A New Neurological Mutant Rat with Symmetrical Calcification of Purkinje Cells in Cerebellum (44419)

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Abstract. **A new neurological mutant has been found in the inbred F344 strain of rats. The mutation is inherited as an autosomal recessive trait and is manifest clinically by a hesitant and wobbling gait with asynergic limbs and slight tremor. These symptoms begin at 16–18 days of age and remain essentially constant thereafter. Histologic examination revealed severe degeneration of the Purkinje cells and symmetrical calcification in these and in their dendritic branches in the cerebellar cortex. Such calcified Purkinje cells were intensely stained with the periodic acid-Schiff (PAS) method. PAS-positive substances in the Purkinje cells and extending diffusely over the lesioned sites in the molecular layer were also evident before calcification took place. We have named this neurological mutant the Cerebellar Calcification (CC) rat with the gene symbol *cc*. This offers a new animal model for the study of the Purkinje cell degeneration and intracranial calcification.** [P.S.E.B.M. 1999, Vol 221]

Spinocerebellar ataxias in humans are usually inherited and associated with neuronal degeneration in specific areas of the nervous system in the brain stem, cerebellum, and/or spinal cord (1). These diseases are classified as three well-known types: cerebellar cortical atrophy, pontocerebellar atrophy, and Friedreich's ataxia (2). Cerebellar cortical atrophy is characterized by severe degeneration of the Purkinje cells (the central functional unit of the cerebellar cortex) and the inferior olivary nuclei. Other human diseases that are characterized by ataxic symptoms associated with intracranial calcification in the cerebral and cerebellar cortex and in the brain stem, are Cockayne's syndrome (3), Fahr's disease (4) and Sturge–Weber syndrome (5).

Previous studies have used numerous mutant mice and rats with neurological disorders as models for human diseases in various fields of research. These have provided valuable insights into the normal and pathological conditions of the central nervous system (6, 7). Selective degeneration of the Purkinje cells has been reported in nervous (*nr*) (8) and Purkinje cell degeneration (*pcd*) (9) mouse, shaker rat (10), and Gunn rat with hyperbilirubinemia (11). However, no pathological model for human hereditary ataxia with intracranial calcification is yet available. We report here a new neurological mutant rat characterized by wobbling gait, slight tremor, and severe Purkinje cell degeneration that progresses in parallel with marked calcification in the cerebellar cortex. In this mutant rat, periodic acid-Schiff (PAS)-positive substances coexisted with calcified deposits in the Purkinje cell bodies and their dendritic branches, and extended diffusely all over the lesioned sites. We have named this mutant the Cerebellar Calcification (CC) rat.

Materials and Methods

Rats. In 1992, one female and two male rats that showed wobbling gaits with a slight tremor appeared in F58 generations in the inbred F344 strain maintained in the

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Laboratory Animal Science and Toxicology Laboratories of Sankyo Co., Ltd., Japan. Rats with the same clinical signs also appeared in successive generations achieved by brother-sister mating. Since this trait was always accompanied by a marked cerebellar calcification in the brain, this mutant was tentatively called CC rat. Reproductivity of the affected males was very low, whereas the affected females were fertile. For this reason CC rats were produced in many cases by brother-sister mating between the affected females and the normal males. These rats were housed in plastic cages and given free access to water and the commercial diet CMF (Oriental Yeast Co., Ltd.). They were maintained in an animal room under a barrier system conditioned to a temperature of 23 ± 1°C, a relative humidity of 55 ± 10%, and a 14-hr illumination. The animals were cared for and used according to humane standards and in compliance with the law and related standards for animal welfare.

Pathological Examination. In this study, 36 clinically affected and 32 normal rats between 3 and 30 weeks of age were used for the pathological examination. Some rats were sacrificed by exsanguination from the carotid artery under deep diethyl ether anesthesia. Their brains were removed immediately and fixed in a 10% neutralized buffered formalin solution. Others were sacrificed by transcardiac perfusion of a phosphate buffered solution containing 4% paraformaldehyde under deep anesthesia with chloral hydrate. According to the Atlas of the Rat Brain and Spinal Cord (12), we made coronal sections of the brain at plates 15, 32, 39, 47 and 72, and of the cervical (C1–2; 114), thoracic (T8; 116), and lumbar (L5; 117) cord segments. We also prepared sagittal sections of the cerebellum at plate 77.

