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Original Article

Cerebellar histogenesis as seen in identified cells of normal-reeler mouse chimeras

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ABSTRACT The potential contribution of cell-cell interactions and extracellular factors to cytoarchitectonic abnormalities in the cerebellum of the reeler mutant mouse was investigated by forming chimeras between the reeler and normal animals. The strain origin of Purkinje cells, granule cells and Golgi epithelial cells was immunohistologically identified with a strain-specific antibody. We analyzed 16 overt coat color chimeras, 10 reeler ↔ C3H and 6 reeler ↔ Balb/c. Abnormal behavioral traits of reeler were rescued in all chimeras. However, cerebellar histology was more affected in reeler ↔ C3H chimeras than in reeler ↔ Balb/c. Purkinje cells from the normal genotype occupy ectopic positions, and reeler genotype cells are arranged appropriately in the same chimeric cerebellum. We also obtained histologically normal chimeras with a significantly high contribution of the reeler genotype in Purkinje cells, Golgi epithelial cells and granule cells. These results clearly indicate that the abnormal cell positioning and cytoarchitecture of neurons and glia in the reeler is caused by a deficiency of extracellular environments, but is not determined cell-autonomously. The present data on chimeric mice suggest that Reelin is one of the important extracellular environmental factors that affects indirectly radial glial cells during cerebellar histogenesis.

KEY WORDS: *reeler, mutant, chimera, C3H strain-specific antigen, cerebellar cortex*

Introduction

Reeler is an autosomal recessive mutation located on chromosome 5 in mice (Falconer, 1951,1952), and it is characterized by abnormal cell positioning mainly in the forebrain and cerebellar cortices (Caviness and Rakic, 1978). In the reeler cerebellum, there are widespread abnormalities in the position and shape of Purkinje cells, granule cells and deep-cerebellar nuclei neurons (Mariani *et al.*, 1977; Goffinet, 1984; Inoue *et al.*, 1990). In addition to these neuronal abnormalities, astroglial cells and their associated molecules have also been reported to be affected in the developing cerebral and cerebellar cortices of the mutant (Ghandour *et al.*, 1981; Benjelloun-Touimi *et al.*, 1985; Terashima *et al.*, 1985; O'Brien *et al.*, 1987; Steindler *et al.*, 1990).

Chronological analyses during development (Mikoshiba *et al.*, 1980a,b), and autoradiographic studies of reeler mice (Caviness, 1982), have revealed anomalies in their inside-out pattern of cortical histogenesis that lead to inverted cells and layers. These observations suggest that the migration of young neurons is affected by the reeler mutation. It is generally accepted that young neurons generated in the ventricular zone migrate on radial glial fibers as guides to the final cortical position (Rakic, 1988). There is growing evidence that the reeler gene either directly or indirectly disturbs cell-cell interactions (Pinto-Lord *et al.*, 1982; Mikoshiba *et al.*, 1983; Goffinet, 1984; Juasa *et al.*, 1993) during migration of young neurons.

Recently, the reeler gene, *reelin* has been isolated, and its expression has been demonstrated by *in situ* hybridization (D'Arcangelo *et al.*, 1995; Hirotsune *et al.*, 1995). The sequence analyses have revealed that the protein encoded by the candidate gene relates to extracellular matrix proteins (D'Arcangelo *et al.*, 1995; Hirotsune *et al.*, 1995). At the same time, a unique monoclonal antibody, CR-50, has been generated by immunizing reeler mice with fetal brain homogenate from wild type mice (Ogawa *et al.*, 1995). This CR-50 reacted specifically with Cajal-Retzius neurons, and has been proved to recognize the reelin gene product, *Reelin* (D'Arcangelo *et al.*, 1997). In the developing cerebellum, both reeler mRNA and CR-50 antigen were detected specifically in the pre- and post-migratory granule cells (D'Arcangelo *et al.*, 1995; Ogawa *et al.*, 1995). Immunocytological analyses further revealed

Abbreviations used in this paper: CSA, C3H strain-specific antigen; IP3, inositol 1, 4, 5-triphosphate; GFAP, glial fibrillary acidic protein.

