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Research report

A behavioral study of the development of hereditary cerebellar ataxia in the shaker rat mutant

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Abstract

shaker mutant rats were first identified by their abnormal motor behaviors and degeneration of cerebellar Purkinje cells and brainstem inferior olivary neurons. After 6 generations of inbreeding 77% of *shaker* rat mutants are mildly ataxic (identified as mild *shaker* mutants) and 23% are ataxic and exhibit a whole body tremor (strong *shaker* mutants) by 3 months of age. This study of *shaker* mutants from birth to 3 months of age was designed to: (1) compare the somatic and motor development of *shaker* mutants with age matched normal rats; (2) identify the temporal onset of motor deficits; and (3) correlate qualitative differences in Purkinje cell degeneration between 3-month-old mild and strong *shaker* rat mutants. *shaker* mutant rats consistently weighed less than age-matched control animals. Analysis of motor development using the hindlimb splay test demonstrated the distance between hindpaws was significantly greater in *shaker* mutant rats than in controls starting at 42 postnatal days (PND) of age. Hindlimb stride width was greater for *shaker* than control rats at 42 PNDs. However, after 42 PNDS *shaker* mutant average hindlimb width was narrower than controls. Forelimb stride width was consistently narrower in *shaker* mutants than in normal rats. Hindlimb placement was impaired in *shaker* rat mutants after 15 PND. Forelimb placement, cliff avoidance and surface righting were only transiently impaired in *shaker* mutants. Mid-air righting, performance of a geotaxic response, and climbing and jumping postural reactions were similar in *shaker* and normal rats. The spatial extent of Purkinje cell survival/degeneration correlated with differences in abnormal motor activity seen in 3-month-old mild and strong *shaker* mutants. In mild *shaker* rat mutants, Purkinje cells appeared to have degenerated randomly throughout the cortex. In strong *shaker* mutants most Purkinje cells in the anterior lobe had degenerated. In the posterior lobe Purkinje cell degeneration appeared to be numerically significant, but many surviving cells were present. Although Purkinje cell loss was not numerically quantified in this study, a strong association between the extent and type of spatial loss of Purkinje cells, and the severity of clinical signs, appears to exist.

Keywords: Cerebellum; Purkinje cell; Ataxia; Mutant; Behavior; Neurodegenerative disease

1. Introduction

The cerebellum regulates posture and voluntary movement and therefore any alterations in cerebellar structure results in impaired motor function [23]. Abnormal motor activity associated with cerebellar cortical Purkinje cell loss occurs most frequently as the result of spontaneous genetic mutations. In various mouse mutants disparities in the spatial and temporal pattern of Purkinje cell degeneration have been correlated with differences in somatic development and abnormal motor behaviors [8,11,33,34,38,43,44,50,51]. In *Purkinje cell deficient* (*pcd*), *nervous*, *lurcher*, and *staggerer* mutant mice, Purkinje cell degeneration is numerically significant. In these mutants 75–90% of Purkinje cells degenerate before 60 postnatal day of age (PND), with a majority dying between 20–40 PND. The loss of Purkinje cells occurs coincident with somatic abnormalities including smaller body size, abnormal audition, poor reproductive capability and death of homozygotes shortly after birth. Behaviorally, these mutants develop a splayed hindlimb stance, hindlimb weakness, head or whole body tremor, reduced levels of activity, ataxic gait and disequilibrium [15,19,20,30,32,34,35].

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Temporally slower and less extensive Purkinje cell degeneration has been reported in *hyperspiney*, *hot-foot*, *weaver* and *leaner* mutant mice [3,14,22,23,29,38,45]. In these mutants only 15 to 30% of the Purkinje cells degenerate over a period of many months. Unlike mouse mutants with severe Purkinje cell degeneration, mildly affected murine mutants' somatic development is relatively normal. Body size and weight are not significantly affected, audition is relatively normal, and most homozygotes survive and reproduce. Similarly, motor behaviors are only minimally affected. These animals display a normal gait (with the exception a slightly shortened stride in *hot-foots*). The *hyperspiney*, *weaver*, *hot-foot* and *leaner* mutants display a mild ataxia, no tremor, and only a slight decrease in activity levels. Analogous to mutants with more severe Purkinje cell loss, these mildly affected mutants demonstrate a splayed hindlimb stance with occasional hindlimb weakness. In these different murine mutations it is therefore apparent that the temporal and numerical loss of Purkinje cells is directly correlated with body size and weight, motor development and reproductive capability [14,21–23].

Studies of Purkinje cell degeneration in mouse mutants have provided important new findings on the mechanisms regulating cell death, cerebellar corticogenesis, and the development of cortical afferent topography [2,11,18,44,49–51]. However, rats, due to their larger size and ability to learn complex behavioral tasks, would be advantageous for other types of studies of Purkinje cell degeneration.

Rat Purkinje cells can be experimentally induced to degenerate by irradiation or chemotoxicity [6,7,20,39]. For example, acrylamide injections cause up to 30% of Purkinje cells to degenerate rapidly and the injected rats to perform poorly in motor tasks [6,28]. Several rat mutants with spontaneous Purkinje cell degeneration have also been identified. In *Gunn* rats, a mutant strain of Wistar rats with autosomal recessive hereditary hyperbilirubinemia, selective Purkinje cell degeneration occurs within 30 and 72 h after birth in homozygote and heterozygote matings, respectively [46]. The number of degenerating Purkinje cells increases steadily from day 7 to 23 postnatal days of age with severely damaged cells dying and disappearing by day 30. In a preliminary characterization of the *shaker* rat mutant, an apparent sex-linked mutation in a line of Sprague-Dawley rats, Purkinje cell degeneration appeared correlated with abnormal motor activity. In postadolescent *shaker* mutants, Purkinje cell loss was reportedly extensive in the cerebellar anterior lobe and moderate in the posterior lobe. Behaviorally these animals were distinguished by their wide-based hindlimb stance, ataxic movements and whole body tremor.

Three studies were initiated concurrently in *shaker* mutant rats to identify: (1) the spatial/temporal patterns of Purkinje cell degeneration in *shaker* mutants [48]; (2) morphological features of degenerating Purkinje cells and the effects of the loss of Purkinje cells on pre- and postsynaptic cerebellar neurons [13]; and (3) the development of somatic and motor behaviors. Results from the first study indicate that there are two distinct spatial and temporal patterns of Purkinje cell degeneration in *shaker* rats at 3 months of age and beyond [48]. Findings from the second study indicate that predegenerating Purkinje cells have abnormal axonal morphologies and that cerebellar pre- and postsynaptic neurons do not appear to be immediately affected by the death of Purkinje cells. This paper reports on: the postnatal, preadolescent and postadolescent (up to 3 months of age) somatic and motor development of *shaker* mutants with age matched normal rats, the temporal onset of motor deficits in *shaker* rats, and correlates qualitatively the abnormal motor behaviors in *shaker* mutant rats with spatial patterns of Purkinje cell degeneration at 3 months of age.

Somatic development of *shaker* mutants was assessed by measuring body weight evolution and ear flap and eyelid opening. Overall somatomotor development was appraised by the auditory startle response. Vestibular and somatosensory systems were examined by various tests of righting and placing including the surface righting test, the geotaxic response, and the mid-air righting response. Additional dynamic placing tests studied were those involving placement of the hindlimbs and forelimbs on a horizontal rod, the vibrissae placing response, the climbing response and the cliff avoidance reaction. The hindlimb splay test was performed as an objective method for early assessment of motor disability. Measurements of gait interstride lengths and widths were used to evaluate locomotor development. Behavioral testing was terminated at 3 months of age and cerebella were evaluated microscopically to correlate the behavioral findings with qualitative histopathologic evaluation of Purkinje cell degeneration.