Blocks of the brains were embedded in paraffin. Sections of 6 μm thick were stained with hematoxylin and eosin (HE) for general pathological observations, with periodic acid-Schiff (PAS) to detect mucopolysaccharides (glycogen and mucin), and with von Kossa's method to detect calcified deposition. The total number of Purkinje cells, excluding those in which the nucleus was not visible, was counted in each HE section. Glycogen was confirmed by loss of stainable PAS-positive substances after diastase digestion. After perfusion-fixation with 4% paraformaldehyde, we viewed calcification, especially in the cerebellum with the digital micro-X-radiography system (Fuji Photo Film Co., Ltd., Tokyo).

Genetic Analysis. Reciprocal mating experiments were made between the affected rats and the control normal F344 rats. Some of the F1 offspring were intercrossed to check the F2 segregation ratio. The rest of them were backcrossed to affected and control rats to determine the N2 segregation ratio.

Results

Clinical Features. The abnormality of CC rats was obvious as early as 16–18 days after birth. They were characterized by a hesitant and wobbling gait with asynergic limbs, a slight tremor, and, in rare instances, a tendency to fall sideways. Once the clinical signs were manifested, they remained essentially constant during the rest of the animal's life. Body weights of affected rats were usually slightly lower than those of normal littermates in the earliest stages, when the aberrant behavior was subtle (Table I). They then declined in both sexes to ≈ 60% of normal weight at 5 weeks of age, and to ≈ 70–80% of normal weight at 10–40 weeks of age. Sporadic deaths occurred in 15% (6/20) of CC males and 65% (13/20) of CC females between 30 and 70 weeks of age without any premonitoring signs.

Pathological Findings. A dorsal view of mutant brains at 3 weeks of age appeared to be normal. However, in 10-week-old affected rats, the cerebellum appeared slightly smaller compared to normal rats (Fig. 1), coinciding with a reduction in cerebellar weight (Fig. 2).

One of the pathological abnormalities in the brain of the affected rats was a rapid loss of Purkinje cells in the cerebellum shortly after birth. The number of morphologically normal Purkinje cells in a midsagittal section of the affected cerebellum was markedly reduced compared to normal rats as early as 3 weeks of age, and numbers continued to decrease with advancing age (Table II). Although a reduction in the number of Purkinje cells was noted equally in most cerebellar lobes, we observed an exceptional case in lobule X, where the Purkinje cells maintained normal number and structure during the course of the experiment. Although we found a slight thinning of the molecular layer in the atrophied cerebellum, the constitution of the layers in the cerebellar cortex, including the molecular layer, the slightly curvilinearly arranged Purkinje cells, and the granule cell layer, appeared normal until the relatively late stages.

With HE staining, the molecular layer was slightly thin

Table I. Body Weight in Normal and CC Rats

Age (weeks)	No. of animals	Normal		Affected	
		Male	Female	Male	Female
3	10	30.4 ± 2.5	28.2 ± 1.9	23.4 ± 1.5[a]	21.3 ± 1.1[a]
5	20	98.9 ± 6.5	87.0 ± 5.4	60.6 ± 8.7[a]	53.9 ± 7.1[a]
10	20	262.7 ± 10.6	162.5 ± 6.4	195.0 ± 14.2[a]	129.2 ± 6.0[a]
20	20	372.4 ± 13.5	208.0 ± 7.5	270.0 ± 18.3[a]	154.9 ± 7.7[a]
40	20	449.1 ± 18.8	245.6 ± 11.1	309.9 ± 23.1[a]	172.2 ± 6.9[a]

Note. All results were presented as mean ± SD.
[a] Statistically significant difference between normal and affected rats ($P < 0.01$) by Student's t test.

