atrophy of the hindlimbs, and the mutants died between 7 and 9 months of age. Although the mutants appeared normal in their cage until about 11 weeks of age, detailed clinical observations showed that the mutants (*gad/gad*) can be distinguished from normal littermates (*gad/+* or *+/+*) as early as 4 weeks of age by the position of the hindlimbs when suspended by the tail. When this is

done, GAD mice stretch their hindlimbs upward spasmodically and/or clasp them tightly together [10].

Pathologically, in the central nervous system the dorsal column, the dorsal spinocerebellar, pyramidal, and spinal trigeminal tracts are affected [4, 6, 10, 11]. Focally swollen axons or spheroids, which are characteristic of GAD mice, are observed in these affected tracts in addition to nerve fiber loss and gliosis. The fasciculus gracilis of the dorsal column, one of the ascending sensory pathways, is the tract which degenerates first and most severely. In the peripheral nervous system, both sensory and motor nerves are affected [7, 8]. Beginning at about 3 weeks of age, the

Table 1. Changes in mean tail-flick latencies± S. E. M. (sec) of male and female GAD and phenotypically normal sib mice from 6 to 11 weeks of age

Sex	Age (weeks)	Phenotype	
		GAD	Normal
Male			
	6	6.6±0.8 (11) ¹⁾	6.4±0.5 (11)
	7	6.9±1.2 (11)	5.4±0.5 (11)
	8	5.4±0.8 (11)	5.1±0.4 (11)
	9	4.3±0.6 (11)	5.0±0.8 (11)
	11	5.0±0.5 (11)	5.6±0.9 (11)
Female			
	6	5.2±0.6 (10)	8.3±1.2 (10)*
	7	4.5±0.4 (10)	5.8±0.6 (10)
	8	4.6±0.6 (10)	7.0±0.9 (10)*
	9	4.2±0.3 (10)	4.8±0.5 (10)
	11	4.7±0.5 (10)	6.5±0.9 (9)

1) Number of mice tested. Asterisks indicate significant differences at P<0.05 between GAD and age-matched normal sib mice according to Student's t-test.

Table 2. Changes in the distribution of GAD and phenotypically normal sib mice according to their rotarod performance times from 5 to 11 weeks of age

Sex	Age (weeks)	GAD					Normal					
		N	0-19	20-39	40-59	60 (sec)	N	0-19	20-39	40-59	60 (sec)	
Male												
	5	10	1	1	2	6	10	0	0	0	10	*
	6	14	2	5	2	5	14	0	0	2	12	**
	7	14	1	3	2	8	14	0	0	0	14	**
	8	14	2	0	1	11	14	0	0	1	13	
	9	14	3	5	3	3	14	0	0	0	14	**
	10	14	8	3	2	1	14	0	0	0	14	**
	11	14	13	0	0	1	14	0	0	0	14	**
Female												
	5	4	1	0	1	2	4	0	0	0	4	
	6	10	2	2	1	5	10	0	0	1	9	**
	7	10	0	2	3	5	10	0	0	0	10	**
	8	10	1	1	1	7	10	0	0	0	10	*
	9	10	1	5	1	3	10	0	0	0	10	**
	10	10	2	5	2	1	10	0	0	0	10	**
	11	10	7	2	1	0	10	0	0	1	9	**

Asterisks indicate significant differences between GAD and age-matched normal sib mice according to the cumulative χ^2 test * P<0.05 ** P<0.01

sensory nerve fibers in the muscle spindles degenerate starting at their distal end, while the motor nerves begin to degenerate from their endplates at about 8 weeks of age. Axonal

degeneration in GAD mice occurs in the preterminal or presynaptic parts of axons and progresses toward the cell bodies, the so-called “dying back” phenomenon. Based on these

clinical and pathological findings, we can divide the clinical course in GAD mouse into two phases: 1) a sensory ataxia stage between about 4 and 8 weeks of age, and 2) a paresis stage after about 9 weeks of age [7]. We believe that GAD mice can serve as an animal model for spinocerebellar degeneration (ataxia), particularly Friedreich's ataxia, and Charcot-Marie-Tooth disease (hereditary motor and sensory neuropathy) in humans [4, 7, 11].