2. Materials and methods

2.1. Animals and postnatal physical development

shaker rats used in this study were in the F_6 generation of inbreeding. Four litters of *shaker* mutant rats (brother-sister mating) were compared to three litters of normal Sprague-Dawley rats. Litter sizes of normal rats were matched to the size of three of the four *shaker* mutant litters. (The fourth normal rat litter and dam were removed from the study due to the dam's poor mothering ability.) At 3 months of age clinical observations revealed that some *shaker* mutants (77%) are only ataxic (identified as mild *shaker* mutants) whereas 23% are ataxic and exhibit a whole body tremor (strong *shaker* mutants). This represented an increase in percentage of strong *shaker* mutant rats compared to the F_2 parent-offspring generation where only 15% of the offspring are charac-

terized as strong *shaker* rats. Animals were housed in microisolator cages and fed Teklad Chow 7001. Rats had free access to water and food.

Somatic development was assessed by measuring body weight gain, eyelid opening and ear flap opening. The auditory startle response, a test of somatomotor development, was also examined. Pups were weighed daily until 3 weeks of age and at weekly intervals thereafter. The initial appearance of ear flap opening, eyelid opening and development of the auditory startle response, as described in Barlow et al. [4], were recorded and compared to controls. A positive response to the auditory startle stimulus (a loud clapping noise) was defined as any visible muscular response by the rat to the noise.

2.2. Righting and placing reactions

A variety of tests are described in the literature to assess neuromuscular development and motor behavior in rats (Fig. 1 and Fig. 2) [1,5,12,16,17,25,26,37,42]. Of these, tests of righting and placing were chosen from those commonly used in both behavioral and clinical assessments of the nervous system and are dependent on intact vestibular and somatosensory systems. Additionally, an intact peripheral motor system is necessary in order to produce a neuromuscular response to these various stimuli [1,4,37]. In early postnatal development, rats surface right via integration of tactile and vestibular stimuli. During the second week of life, the vestibular righting system is the dominant form, while the tactile system is expressed only when the vestibular system is disabled [40]. Newborns can right themselves on a surface by day 1, but speed of righting improves with age. Normal rats display a response to negative geotropism by 5 PND. This is the geotaxic reaction, a special case of the surface righting response. Rats are successful at mid-air righting by 17 PNDs, at which time vestibular, not tactile input, elicits rotation of the shoulders which then carry the head and neck to an upright position [41]. Simple placing reactions normally develop by 9–10 PNDs and can be assessed using the following tests [16].

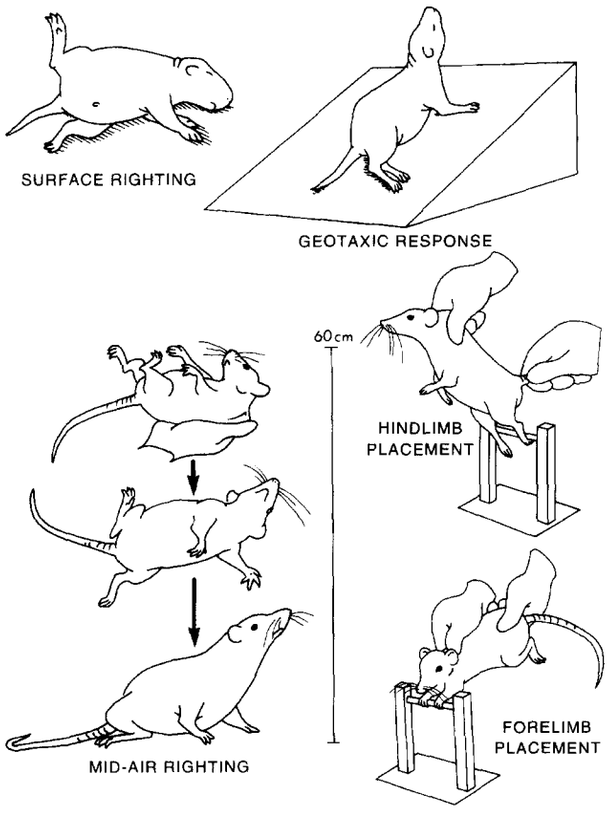

Fig. 1. Diagrams illustrating the behavioral tests used in this study.

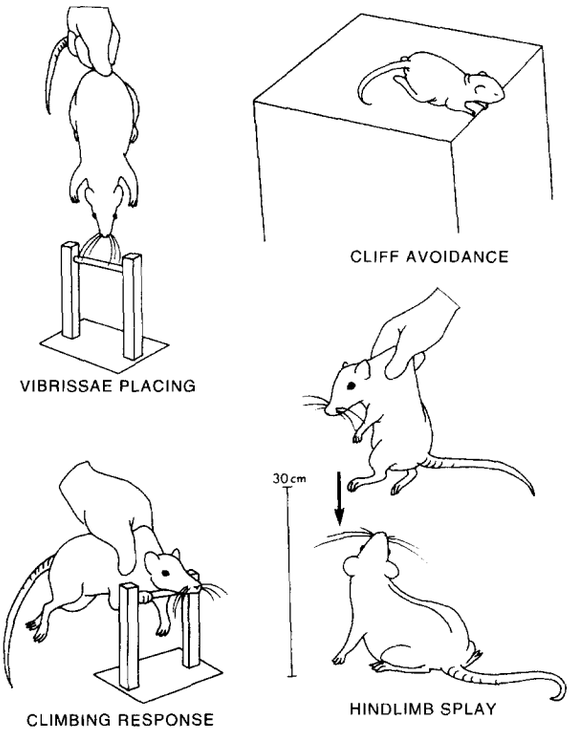

Fig. 2. Diagrams illustrating the behavioral tests used in this study.

The surface righting test consists of placing a newborn rat on its back on a flat surface and recording the amount of time elapsed until it turns over (Fig. 1). Surface righting was assessed daily from PND 1 until a normal reflex was attained (1 to 10 PND) and weekly thereafter to 3 months of age.

Geotaxic reactions are orientations of an animal in response to a gravitational force [4]. It is a special case of the surface righting response and is primarily dependent on an intact central vestibular system. Rats were placed on a 25 degree wooden incline with the head pointing downward. A normal rat placed in this position will turn and face upward (Fig. 1). Rats were given a single trial a day with a turn of 180 degrees within 3

min scored as successful. Rats were tested daily from 2 to 21 PND and weekly thereafter until 3 months of age.

Mid-air righting was assessed by holding the rat at a height of 60 cm above a well padded surface and releasing it (Fig. 1). Rats were tested starting at 15 PND and daily until 21 PND and then weekly until 3 months of age. Mid-air righting was scored as positive or negative. A positive response occurred when rats landed on all four feet.

Several placing/postural reactions were measured daily from 7 to 21 PND and then weekly until 3 months of age. Four reactions were tested: forelimb/hindlimb placement, the vibrissae placing response, the climbing response and the cliff avoidance response. Fore- and hindlimb placing responses are strongly dependent on an intact peripheral and central nervous systems [1,10]. Fore- and hindlimb placing was tested by supporting the rat's body by holding the base of the tail and nape of the neck and moving the animal backward so that the backs of the feet touched the edge of a stationary horizontal rod (Fig. 1). The rats were prevented from seeing the rod or touching it with their vibrissae. Normal adult rats immediately raise their feet and place them on the rod in response to the stimulus. Rats were given three trials daily, testing both the forelimbs and hindlimbs. Reactions were scored from 0 to 3 as follows: a score of 0 indicated no response. A score of 1 was recorded to a slight lifting of the limbs without contacting the rod. A score of 2 was recorded to an uncertain response with extended paws and a spread of digits. A 3 was scored when there was an immediate and accurate placing response.