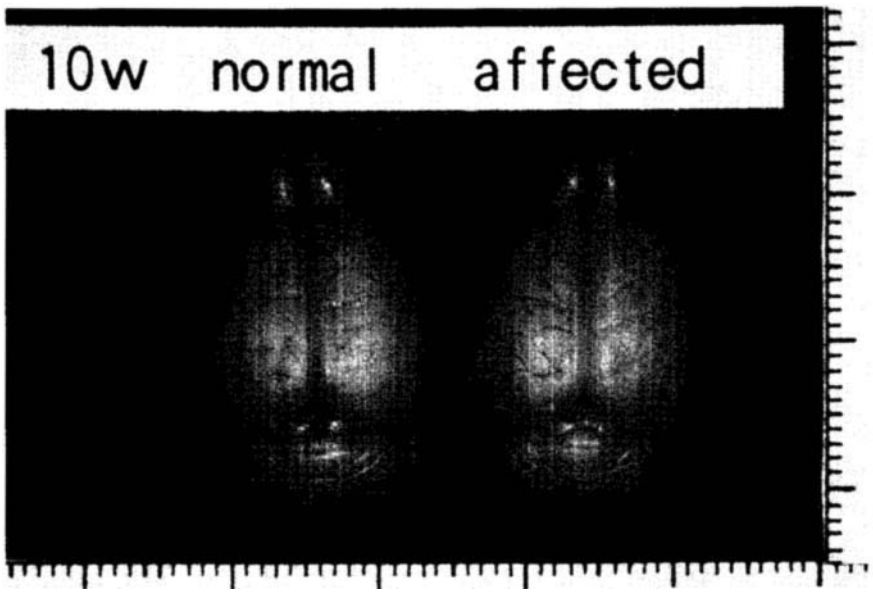

Figure 1. Dorsal views of brains from a 10-week-old normal (left) and an age-matched affected CC rat (right). The brains were fixed in a 10% neutralized buffered formalin solution. The affected cerebellum was slightly smaller than the normal one.

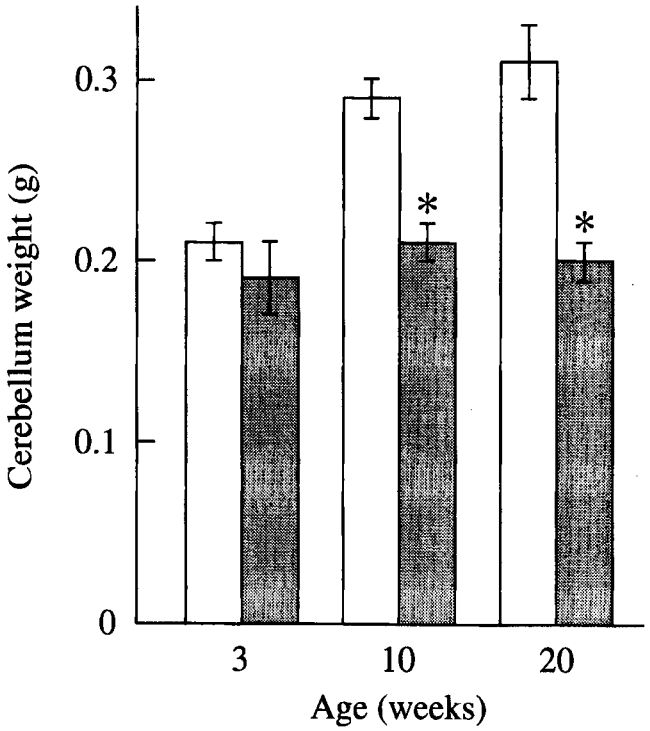

Figure 2. Cerebellum weight in CC rats. Open and solid columns indicate the normal and affected rats, respectively. Weight of cerebellum fixed in 10% neutralized buffered formalin solution was measured. All results are presented as mean ± SD of four males. The asterisk indicates a statistically significant difference between normal and affected rats ($P < 0.01$) by Student's t test. The cerebellum weight of the affected rats remained nearly constant after 3 weeks of age.

and spongy, and it appeared to be distorted near the Purkinje cell layer (Fig. 3B). Most of the Purkinje cells had disappeared, and the remaining cells were shrunken or hydropic, with an obscure nucleus. The lesioned sites in the cerebellum appeared dark blue in HE sections. The tissue appeared

Table II. The Number of Cerebellar Purkinje Cells in CC Rats

Age (weeks)	No. of Purkinje cells[a]	
	Normal	Affected
3	868 ± 98	188 ± 21[b]
5	1026 ± 76	41 ± 12[b]
10	893 ± 78	16 ± 3[b]
30	955 ± 74	15 ± 1[b]