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that the extracellular *Reelin* in the developing cerebellum was deposited only in the external granular and molecular layers, and concentrated especially at the interface between granule cells and Purkinje cells (Miyata *et al.*, 1996), suggesting that *Reelin* plays an important role in the positioning and maturation of Purkinje cells. Moreover, Del Rio *et al.* (1997) showed by an *in vitro* experiment that Cajal-Retzius neurons and Reelin are essential for the formation of layer-specific axonal connections between entorhinal cortex and hippocampus. In spite of these molecular and cellular data, the role of Reelin in neuronal cell migration and neuron-radial glia interactions is still unclear.

Chimeric mice are unique experimental tools to analyze the effect of cellular environments on the developmental fate of component cells (McLaren 1986). Terashima *et al.* (1986) demonstrated that cerebellar malformations in reeler were counterbalanced by forming chimeras with normal mice, but they were not able to histologically demonstrate chimerism. Thus, it has remained necessary to precisely identify genetically mutant cells that are able to differentiate into every neuronal and glial cell types in the normalized chimeric cerebellum. This study was undertaken to elucidate the potential contribution of cell-cell interactions and extracellular factors to cytoarchitectonic abnormalities in the cerebellum of the reeler mutant mouse. We have produced chimeric mice between the mutant and normal mice, and applied a strain-specific antibody (Kusakabe *et al.*, 1988; Yoshiki *et al.*, 1993) to identify the strain origin of neurons and glia in chimeric cerebella. Interestingly, significant numbers of Purkinje cells, Golgi epithelial cells and granule cells derived from reeler genotype were appropriately positioned in chimeras. Besides, a number of Purkinje cells of both normal and mutant genotypes stayed in the deep cerebellar region, and seemed unable to migrate to their appropriate position. These findings are discussed in relation to how the reelin gene might act during cerebellar histogenesis.

Results

We have produced sixteen overt coat color chimeras, ten *rl/rl* ↔C3H and six *rl/rl* ↔ Balb. All of these chimeras, with different coat color ratios, were normal in their locomotion. This indicates that ataxia, tremor and dystonia, which are characteristic abnormalities in behavior of the reeler mutant, can be rescued by forming chimeras with normal mice.

To detect strain origin of cells in the tissue, we used anti-C3H strain-specific antigen (CSA) antibody. The anti-CSA antibody can recognize one of the strain variants, hsc 74^a of C3H, but does not react with hsc74^b of C57BL/6 and Balb (Dvorak *et al.*, 1995). As homozygous reeler mice *(rl/rl)* used in this study were heterozygous for the hsc74, three genotypes, *hsc74^a/hsc74^a*, *hsc74^a/hsc74^b* and *hsc74^b/hsc74^b*, segregated among *rl/rl* embryos. Therefore, six possible genotypic combinations in chimeric mice are generated as listed in Table 1.

Three cases out of six can be analyzed by the anti-CSA antibody for chimerism: 1) When the reeler embryos of *hsc74^b/hsc74^b* are aggregated with C3H, only cells from C3H are positive for anti-CSA antibody (Table 1, No. 3). 2) When the reeler embryos of *hsc74^a/hsc74^a* or *hsc74^a/hsc74^b* are aggregated with Balb, only cells from the reeler mutant are positive for the anti-CSA antibody (Table 1, No. 4 and 5). The sixteen coat color chimeras were examined to determine whether the strain origin of cells could be distinguished by the anti-CSA Mab before performing immunohistological analyses of chimerism. It was found from culture of tail fibroblasts that one *rl/rl* ↔ C3H and six *rl/rl* ↔ Balb chimeras consisted of both CSA-positive and negative cells. These chimeras were analyzed by using anti-IP3 receptor, anti-GFAP and anti-CSA antibodies. The other chimeras were only analyzed with anti-IP3 receptor and anti-GFAP antibodies to detect the morphology and distribution of Purkinje cells and astroglial cells. Estimation of chimerism in coat color and Purkinje cells, cerebellar foliation pattern and existence of ectopic Purkinje cells in the chimeras are summarized in Table 2.