In the vibrissae placing response, rats were suspended by the base of their tails and allowed to touch a stationary horizontal rod with their vibrissae (Fig. 2). Normal adult rats extend and adduct the forelimbs and extend the head. All rats were tested daily from 7 to 21 PND and then weekly until 3 months of age. This test simulates movements seen in rats attempting to jump down from an elevated area and is most strongly dependent on an intact exteroceptive (tactile) system. Absence of limb adduction and head extension was scored as 0. Slow sideways movement of the head with extension of the forelimbs were scored as 1. Slow extension and swaying of the trunk and head was scored as 2. When all 3 components were present, and the rat successfully touched the rod with either forelimb, the score was recorded as 3.

The climbing response incorporates vestibular and tactile input and simulates behavior seen when an animal climbs. This includes raising the shoulders, flexion of the forelimbs and extension of the hindlimbs. The rats were suspended by the nape of the neck and their chin touched to a stationary horizontal rod (Fig. 2). A normal adult rat will immediately raise its front feet and place them behind the jaws and extend its rear limbs as if attempting to stand. Testing was done daily from PND 7 through 21, and weekly thereafter. Absence of a response was graded as 0. Slight forelimb flexion without contacting the rod was scored 1. Slow, uncertain forelimb flexion with extended paws and spreading digits received a score of 2. When all components were swiftly performed the score of 3 was recorded.

Cliff avoidance has a strong exteroceptive component (vibrissae) and minimal vestibular involvement. When placed on the edge of a wooden platform with nose and forepaws over the edge, a normal rat will move away from the cliff by backing up and moving sideways (Fig. 2) [4]. The platform was 15 cm × 15 cm and stood 15 cm off the ground. In other normal rat strains, a swift response is seen by 7 days of age. Rats were tested daily between 5 and 21 PND and weekly until 3 months of age. Responses were graded as follows: 0-no response, 1-response in 11–15 s, 2-response in 6–10 s, and 3-response in 5 s or less.

2.3. Hindlimb splay

In the hindlimb splay test an increase in hindlimb landing foot space is a simple and objective method for early assessment of motor disability in rats [17,26]. The hindpaws were marked with stamp pad ink and the rat released 30 cm above a sheet of blotting paper. The distance between the position of the fourth digit of each hindpaw print was measured. Rats were given three trials daily from 14–21 PND and weekly thereafter until 3 months of age.

2.4. Locomotor behavior

Gait topography has been used to evaluate the functional effects of peripheral nerve damage [17] and to measure motor dysfunction with age [42]. These studies were modeled after those of Parker and Clark who studied gait topography in normal rats [37]. Previous studies have shown that typical walking movements begin shortly after day 11 in normal rats [4]. The hindpaws were marked with stamp pad ink and the rat allowed to make a single transit of an opaque tunnel (1 m long × 7 cm high × 10 cm wide). The floor was covered with a strip of plain white paper. Rats traversed the tunnel spontaneously. Stride length was measured between ipsilateral foot prints using the second and third interdigital pads as reference points. Stride width was measured between consecutive fore or hindpaw prints and at right angles to the direction of travel. Gait topography measurements were done at 15, 17, 21, 25 and 28 PNDs and weekly thereafter until 3 months of age.

2.5. Collection of tissues and histopathologic evaluation

At the conclusion of the behavioral testing (3 months) 4 *shaker* mutants were retained for breeding purposes and 31 were prepared for histopathologic examination. These latter animals were deeply anesthetized with sodium pentobarbital, a thoracotomy performed to expose the heart, and the animals perfused transcardially first with normal saline followed by 10% buffered neutral formalin. The brains and spinal cords were removed immediately and stored in the fixative/perfusate for a variable length of time. Prior to freezing microtomy, cerebella were cyroprotected with 30% sucrose and then sectioned sagittally or transversely at 32 μm with a sliding microtome. Serially spaced sections were mounted onto gelatin subbed slides and stained with 1% cresyl violet or processed for calbindin immunoreactivity. Calbindin is a calcium binding protein present in the somata and processes of all Purkinje cells [9,27]. Briefly, slide mounted serially spaced sections were first incubated in 5% horse blocking serum, followed by the primary anticalbindin antibody (1 : 20,000 dilution, Sigma) [48]. The sections were then incubated in biotinylated secondary antibody and then antigen-antibody complex visualized using a peroxidase avidin-biotin complex reaction (ABC kit Vector). Calbindin immunoreactive PC somata were dark brown to black in color while their processes appear lighter brown in color. In this study the severity of clinical signs and locomotor deficits were qualitatively compared to the spatial degeneration of Purkinje cells. In companion studies the temporal-spatial loss of Purkinje cells, abnormal Purkinje cell morphology, and effects on pre- and postsynaptic cerebellar neurons were accessed quantitatively [13,48].

2.6. Statistical analysis

Measurements of body weight, distance between digits and other parametric data were analyzed using Student's *t*-test. Nonparametric data were analyzed using χ^2-square analysis.

3. Results

3.1. Postnatal physical development

Body weights were monitored in *shaker* and control animals and used as a general indicator for comparison of somatic growth (Fig. 3). In normal rats there was no significant difference in body weights between males and females until weaning (21 PND). After weaning the rate at which normal males gained weight was slightly greater than normal females such that at 8 weeks of age normal males weighed approximately 35% more than female littermates. Female *shaker* rat mutants weighed 26–30% less than normal female rats at 3 PND and throughout most of the period of observation. Male *shaker* mutants at birth and prior to 7 PND were comparable in weight to normal males. After 7 PND male *shaker* mutants consistently weighed 20–28% less than age matched normal males and 19–24% less than similar aged normal

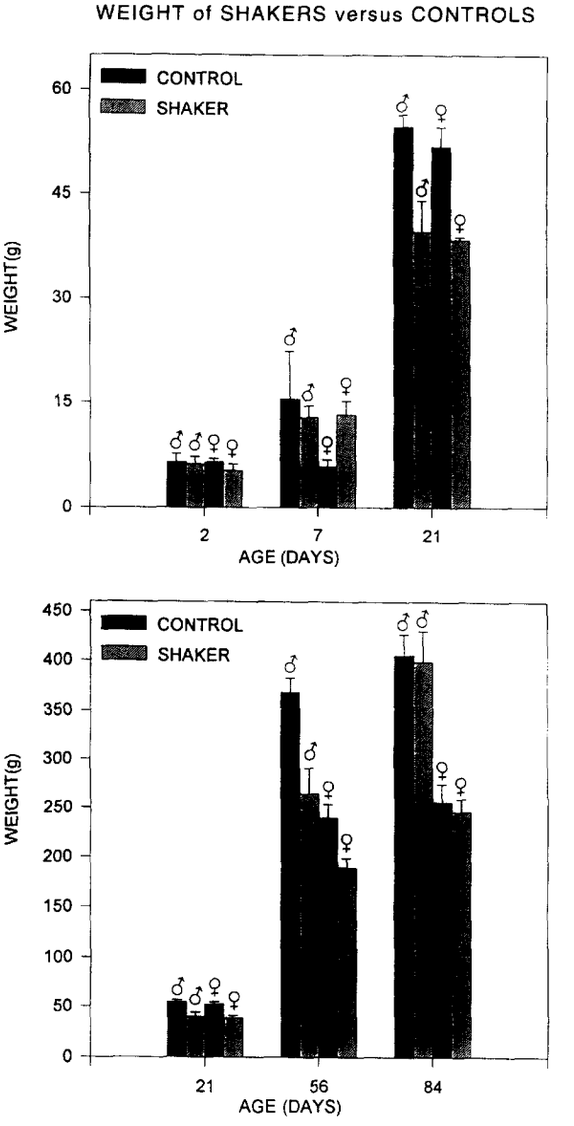

Fig. 3. Body weights in grams of normal and *shaker* mutant rats. In normal rats there was no significant difference in body weights between males and females until weaning (21 PND). After weaning, the rate that normal males gained weight was slightly greater than females. Female *shaker* rats weighed less than normal rats after 2 days of age and throughout most of the period of observation. Male *shaker* rats at birth and prior to 7 PND of age were comparable in weight to normal males. After 7 PND male *shaker* mutants consistently weighed less than age matched normal males and females. *shaker* rat males and females exhibit poor weight gain as compared to normal rats which is similar to the retarded growth documented in most strains of mutant mice with Purkinje cell abnormalities. (Student's *t*-test)

females. Strong versus mild *shaker* mutants body weights were not consistently different.