Note. All results were presented as mean ± SEM of four rats.
[a] The number of Purkinje cells counted on a midsagittal section stained with hematoxylin and eosin per rat.
[b] Statistically significant difference between normal and affected rats ($P < 0.01$) by Student's t test.

fractured in some areas because the mineralization had made the tissue brittle, and artifactual tearing during sectioning occurred (Figs. 3B, 3D, 3F, 3G, and 3H). The granule cell layer appeared relatively normal in thickness and cellularity. PAS-positive substances were detected in the cell bodies of the Purkinje cells in the initial stages of degeneration. In the molecular layer, these appeared as collections of tiny granules, and as amorphous rounded and club-shaped bodies, and had an extensively diffused bushy appearance (Fig. 3D). Furthermore, we detected a dot-like calcified deposition with von Kossa's staining, initially in the Purkinje cell bodies (Fig. 3F), expanding subsequently to their dendritic branches (Fig. 3G). Calcification appeared to overlie the PAS-positive areas that we detected earlier than in those of the calcified deposition. These pathological changes were already conspicuous at 18 days of age in the posterior lobules of cerebellum in a rather patchy pattern, and they spread rapidly over other lobules except for the lobule X. Lobules VII and VIII were more severely affected than the other lobules. With the progression of the disease, calcified deposits formed many large aggregates, leading to