Since there have been few quantitative studies on behavior or pain sensation in the GAD mouse, we tried to measure both, using two methods. The main purpose of the present study was: 1) to find (a) good behavioral method(s) of quantifying and characterizing the motor incoordination or abnormal sensation of the GAD mouse, particularly between 6 and 11 weeks of age when the clinical signs change qualitatively, and 2) to compare the behavioral data obtained with pathological changes.

The animals used were GAD (*gad/gad*) mutant mice and phenotypically normal littermates (*gad/+* or *+/+*) from a C57BL/6-*gad* (B6-*gad*) congenic strain. The B6-*gad* strain, originally introduced from the Laboratory of Animal Genetics, Faculty of Agricultural Sciences, Nagoya University, Japan, was maintained at Tsukuba Research Laboratories, Eisai Co., Ltd., Ibaraki, Japan. Mating procedures to maintain the strain and to produce the mice have been described previously [9]. The mice were provided with a commercial diet (CE-2, Nihon CLEA Inc., Tokyo, Japan) and water *ad libitum* under controlled temperature, humidity, and light conditions ($22 \pm 2^\circ\text{C}$, $55 \pm 5\%$, and a 12-hr light/dark cycle with the lights on at 07:00).

The tail-flick test was conducted between 09:00 and 10:00 in order to investigate nociception in GAD mice by the method of D'Amour and Smith [2], recording the tail-flick latency. The instrument (IITC Inc., N. J., U. S. A.) was adjusted as follows: sensitivity=3.0; beam=85. To minimize tissue damage, we set the cutoff time at 15 sec, and cooled the tail in tap water after measurement.

We used the rotarod test [3] to quantify motor incoordination in GAD mice, conducting it between 10:00 and 12:00. The mice were trained and accustomed to movement on a rotarod at beginning weeks of age in the

experiment. The rotarod apparatus (Natsume Seisakusho Co., Ltd., Tokyo, Japan) was set at 10 rotations per min. The time taken for each mouse to fall from the rotarod was recorded. Measurements were made three times for each mouse, and the longest time was recorded. The cutoff time was set at 60 sec.

Comparison between GAD and age-matched normal sib mice in the tail-flick test was made using the F-test followed by Student's t-test (when $P \geq 0.05$ in the F-test) or Aspin-Welch's test (when $P < 0.05$ in the F-test). In the rotarod test, the comparisons were made using the cumulative χ^2 test. P values less than 0.05 were considered to represent significant differences.

Table 1 shows the mean tail-flick latencies of GAD and normal mice from 6 to 11 weeks of age. No prolongation of the latencies was observed at any age in either sex, suggesting that C fibers, which are excited by thermal stimuli [1], escape degeneration. Rather than prolongation, slight but significant shortening of the latencies was seen in female GAD mice aged 6 and 8 weeks ($P < 0.05$). According to the gate control theory proposed by Melzack and Wall [5, 12], the transmission of pain messages at spinal relays is controlled by collaterals of primary afferent fibers in the dorsal column passing into the gray matter. The theory was corroborated by the fact that inhibition of pain was obtained by dorsal column stimulation [1]. The fasciculus gracilis of the dorsal column degenerates first and most severely in GAD mice, beginning at about 4 weeks of age. Moreover, spheroids are observed in the gray matter of the lumbar cord particularly the dorsal part, where A δ and C fibers terminate [1], from about 11 weeks of age onwards [4]. The reduction in latencies may be related to dysfunction or degeneration of collaterals of primary afferents controlling the transmission of nociceptive information in the fasciculus gracilis.