Ear flap and eye opening are easily assessed epochs for evaluation of growth. In both normal and mutant rats ear flaps opened between days 2–4 PND and eyes opened between 15–17 PND. The timing of both events was consistent with previously reported data by Barlow et al. [4].

The auditory startle response is an indicator of reflexic auditory-somatomotor development and integrity. An auditory startle response could be elicited in both normal and *shaker* pups between 11–13 PND. In most *shaker* mutant pups the auditory response was present at 11–12 PND, whereas in most control pups the response was elicited on 12–13 days of age. The auditory startle response persisted through the testing period (3 months of age) in both normal and *shaker* rat mutants, confirming the normal integrity of auditory-somatomotor circuitry.

3.2. Righting and placing reactions

3.2.1. Surface righting

The ability to right from a supine to a prone position on a horizontal flat surface is a test of integrated tactile and vestibular development in neonatal rats [25,40]. Between 4 and 9 PND the total time for supine-prone righting was consistently less for normal rats than for the mutants and normal rats righted themselves on the average, 3–10 times faster than *shaker* rats (Table 1). After 9 PNDs there were no significant differences in righting times, all pups righted themselves in less than 1.5 s. There were no significant differences in righting between strong and mild *shaker* mutants at any age tested.

3.2.2. Geotaxic response

When neonatal rats are placed on an incline with their heads pointing downwards they will turn to face upwards performing a postural response called negative geotropism. It is similar to the surface righting response in that integration of vestibular and somatosensory development are assessed. No significant difference between the percent of *shaker* and control animals passing the geotaxic response test was detected. At and after 10 days of age, all rats successfully produced the 180 degree turns in less than 3 min.

Table 1
Time in seconds for surface righting of control and *shaker* mutant rats

Day	*Shaker* rats	Control rats	*P* value
4	34.4±32.0	8.8±8.6	<0.001
5	23.1±26.3	2.4±2.0	<0.01
6	13.2±30.8	4.3±7.2	NSD
7	8.5±12.1	2.1±2.3	<0.05
8	3.3±3.2	0.9±0.9	<0.05
9	3.3±3.2	1.0±0.0	<0.01
10	1.3±0.5	1.0±0.0	NSD

Total time taken to right on a surface was consistently less for normal rats compared to the *shaker* mutants at 4–9 PND, suggesting delayed integration of vestibular, somatosensory and neuromuscular development in the mutant. All rats righted themselves in less than 1.0 s after 10 PND. (Student's *t*-test)

3.2.3. Mid-air righting response

The mid-air righting response involves a sequence of rostrocaudal rotational movements reflecting an integration of the sensory (vestibular) and motor systems. In mid-air righting, all normal and mutant rats successfully righted themselves in the air and landed on all four feet by 21 PND. There were no significant differences in the mid-air righting ability between normal and *shaker* rats at any age.

3.2.4. Forelimb and hindlimb placing responses

Forelimb and hindlimb placing responses are strongly dependent on intact proprioceptive systems. Normal and mutant rats demonstrated no significant difference in scoring for hindlimb placement from the first day of testing (day 7) through day 12. On day 12, 65% of the mutant and 73% of the normal rats achieved a score of '3' (immediate response) for hindlimb placement, 20% and 28% respectively scored a '2' (a slow uncertain response) and 14% of *shaker*s scored a '1' (a slight lifting of the limbs) (Fig. 4). After 12 PND normal rats obtained significantly higher scores for hindlimb placement whereas the scores decreased for *shaker* mutant rats. On day 13, 95% of normal rats immediately and accurately placed their feet on a rod and scored a 3. Conversely, the mutants performed comparatively worse than their age matched controls with only 37% obtaining a score of 3 (Fig. 4). After 13 days of age, *shaker* rats performed inconsistently but always comparatively worse than their age matched controls. At 6 weeks of age 100% of control animals scored a '3', whereas only 17% of *shaker*s scored at this level. By 10 weeks of age control animals maintained their high performance with 100% of the rats scoring a '3', while only 13% of *shaker*s scored at this level. These data indicate that there is a continued worsening of *shaker* rats ability to use their hindlimbs for placing, which is consistent with observations made in Purkinje cell deficient mouse mutants.

Strong *shaker* rats demonstrated some delay in their ability to hindlimb place as compared to mild *shaker* rats. Mild *shaker* rats obtained higher scores for hindlimb placement compared to strong *shaker* rats at day 13. On day 13, 48% of mild *shaker* rats scored at the level of '3', while 0% of strong *shaker* rats obtained this score. After day 13, scores for strong and mild *shaker* rats were not significantly different. Hindlimb placement was not a consistently sensitive discriminator of mild versus strong *shaker* rats after PND 13.