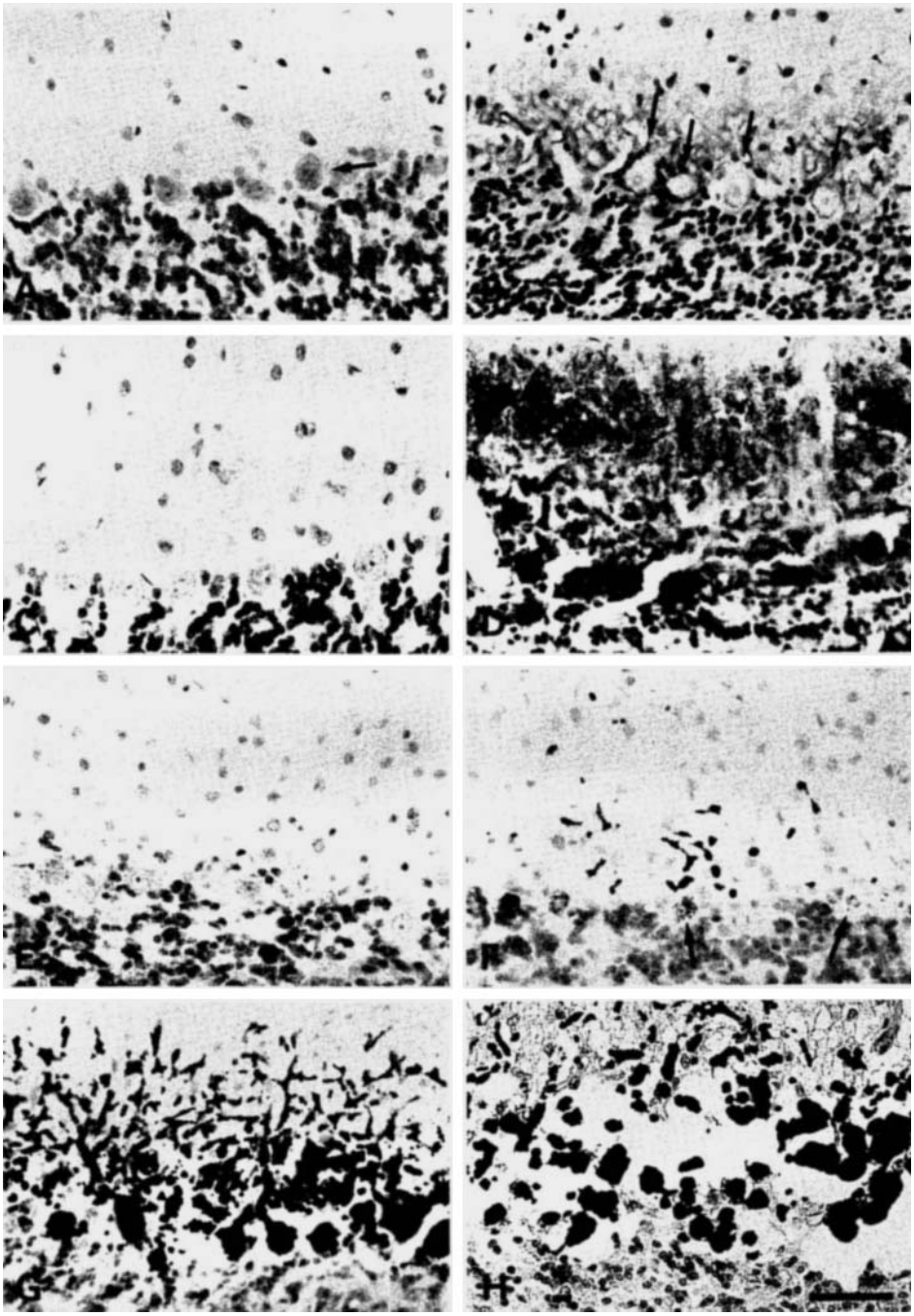

Figure 3. Sagittal sections of the cerebellar cortex in CC rats. In each micrograph, the molecular layer is at the top, the Purkinje cell layer in the middle (arrow in **A**), and the granule cell layer at the bottom. (**A, B**) The sections were stained with hematoxylin and eosin. **A** is the cerebellum from an 8-week-old normal rat. **B** is the affected cerebellum from an age-matched CC rat, showing that many Purkinje cells had degenerated, while others were in the process of degenerating (arrows). The molecular layer is slightly spongy. (**C, D**) The sections were stained with periodic acid-Schiff (PAS). **C** is from an 8-week-old normal rat. In an age-matched affected rat **(D)**, PAS-positive reactions were detected in degenerating Purkinje cells, and spottily and diffusely all over the molecular layer. (**E, F, G, H**) The sections were stained with von Kossa's methods. **E** is from an 8-week-old normal rat. In an age-matched affected rat (**F**), dot-like calcification was recognized initially in the Purkinje cell bodies (arrows). The calcification was detected in the dendrites derived from other Purkinje cells, but not in the dendrites near the Purkinje cells with dot-like calcification. In a more advanced site (**G**), the calcification expanded to the dendritic processes of the Purkinje cells. In the terminal stage (30-week-old) (**H**), the calcified deposits aggregated in the spongy formed regions in the molecular and Purkinje cell layers. The scale bar in H represents 50 μm.

a loss and thickening of the dendritic branches of the Purkinje cells, which caused thinning of the molecular layer (Fig. 3H).

X-ray radiographs from the dorsal side of the cerebellum showed bilateral symmetrical distribution of the calcified deposits, which lay linearly or spottily along the lobules, as shown in Figure 4B. In the posterior views, spotty deposits appeared symmetrically in the vermis, and were