Table 2 shows changes in the distribution of GAD and normal mice with regard to their performance times in the rotarod test from 5 to 11 weeks of age. Throughout this testing period phenotypically normal mice remained on the rotarod beyond the cutoff time of 60 sec. The five exceptions were 48 sec (2 mice) and 59 sec in the males and 49 sec and 59 sec in the females.

At 5 weeks of age, GAD mice whose performance times were under 40 sec (21.4% ; 2 of the 10 males and 1 of the 4 females) first appeared. Reportedly, many spheroids appear in the gracile nucleus of the medulla oblongata, and sensory nerve terminals in the muscle spindles degenerate at 3 to 4 weeks of age [7, 8]. Thus, clinically, sensory ataxia occurs at about 5 weeks of age. Hence, the rotarod test can detect the development of motor incoordination in some GAD mice at 5 weeks of age as a decrease in the performance time.

Statistically significant differences between GAD and normal littermates were detected using the cumulative χ^2 test beginning at 5 and 6 weeks of age in males and females, respectively (Table 2). At 8 weeks of age, there was an increase in the number of GAD mice whose performance times were over 60 sec. As a result, the significant differences disappeared in the males, and the P value increased ($P < 0.01$ to $P < 0.05$) in the females in the cumulative χ^2 test. This phenomenon may be explained by the following hypotheses: 1) GAD mice can become accustomed to walking on the rotarod, or 2) sensory ataxia is slightly ameliorated by regeneration of sensory fibers in muscle spindles following their degeneration [7, 8].

Significant difference again appeared in males and the P value decreased ($P < 0.05$ to $P < 0.01$) in females at 9 weeks of age, after which the performance times dropped sharply, especially in male mice (Table 2). At 11 weeks of age, almost all GAD mice (91.7% ; 13 of the 14 males and 9 of the 10 females) had performance times of less than 40 sec. The age when performance times begin to decrease almost perfectly coincides with the age at which the motor nerves start to degenerate from their terminals, i.e., from the motor endplates, in the hindlimbs [7]. Because of this denervation, grouped muscle fiber atrophy and paresis occur, resulting in difficulty in moving. Thus, a sharp drop in performance time can be caused by both concurrent motor dysfunction and paresis.

GAD mice clearly exhibit tremor and abnormal gait, dragging their hindlimbs, after

about 11 weeks of age. One can thus distinguish between GAD and normal mice after this age. Before about 8 weeks of age, however, one cannot select GAD mice without some degree of experience in distinguishing them by suspending mice by the tail. Under the rotarod test conditions in this study, the rotarod performance times of almost all normal mice were over 60 sec, and the times were within 48 to 59 sec even in the case of the 5 exceptions. Accordingly, mice whose performance times are less than 40 sec must be GAD mice, and the rotarod test can be used to select GAD mice before 8 weeks of age more objectively than by suspending them by the tail. Moreover, the test can serve as a good method of evaluating drug candidates for the treatment of ataxia or paresis.

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Gracile axonal dystrophy (GAD)

マウスに関する行動学的研究

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Tail-flick test 及び rotarod test を用いて、5～11 週齢の神経変性ミュータント gracile axonal dystrophy (GAD) マウスに関する行動学的検討を行った。Tail-flick test により、6 および 8 週齢の GAD 雌マウスにわずかながら tail-flick latency の有意な減少が認められた。これは痛覚を制御していると考えられている後索線維の側枝の変性に関与している可能性がある。Rotarod test では、rotarod performance time の有意な減少が GAD 雄マウスで 5 週齢から、GAD 雌マウ

スで 6 週齢から累積 χ^2 検定法により示された。これは rotarod test が GAD マウスの感覚性失調を 5～6 週齢から検出できることを示唆する。GAD マウスの rotarod performance time は 9 週齢以後急速に低下したが、これは GAD マウスの運動麻痺の進行と一致する。今回の検討により、rotarod test は GAD マウスの早期の客観的選抜、あるいは GAD マウスに対する運動失調、運動麻痺の薬物評価に応用できることが示唆された。