In forelimb placement normal rats scored higher than

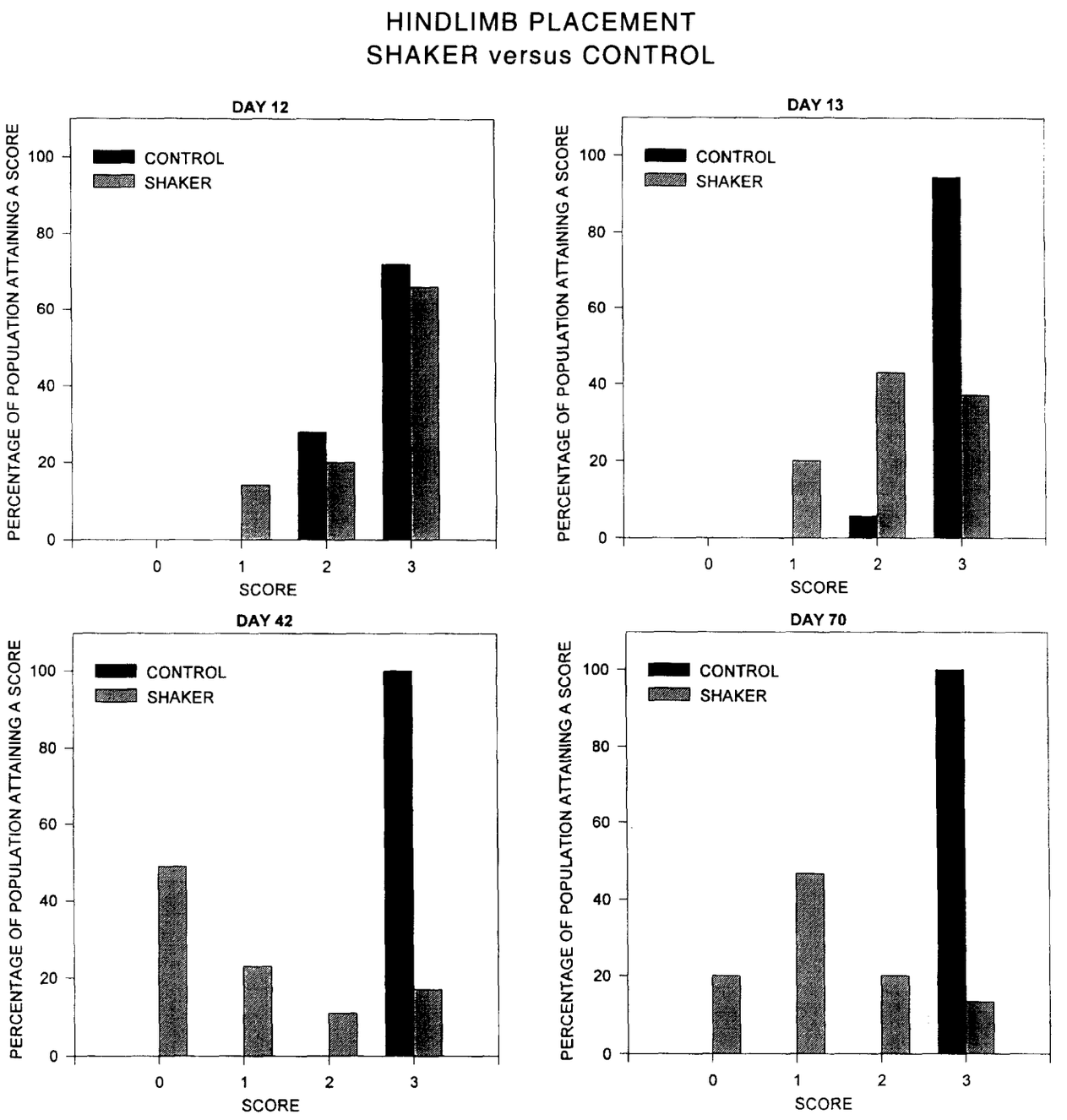

Fig. 4. Hindlimb placement scores for normal versus *shaker* mutant rats on day 12 where the number of animals achieving scores were not significantly different. On day 13 control rats improved their ability to use their hindpaws to contact a rod, but *shaker* mutants performed inconsistently from day to day, and comparatively worse than their age matched controls. At 42 PND, 100% of control rats completed the placement task flawlessly as compared to 17% of the mutants. Hindlimb placement scores at 70 PND were no different than scores at 42 PND for normal rats. *shaker* mutants continued to score significantly worse than age matched controls. Overall, *shaker* mutant rats performed progressively worse than age-matched controls when placing their hindlimbs on a stationary horizontal rod indicating a degenerative deficit in proprioceptive and exteroceptive (tactile) systems regulating the hindlimbs. (See text for scoring methods.)

shaker rats between 7 to 21 days of age (Table 2). On day 7, 11% of *shaker* rats compared to 83% of control animals attempted forelimb placing. Over the next 3 days forelimb placing improved in both groups of animals such that at day 10, 89% of controls and 57% of *shaker* mutant rats scored either at the '2' or '3' level. On day 14, all control animals performed forelimb placing flawlessly (100% scoring '3'), while only 83% of *shaker* rats scored at this level. At 21 days *shaker* rats performed at the same level as normal rats. In contrast to hindlimb placement results, there was no indication that *shaker* rats lose the ability to place their forelimbs

Table 2
Forelimb placement scores for normal and *shaker* mutant rats.

Day		Score 0	Score 1	Score 2	Score 3	*P* value
7	Control	17	83	0	0	<0.01
	Shaker	89	11	0	0	
10	Control	0	11	50	39	<0.01
	Shaker	9	34	17	40	
14	Control	0	0	0	100	<0.05
	Shaker	0	3	14	83	
21	Control	0	0	0	100	NSD
	Shaker	0	0	0	100	

On PND 7, *shaker* mutant rats performed significantly worse than normal rats with a majority of the mutants failing the test completely. At day 10, a greater percentage of control animals scored at the upper levels than the mutants, indicating normal rats continued to have superior forelimb placing ability as compared to age-matched mutants. On PND 14 normal rats performed the placing task flawlessly, while fewer *shaker* mutants scored at this level. At and following 21 PND scores for normal rats and *shaker* mutant rats were not significantly different as all animals consistently scored at the highest level (χ^2-test).

as they age. Strong and mild *shaker* rats attained similar scores for forelimb placement throughout the testing period.

3.2.5. Vibrissae placing response

In the vibrissae placing response, normal adult rats while suspended by their tail, will extend and adduct the forelimbs and extend the head in response to touching the vibrissae to an underlying stationary object. This response simulates movements seen in rats attempting to jump from a higher to a lower platform. In the climbing response, normal rats will raise their shoulders, flex their forelimbs and extend their hindlimbs after having their chins placed on a horizontal surface. There were no significant differences in the vibrissae placing response scores or climbing scores between normal and *shaker* rats, or between strong and mild *shaker* mutants at any age tested.

3.3. Cliff avoidance response

Cliff avoidance ability primarily involves the somatosensory system and to a lesser extent the vestibular system. In this study both normal and mutant pups failed to avoid a cliff edge at day 5. Animals became progressively more successful at avoiding a fall between 6 and 9 days of age with control and *shaker* rats displaying equal ability (Fig. 5). On day 8, 45% and 40% of control and *shaker* mutant rats respectively scored at the level of '2' or '3' suggesting little difference in avoidance ability. On day 10, a significantly greater percentage of normal rats scored higher in cliff avoidance ability than *shaker* mutants. A total of 83% of control animals performed the task flawlessly (at a score of '3') as compared to only 40% of *shaker* mutants scoring at that level. Similar results were found on PND 11. *shaker* and normal rats successfully achieved the highest score for cliff avoidance after day 11, with no difference in ability present between groups. Results for day 15 are representative of these scores (Fig. 5). None of the rats lost this ability throughout the testing period. *shaker* rats demonstrated only a temporary delay in cliff avoidance ability at PNDs 10–11 suggesting a slight delay in the development of their exteroceptive system. As all rats maintained the ability to cliff avoid after day 11, no degeneration in this system could be detected in *shaker* rats. No significant difference in cliff avoidance ability was found in strong compared to mild *shaker* rats at any age.

3.4. Hindlimb splay

Hindlimb splay is a measure of the distance between the position of the fourth digit of each hindlimb on landing resulting from a drop of 30 cm onto a flat padded surface. The distances between hindpaw prints were generally significantly greater for normal rats over *shaker* mutants from the first day of testing (day 14) through day 35 (Fig. 6). At day 35, the width of splay was 78 mm for normal rats and 67 mm for *shaker* mutants. (Note rats were tested daily from PND 14–21 and weekly thereafter.) Normal rats' splay was significantly greater than that of the *shaker* mutants at days 16, 17, 28 and 35, but not at day 21 where *shaker* mutants had a slightly greater splay. The reason for this mild departure in trend could not be determined. As the average weight of normal rats was greater than that of *shaker* rats throughout the period of observation, a consistently greater splay was expected for normal rats over *shaker* rats at all times due merely to the normal animals' greater weight. Despite this weight difference, the distance between hindpaw prints became significantly greater for *shaker* rats over normal rats from day 42 (week 6) to the end of the testing period. At day 42, the average splay for normal rats was 81.8 mm, but was 94.0 mm for mutants. As greater hindlimb splay is an indicator of increased ataxia in rats in neuropharmacologic studies [17,26], it was concluded that *shaker*