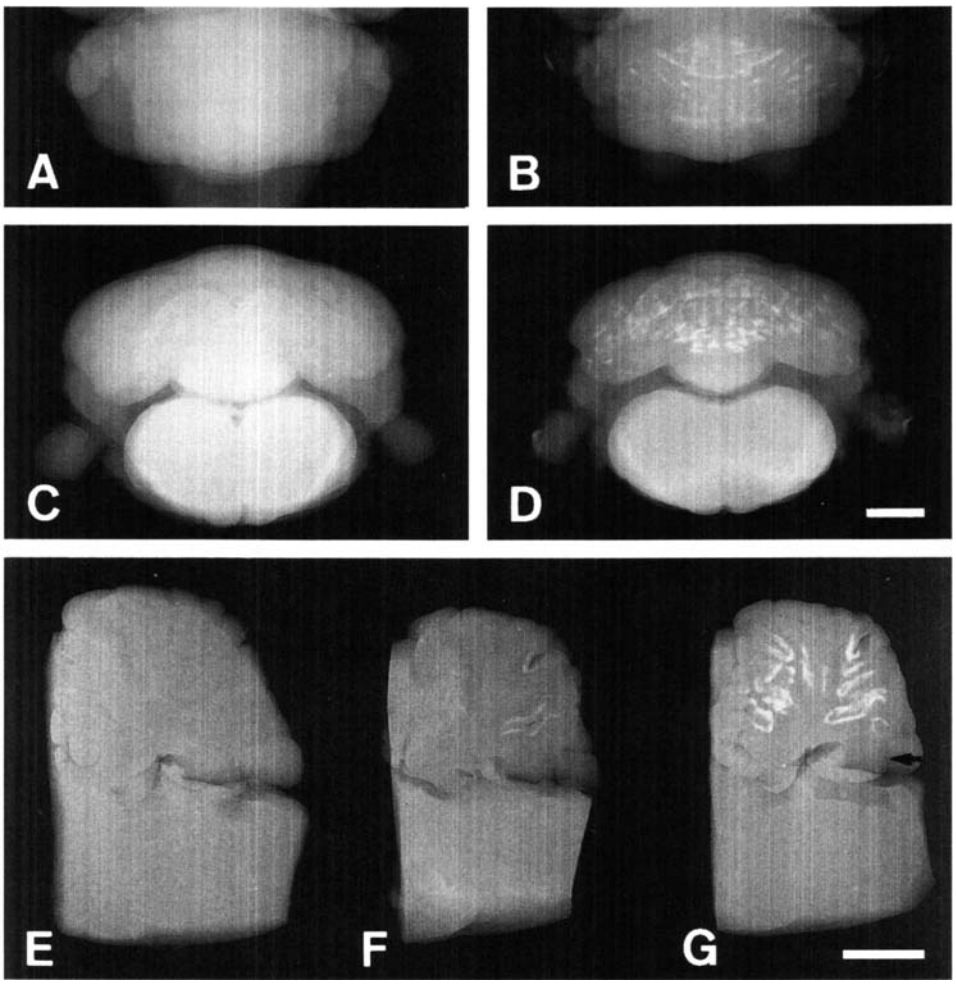

Figure 4. X-ray radiographs of the cerebellum in CC rats. In each radiograph, after exposure of the whole cerebellum with a part of the medulla to an X-ray of 40 kV, 100 mA for 4 sec, the digital images were visualized by the Digital Microradiography (DMR) System (Fuji Photo Film Co., Ltd.). The brains were fixed by a phosphate buffered solution with 4% paraformaldehyde. (**A, B**) The X-ray was exposed from the dorsal side. **A** is from the 19-week-old normal rat. In an age-matched affected rat (**B**), calcified deposits can be detected linearly parallel to or spottily along the lobules, and the distribution is symmetrical to the midsagittal plane. (**C, D**) The X-ray was exposed from the posterior side of the cerebellum. In both radiographs, the cerebellum is in the upper and the medulla in the lower. **C** is from a 19-week-old normal rat. In an age-matched CC rat (**D**), we have recorded spotty symmetrical calcification in the whole area corresponding to the cerebellar cortex, and focal calcification in the deep area of the vermis. (**E, F, G**) The X-ray was exposed from the left side of the midsagittal section (2-mm thickness) of the cerebellum. In each radiograph, the cerebellum is in the upper and the medulla in the lower. **E** is from a 19-week-old normal rat. In a 7-week-old affected CC rat (**F**), we found a slight deposition, especially in the sulci of the posterior lobules. In a 19-week-old affected CC rat (**G**), focal calcification appeared in the sulci between all lobules except for the lobule X (arrow). The scale bars represent 2 mm.

more obvious than in the hemisphere. We found these in both the shallow and deep regions in the vermis, but only in the shallow region of the hemisphere (Fig. 4D). We began to detect a slight calcification in the vermis in the sulci between lobules VI and VII, and between lobules VIII and IX, at 7 weeks of age (Fig. 4F). At 19 weeks of age, they appeared to be deposited focally near the bottom of the sulci between lobules, but not near the tip of each lobule (Fig. 4G). We never observed calcification in the normal cerebellum (Figs. 4A, 4C, and 4E) and the lobule X (arrow in Fig. 4G).

We detected no pathological changes, such as calcification and demyelination, in the cerebellar white matter and in the deep cerebellar nuclei. Nor did we find abnormalities in other main tissues, such as the liver and kidney. General biochemical tests on blood samples showed nearly normal levels in the affected rats, except for the decrease of triglyceride (data not shown).