Immunohistological analyses of the cellular origin of Purkinje cells and astroglial cells was performed by double-immunostaining in the chimeras appropriate for CSA analysis. In the No. 3412 *rl/rl* ↔ C3H chimera at P11, the cerebellum had more distinct cerebellar layers in comparison to the mutant, but less developed foliation. In this chimera, the presumptive lobules VI and VII were not

TABLE 1

GENOTYPIC COMBINATION OF REELER — NORMAL CHIMERAS

	Component strain		Immunohistological staining pattern with anti-CSA ab	
	reeler	normal counterpart	reeler	normal counterpart
1	B6C3Fe-*rl/rl*, *hsc74^a/hsc74^a*	C3H-*hsc74^a/hsc74^a*	positive	positive
2	B6C3Fe-*rl/rl*, *hsc74^a/hsc74^b*	C3H-*hsc74^a/hsc74^a*	positive	positive
3	**B6C3Fe-*rl/rl*, *hsc74^b/hsc74^b***	**C3H-*hsc74^a/hsc74^a***	**negative**	**positive**
4	**B6C3Fe-*rl/rl*, *hsc74^a/hsc74^a***	**Balb-*hsc74^b/hsc74^b***	**positive**	**negative**
5	**B6C3Fe-*rl/rl*, *hsc74^a/hsc74^b***	**Balb-*hsc74^b/hsc74^b***	**positive**	**negative**
6	B6C3Fe-*rl/rl*, *hsc74^a/hsc74^b*	Balb-*hsc74^b/hsc74^b*	negative	negative

Bold letters indicate genotypic combination in which strain origin of component cells can be distinguished by anti-CSA antibody. In no. 3, cells derived from *rl/rl* are negative for anti-CSA and cells from C3H are positively detected. In no. 4 and 5, cells from rl/rl can be detected as anti-CSA positive cells, in contrast to negative cells from Balb.