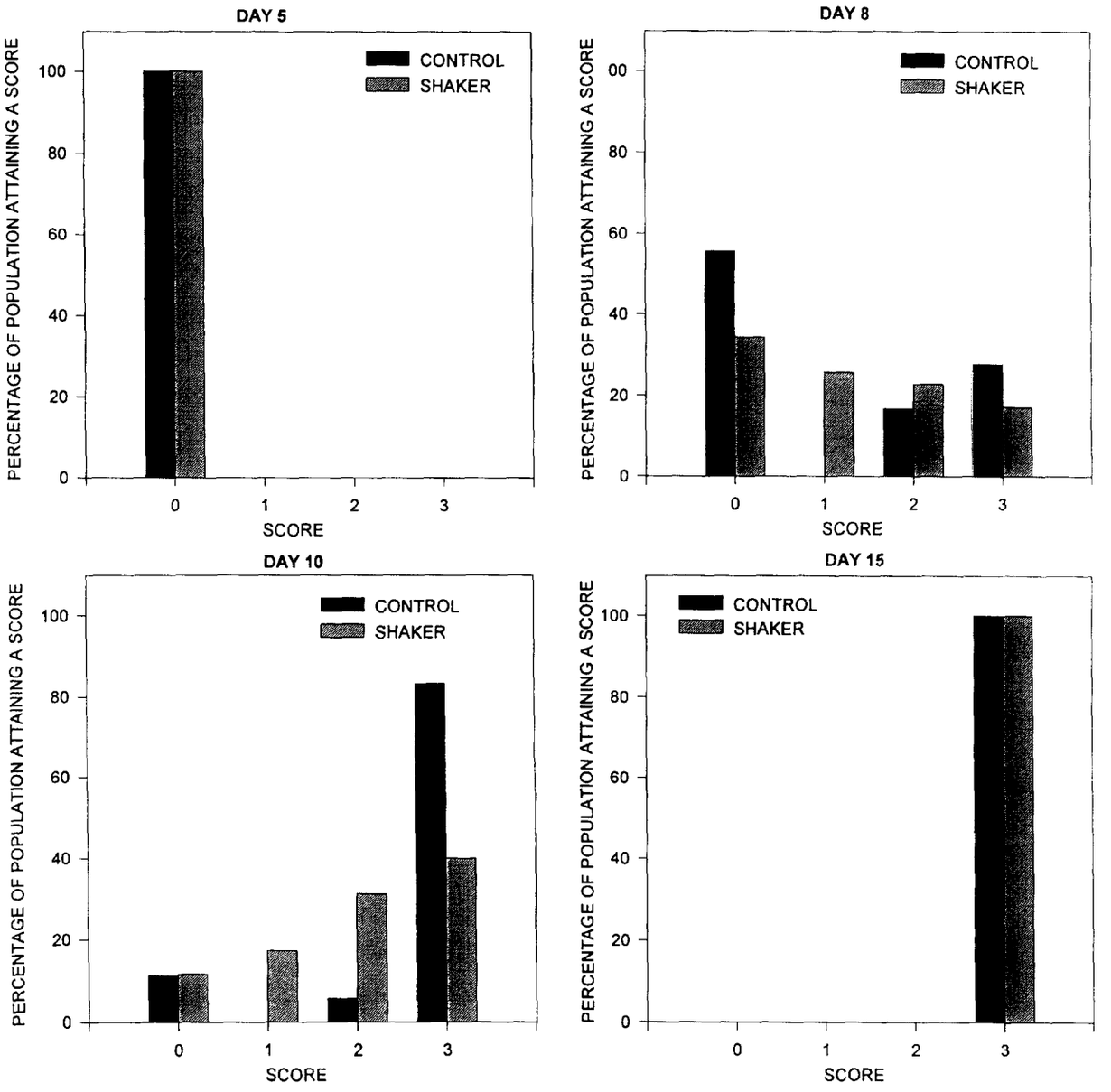

Fig. 5. At 5 days of age, all rats failed to avoid falling off the edge of cliff attaining a score of '0'. On days 6–9, the animals cliff avoidance ability improved and was comparable for *shaker* and control rats. The rats' performance on day 8 is depicted here as it analogous to rats performance during days 6–9. On day 10, as on day 11 not shown here, control animals performed the task flawlessly (at a score of 3) as compared to significantly fewer *shaker* mutants scoring at that level. After day 11, *shaker* and normal rats successfully achieved the highest score for cliff avoidance (as depicted here by responses recorded on day 15), with no difference in ability present between groups. (See text for scoring methods.)

mutants were first consistently showing clinical signs consistent with ataxia at 42 PND.

In the hindlimb splay test, distances between hindpaw prints were not significantly different between strong and mild *shaker* mutants until 4 weeks of age (Fig. 6). At 28 days, mild *shaker* mutant rats presented with a significantly greater splay of 71.8 mm as compared to the 61.0 mm of the strong *shaker* rats. The weights of mild and strong *shaker* rats were not significantly different at any age, thus similar splay widths had been expected at all ages. At day 35, hindlimb splay was once again not significantly different between mild and strong

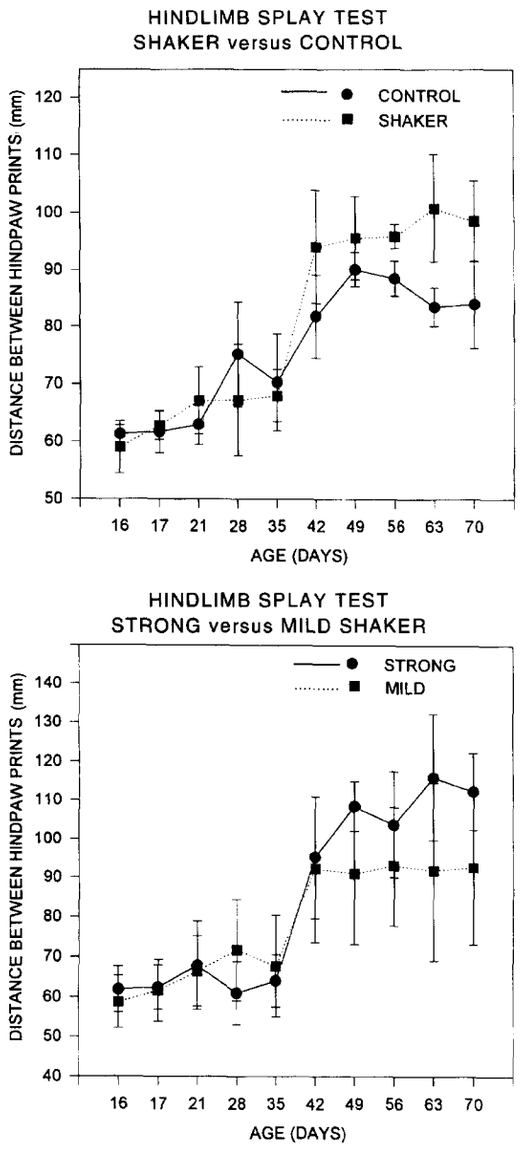

Fig. 6. The distances between hindpaw prints were significantly greater for normal rats over *shaker* mutant rats through day 35. The normal rats' greater splay was attributed to their greater size as demonstrated by their greater weight (see Fig. 3). Despite the normal rats' greater size, and continued expectation of greater splay, the distance between hindpaw prints became significantly smaller for *normal* rats over *shaker* rats from day 42 (week 6) to the end of the testing period. A greater splay at week 6 suggests a degenerative pathology significant enough to produce ataxia. (Student's *t*-test), Normal rats' splay was significantly greater than *shaker* rats at days 16, 17, 28 and 35, but not at day 21 where *shaker* mutants had a slightly greater splay. The reason for this mild departure in trend could not be determined. Distances between hindpaw prints were not significantly different between strong and mild *shakers* until day 28 (4 weeks of age). At 28 days, mild *shakers* presented with a significantly greater splay. The weights of mild and strong *shaker* rats were not significantly different, thus similar splay widths had been expected at all ages. At day 35, hindlimb splay was again the same for mild and strong *shaker* mutants. At day 42, strong *shaker* rats presented with a slightly greater splay, but differences were not yet statistically significant. At, and after day 49, however, strong *shaker* rats presented with a significantly greater splay despite their lesser weight, and this trend continued throughout the end of the testing period. (Student's *t*-test)