Inheritance. In Figure 5 we show the pedigree of CC rat. The appearance pattern of the affected animals is consistent with an autosomal recessive inheritance. In Table III,

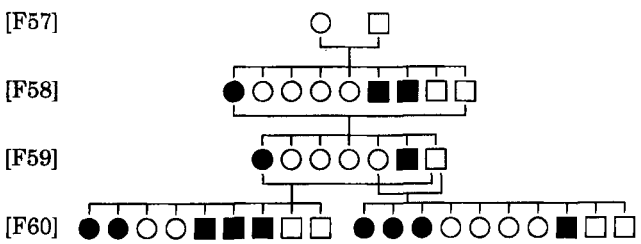

Figure 5. The pedigree of CC rat. Open squares and circles indicate normal males and females, and solid squares and circles indicate affected males and females, respectively.

Table III. Results of Test Crosses Between CC and F344 Rat

Mating		No. of progeny	Phenotypes of progeny		Expected segregation ratio	χ^2
Dam	Sire		Normal	Affected		
Normal[a] (+/+)	affected[b] (*cc/cc*)	13	13	0	1:0	
Affected (*cc/cc*)	normal (+/+)	41	41	0	1:0	
Carrier[c] (*cc/+*)	carrier (*cc/+*)	78	57	21	3:1	0.154 $0.50 < P < 0.70$
Carrier (*cc/+*)	normal (+/+)	51	51	0	1:0	
Affected (*cc/cc*)	carrier (*cc/+*)	56	30	26	1:1	0.286 $0.50 < P < 0.70$

[a] F344 used.
[b] CC used.
[c] F1 between F344 and CC used.

we showed the results of test crosses between CC and F344 rats. None of the F1 offspring were affected. The segregation ratios of F2 and N2 offspring agreed with our expected ratios of 3:1 and 1:1, respectively. These results confirmed that a single autosomal recessive gene controlled the trait. We have named this mutation the Cerebellar Calcification (CC), with the gene symbol *cc*, based on a unique pathological change in the affected cerebellum.

Discussion

Our pathological and genetic studies on the CC rats have shown that an autosomal recessive gene, *cc*, causes cerebellar hypoplasia, thinning of the molecular layer, due to a rapid degeneration of the Purkinje cells, and a symmetrical calcification in the cerebellum. Rodents and human diseases associated with the phenotypes of CC rats are shown in Table IV.

It is known that two neurological mutant mice, nervous (*nr*) and Purkinje cell degeneration (*pcd*), inherit in an autosomal recessive mode and exhibit a severe loss of the Purkinje cells (8, 9). In *nr* mice, Purkinje cell death occurs rapidly after birth, with only 10% of the Purkinje cells surviving to the second postnatal month, and is observed more extensively in the hemispheres than in the vermis (8). These abnormalities seem to be followed by partially degenerating the cells of the external granular layer at 6–8 days of age, which associate to maturate Purkinje cells (13). In *pcd* mice, degeneration of the Purkinje cells occurs at ≈ 15–18 days of age and progresses rapidly over the next 2 weeks in any folium except for the most ventral aspects of the cerebellum (9). In fusion chimeras between *pcd/pcd* and +/+ embryos, it was shown that the *pcd* gene acts directly on the Purkinje cells (14). Homozygotes reveal a moderate ataxia beginning at 3–4 weeks of age.

The CC rat was characterized by a rapid degeneration of most of the Purkinje cells at approximately 2–3 weeks of age in any folium except for lobule X in the vermis, and by a moderate ataxic behavior appeared at the same age. Therefore, the situation of the Purkinje cell degeneration in CC rats is similar to that in *pcd* mice. The major clinical sign of