shakers at 67.8 and 64.1 mm, respectively. At day 42 (week 6), however, strong *shaker* mutant rats presented with a minimally greater splay of 95.4 mm as compared to 92.4 mm for the mild *shaker* rat mutants. Widths became significantly greater for strong *shaker* (108.6 mm) over mild *shaker* (91.1 mm) mutants at PND 49 and this trend continued through the end of the testing period. This consistently greater splay in strong *shaker* mutants was attributed to greater ataxia in strong over mild *shaker* rat mutants at and after 49 PND of age.

shaker rat mutants, and specifically the strong *shaker* mutants, exhibit increased incoordination with a greater than normal hindlimb splay, by postnatal days 42 and 49, respectively. This suggests a degenerative process resulting in disability which may be correlated with Purkinje cell degeneration at these ages. The hindlimb splay test therefore appears to be an objective method for early assessment for ataxia possibly due to Purkinje cell degeneration in the *shaker* mutant rat.

3.5. Locomotor behavior

Stride lengths and widths were used to compare gait topography between normal and mutant rats. Hindlimb stride length varied inconsistently between *shaker* and normal rats at all ages tested (15, 17, 21 and 25 PNDs and weekly until 3 months of age) and therefore not a good discriminator of *shaker* mutant rat behavior from that of normal rats. Hindlimb stride length was not significantly different between strong and mild *shaker* mutants at any age. Forelimb stride lengths were similar for *shakers* and age-matched controls at all ages tested.

shaker mutant's average hindlimb stride width at 42 PNDs was 50.0 mm and was greater than controls (45.1 mm) at this age (Fig. 7). Later at 49, 56 and 63 PNDs, *shaker* mutant average hindlimb stride width was narrower than controls. At PND 49, *shaker* stride width was 43.0 mm as compared to 47.5 mm in controls. At PND 56 and 63 PNDs, stride widths for *shaker* mutants were 41.3 mm and 39.0 mm, respectively, while stride widths for controls at these ages were 44.4 mm and 48.7 mm, respectively. Hindlimb stride widths were not significantly different between mild and strong *shaker* mutants or between mutant and normal rats.

Forelimb stride widths were a reasonable discriminator between *shaker* and normal rats. Mutant rats demonstrated a narrower forelimb width after 42 PND (Fig. 7). At 49 PND, control rats had a significantly greater forelimb width of 36.5 mm as compared to 25.0 mm for *shaker* mutants. At 56, 63 and 70 PNDs, *shaker* mutant forelimb stride widths were 26–39% narrower than age-matched controls. This difference is unlikely due to weight or size differences between normal and mutant rats as size differences should first affect stride lengths before affecting stride widths [37]. Generally strong

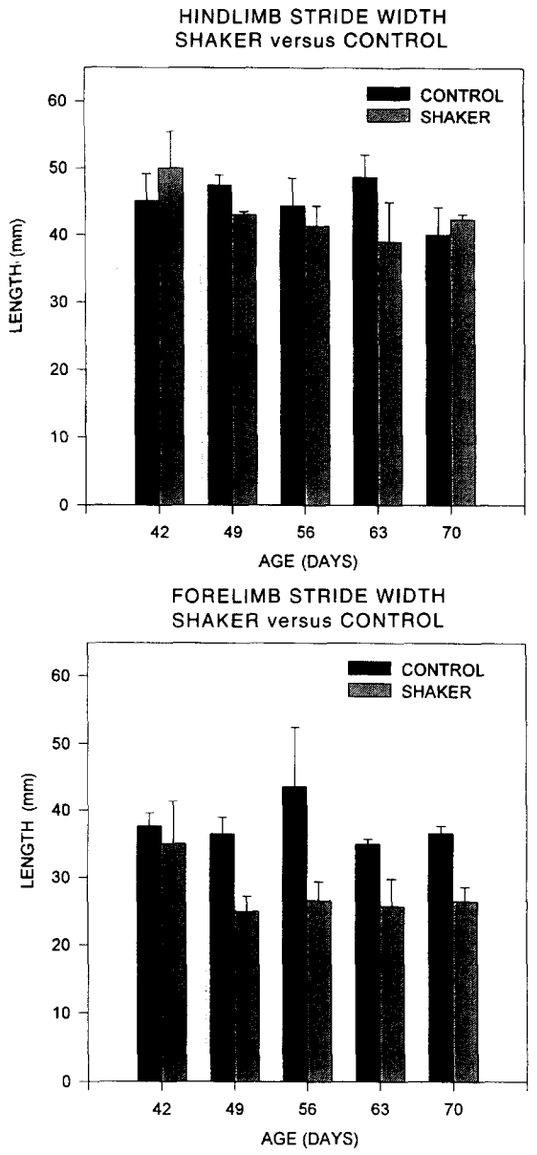

Fig. 7. Hindlimb stride width in *shaker* mutants was found to have an unusual pattern of wider stride at 42 days, and then a narrower stride at 49, 56 and 63 PND. This narrowing of hindlimb stride with age was not evident in control rats and is not typical of mouse Purkinje cell deficient mutants. A more detailed characterization of this gait abnormality is necessary to explain the motor abnormality causing this narrowing of hindlimb width. Forelimb stride width was consistently narrower in the *shaker* mutant rat compared to the control rat after PND 42. The reason for this narrower stride width in the forelimbs may be due to the *shaker* mutant rats tendency to hop while walking. (Student's *t*-test)

shaker mutants demonstrated similar forelimb stride widths comparable to mild *shaker* mutant rats.

3.6. Histopathologic evaluation

Sections stained with cresyl violet were evaluated for evidence of loss of Purkinje cells in the mutants and compared to similarly stained sections from normal rats. However, the sections processed for calbindin immunoreactivity greatly facilitated qualitative comparisons of cerebella from normal and *shaker* mutant rats in this study and for quantitative analysis in a companion study [48]. In normal rats calbindin immunostaining was pronounced in the molecular and Purkinje cell layers (Fig. 8a). Purkinje cells underlying the molecular layer were in a monolayer with uniform spacing between the calbindin immunoreactive somata in many areas. In sections of cerebella from mild *shaker* mutant rats, calbindin immunostaining of the molecular layer was extensive, but appeared interrupted in random areas of lobules I–IX (Fig. 8b). These numerous small gaps in calbindin immunoreactivity in the molecular layer were associated with the absence of underlying calbindin immunopositive Purkinje cell somata. Analysis of adjacent sections stained with cresyl violet confirmed the absence of Purkinje cells in these areas. Beneath the gaps of calbindin immunostaining in the molecular layer 2 or 3 Purkinje cells appeared to have degenerated. In strong *shaker* mutant rats, calbindin immunostained Purkinje cells in the anterior lobe cortex was almost completely absent (Fig. 8c). In the posterior lobe, there were numerous areas with wide gaps in immunostaining of the molecular and Purkinje cell layers. Calbindin immunostaining of the flocculonodular lobe (lobule X) appeared very similar to that seen in normal animals.