Table IV. Rodents and Human Diseases Associated to the Phenotype of CC Rats

Species		Rat			Mouse		Human		
Diseases		Cerebellar calcification (*cc*)	Shaker	Jaundice	Nervous (*nr*)	Purkinje cell degeneration (*pcd*)	Cockayne's syndrome	Fahr's disease	Sturge-Weber syndrome
Inheritance		Autosomal recessive	Hereditary	Autosomal recessive	Autosomal recessive	Autosomal recessive	Hereditary	Autosomal recessive	—
Principal clinical signs		Dwarfism, ataxia	Dwarfism, ataxia, tremor	Dwarfism, ataxia, hyperbilirubinemia	Dwarfism, ataxia	Dwarfism, ataxia	Microcephaly, dwarfism, retinal pigmentation, cataract, deafness, mental deficiency	Convulsions, dementia, speech difficulties, mental retardation	Facial naevus, convulsions, mental retardation, angioma
Age of onset		16–18 days	By 3 months	2–3 weeks	2–3 weeks	3–4 weeks	Childhood	Adulthood	Childhood
Cerebellum	Purkinje cell loss	○	○	○	○	○	○	×	×
	Calcification/ PAS positivity	○	×	×	×	×	○	○	○
Other affected areas		—	Brain stem	—	Retina	Retina, olfactory bulb, sperm	Cerebrum, brain stem, spinal cord, retina	Cerebrum	Cerebrum

CC rats is a moderate ataxia of gait throughout life as observed in *pcd* mice, although it appears earlier in CC rats than in *pcd* mice. Despite the similarity in pathological changes in the cerebellum between *pcd* mice and CC rats, *pcd* mice are distinct from CC rats in that no calcification was observed in the Purkinje cells and in their dendrites in the molecular layer in *pcd* mice. Furthermore, PAS-positive substances, which formed prior to calcification in CC rats, were not observed in *pcd* mice. The hereditary Purkinje cell degeneration and ataxia were also reported in *shaker* (10) and jaundiced (*Gunn*) rat (11). However, those mutant rats are distinct from CC rats in that the former is characterized by the adult-onset (by 3 months of age) (15), and the latter is caused by hyperbilirubinemia due to the deficiency of bilirubin-UDP-glucuronosyltransferase (16).

Up to the present, no studies have found substantial calcification in the central nervous system in rodents with spontaneous mutations that cause cerebellar ataxia. However, in humans calcification in the brain has been reported in patients who suffer from encephalopathy with ataxia, infectious diseases such as congenital toxoplasmosis, and endocrine disorders such as hyperparathyroidism (17, 18). It has also appeared in Cockayne's syndrome (3, 19), Fahr's disease (4, 20) and Sturge–Weber syndrome (5, 21). Calcification in CC rats was not caused by infectious disease, since the disease was genetically controlled, and the rats were maintained under specific pathogen-free conditions. Endocrine disorders like hyperparathyroidism are not related to calcification in CC rats, since they display normal levels of blood calcium concentration. Neuropathological findings in Cockayne's syndrome are similar to the cerebellar calcification in CC rats in that there is a reduction in the number of the Purkinje cells, a thinning of the molecular layer, and calcification in the branching dendrites and the body of the Purkinje cells in the cerebellum, although the calcification is observed all over the intracranial region (3, 19). Although Fahr's disease (4, 20) and Sturge–Weber syndrome (5, 21) are not identical to the case of CC rats, histochemical studies of calcification in humans indicate that the calcification sites were composed of a mixture of polysaccharides, glycoproteins, mucopolysaccharides, calcium salts, and some metals, and were also positive with PAS and von Kossa's reaction. Those results correlate well with our histochemical findings in the cerebellum of CC rats.

Furthermore, pathological phenotypes similar to CC rats were shown in experimental studies on rats by intracerebral injection of alum phosphate or by administration of lead carbonate *via* maternal milk. The rats treated in this manner could develop the spongy degeneration of the molecular layer, the hydropic degeneration of the Purkinje cell bodies, and calcification at the dendrites and cell bodies of the Purkinje cells (22). It was suggested that abnormal vessel permeability into tissues from blood vessels might play some role in the appearance of the disintegration and calcification of the Purkinje cells. Taken together, we find it likely that the degeneration and calcification in the cerebellar tissues in CC rats may be related to impairment in the blood vessels or in their pericytes, or to abnormal vessel permeability. We would expect also that extensive PAS-positive reactions in the molecular layer, succeeding accumulation of calcified deposits with advancing age, and symmetrical distribution of calcification sites of the cerebellar cortex in CC rats may be associated with abnormalities in the vascular system in the cerebellum.

Further studies are needed to clarify the pathogenesis of the Purkinje cell degeneration and calcification in the cerebellum of CC rats, and to relate these pathological changes in CC rats to human diseases. We are now conducting gene mapping in our laboratory to locate the *cc* gene that controls the inherited cerebellar degeneration in rats.

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