4. Discussion

Somatic development of *shaker* mutants was comparable to control animals as indicated by analogous times of onset for eyelid opening and ear flap opening. The rate of weight gain was significantly impaired in *shaker* mutant rats compared to age-matched normal rats. Female *shaker* mutant rats weighed an average 28% less than normal female rats and male *shaker* mutants consistently weighed about 24% less than normal male rats. In *pcd*, *nervous*, *lurcher*, *staggerer*, and *weaver mutants* Purkinje cell degeneration is concurrent with somatic abnormalities including smaller body size [15,19,29–32,34,35]. Poor weight gain in the *shaker* mutants is therefore a similar trait to that found in mouse mutants with hereditary Purkinje cell degeneration. The auditory startle response was another aspect of somatomotor development which proved to be similar in mutant and normal rats. Both groups produced the auditory startle response between 11–13 days of age. The response persisted indicating no loss of auditory-somatomotor integration and was not exaggerated as in some murine mutants such as the *hot-foot* [22].

shaker mutants demonstrated a slight delay in their development of integrated vestibular, somatosensory and neuromuscular systems interactions. This was demonstrated by prolonged times to surface right before PND


a
b
c

9. While this delay was evident, it was not possible to determine if this difference was due to a deficient vestibular or tactile system, or both. Additional testing is required to identify the nature of this deficit. One way to determine if vestibular righting is effected would be to place pups supine in a warm water bath, such as was done by Pellis [41].

shaker mutants did not demonstrate a significant difference in ability to land on all four feet while mid-air righting when compared to normal rats. This suggests that the dynamic vestibular response is not significantly affected in the *shaker* rat between the ages of 15 PND and 3 months of age. A lag in early somatosensory and proprioceptive development was characterized by a delay in the ability to forelimb place before PND 21, and exteroceptive ability was delayed as demonstrated by poor cliff avoidance ability at PNDs 10 and 11. *shaker* mutants did not simply trail in their ability to hindlimb place beginning with PND 12, but displayed a persistent impairment in their ability to perform this simple neuromuscular response. This disability was presumed to be correlated with progressive Purkinje cell degeneration in the mutants. Similar to the behavior of several mouse mutants including *pcd*, *nervous*, *lurcher*, *staggerer* and *weaver* [15,20,26–32,34,35], hindlimb weakness and impaired balance are common clinical signs when Purkinje cells are undergoing degeneration. Cerebellar deficits in rodents are often linked with hindlimb abnormalities. Lack of hindlimb coordination has been reported to be one of the most pronounced deficits with cerebellar growth retardation resulting from neonatal X-ray irradiation [47]. *Staggerer* and *hot-foot* mutant mice exhibit abducted hindlimbs and hold the toes of their hindlimbs at an outward angle, unlike normal mice [21,22].

Stride width, but not stride length, also proved to be a valuable parameter of locomotor behavior to distinguish mild and strong *shaker* mutant rats. Stride lengths varied inconsistently in *shaker* mutants, compared to normal rats, which likely resulted due to spontaneously ambulating rats varying their speed of progression down a narrow tunnel. After 42 PNDs, *shaker* mutant rats presented with toe-ed out hind feet and a narrower forelimb and hindlimb stride width than controls. This difference was not due merely to weight differences between groups, as hindlimb stride lengths were not similarly affected. This mild affect on gait has not been reported in mouse Purkinje cell mutants to date, nor was it evident in normal rats during maturation.

It was not surprising that a reduction in the number of Purkinje cells had only a slight affect on the *shaker* rats' gait. Mutants such as *pcd* mice have almost a total absence of Purkinje cells, yet gait is only mildly affected [33]. *Reeler* mutants, on the other hand, have a more severely affected gait. They sway from side to side when walking, demonstrate a significant foot tremor when each foot is lifted off the ground, and place their hind feet far apart when standing or walking [14]. *Reeler* mutants have a displacement of Purkinje cells, as opposed to Purkinje cell loss, and a considerable reduction of the granular layer [24]. A significant change in gait, therefore, does not seem dependent on a mere loss of Purkinje cells in these mutants, but from some other abnormality such as displacements of Purkinje cells, or a reduction in size of the granular cell layer. The reason for this abnormality in stride width in the *shaker* mutant can be explored further and characterized more quantitatively by kinematic analysis. Movement analysis may also identify more subtle aspects of *shaker* mutant hindlimb stride length undetectable by the simple, relatively unrefined method of gait analysis used in this study.

Hindlimb splay is a fairly coarse method of assessing neuromuscular function in the rat [17,26], and not a practical method in the mouse, but findings from this study prove that it is a simple and accurate test for cerebellar impairment in *shaker* mutants. Initially (14–35 PNDs) normal rats had a wider landing splay attributed to their greater body size, but at and after 42 PND, *shaker* mutants had a significantly greater landing footspread (Fig. 6). This first measurable clinical sign of ataxia in the *shaker* mutant we attribute to the degeneration of a critical number of Purkinje cells by in the sixth postnatal week of development. Strong *shaker* mutant rats were more ataxic than mild *shaker* mutants, as indicated by wider landing splay at and after PND 49 (Fig. 6). This suggests a significant ongoing degenerative process in the strong *shaker* mutants compared to a delayed or halted process of Purkinje cell death in the mild mutant. It is therefore possible, using the hindlimb splay test to distinguish strong *shaker* mutants from mutants with mild ataxia without tremor, and a predictor of progressive from restricted Purkinje cell degenera-

Fig. 8. Low power bright-field photomicrographs of sagittal sections through the midline cerebellum of normal rat (a), and mild (b) and strong *shaker* (c) mutants. The primary fissure separating the anterior and posterior lobes is indicated by the large arrows in each figure. In normal rats calbindin immunoreactivity in Purkinje cells and their processes resulted in a continuous and homogenous staining of the molecular and Purkinje cell layers. In mild *shaker* mutants random degeneration of Purkinje cells resulted in gaps of calbindin immunostaining in the molecular and Purkinje cell layers (small arrows). In strong *shaker* mutants almost all Purkinje cells in the anterior lobe have degenerated. Some of the surviving Purkinje cells are indicated by small arrows. In the posterior lobe moderate numbers of Purkinje cells have degenerated resulting in wide gaps in the calbindin immunostaining of the molecular layer (small arrows). Scale bar = 400 μm.

tion between the strong and mild mutants at 3 months of age.

Lalonde et al. have shown that *staggerer*, *weaver*, *pcd*, *nervous* and *lurcher* mutants, each with cerebellar deficits including Purkinje cell loss, show impairment in a variety of tasks which incorporate spatial learning or memory such as spontaneous alternation [31,33–35]. Spontaneous alternation is an instance of spatially guided behavior in which animals with cerebellar damage exhibit deficits. An investigation of the *shaker* rats' ability to spontaneously alternate is suggested for a future study.

Histopathologic findings suggest a strong association between the Purkinje cell degeneration and ataxia in mild and strong *shaker* mutants. Strong *shaker* mutant rats exhibited extensive degeneration of Purkinje cells in the anterior lobe compared to mild *shaker* mutants. Clinically, strong *shaker* mutants also demonstrated significant whole body tremor in addition to a wider hindlimb stance and difficulty in hindlimb placement as compared to the mild *shaker* mutant rats. This is analogous to mouse mutants with severe Purkinje cell loss which have tremor, a splayed hindlimb stance, and hindlimb weakness [15,19,20,29–32,34,35]. Although Purkinje cell loss was not numerically quantified in this study, severe and early loss of Purkinje cells in *shaker* mutant rats appears to be strongly associated with poor hindlimb motor activity.

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