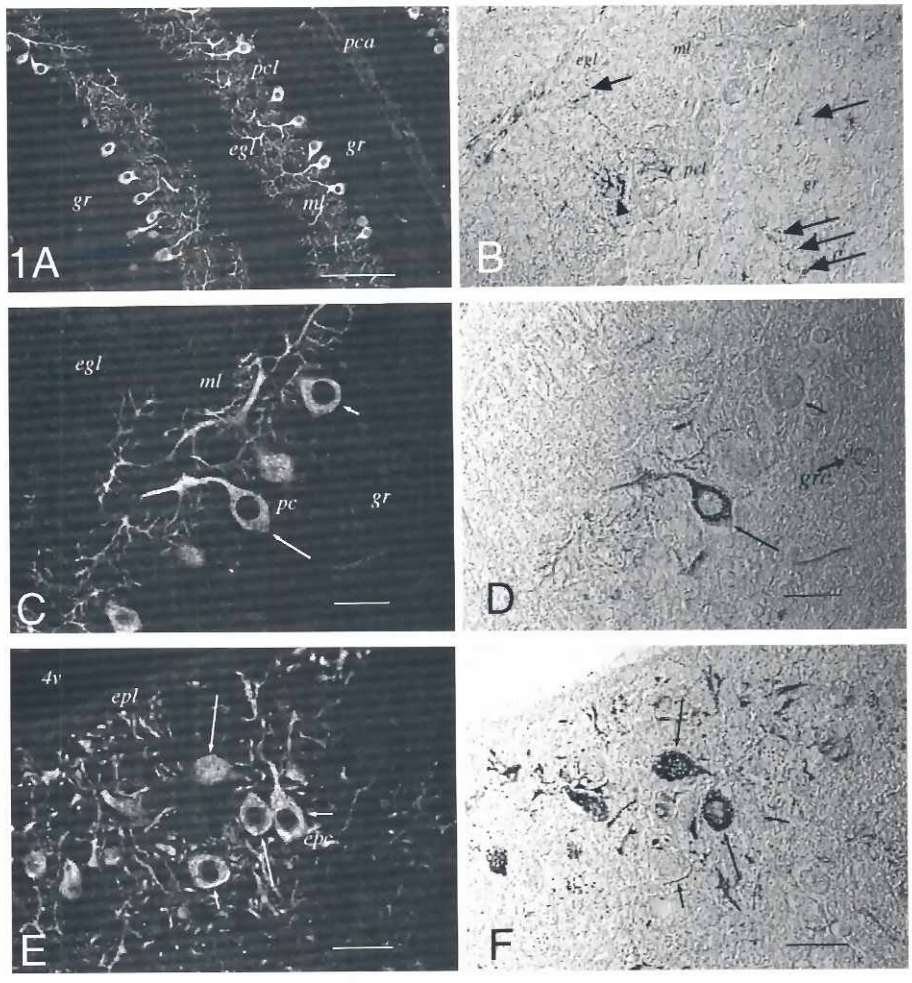

Fig. 1. Immunohistological analysis on the cerebellum of No. 3412 rl/*rl* ↔ C3H chimera. **(A)** *Indirect immunofluorescent staining with anti-IP_3 receptor protein. Most Purkinje cells were arranged regularly at the interface of molecular and granule cell layers. Bar, 100 μm.* **(B)** *Cerebellar cortex immunostained with anti-CSA. CSA-positive and negative cells are derived from normal and mutant strains, respectively in this chimera. Purkinje cells were all anti-CSA-negative (white * mark). One external granule cell (short arrow), several inner granule cells (long arrows) and a few Golgi epithelial cells were derived from the normal C3H in this region. Bar, 50 μm.* **(C-D and E-F)** *Double-immunolabeling by anti-IP_3 receptor protein and anti-CSA Mab. Large and small arrows in C-D and E-F indicate the same Purkinje cells derived from the normal genotype C3H and the mutant, respectively. (C-D) Only a few Purkinje cells in a normal position are CSA-positive. One inner granule cell was found to be CSA-positive in this region. The other inner and outer granule cells were CSA-negative (derived from the reeler) in this region (D, thick arrow, grc).* **(G-H)** *Purkinje cells in the deep cerebellum were composed of both CSA-positive and negative cells. Bars, 25 μm. gr; granule cell layer; egl; external granular layer; pcl; Purkinje cell layer; ml; molecular layer; pca; Purkinje cell axons in white matter; epl; ependymal cell layer.*

separated (Table 2). Some Purkinje cells were arranged in a monolayer between the molecular and granule cell layers (Fig. 1A), but a number of Purkinje cells also remained in ectopic sites, especially in the deep cerebellum. In the cerebellar cortex of this chimera, we could detect only several normal genotypic (CSA-positive) cells among the external granule cells, Golgi epithelial cells, inner granule cells (Fig. 1B) and Purkinje cells (Fig. 1C and D). In these cellular layers, mutant (CSA-negative) cells apparently predominated over the normal genotypic cells. The ectopic Purkinje cells in the deep cerebellar mass were composed of both CSA-positive and negative cells (Fig. 1E,F), indicating that Purkinje cells of both normal and mutant genotypes were located in abnormal positions.

In contrast, the lamination and foliation of cerebellar cortex in *rl/rl* ↔Balb chimeras seemed to be quite similar to those of normal control. Ectopic Purkinje cells forming deep cerebellar masses as seen in *rl/rl* ↔C3H chimeras were not detected in any *rl/rl* ↔Balb chimeras (Table 2). In the No. 4222 *rl/rl* ↔ Balb chimera, Purkinje cells were arranged in a single layer at the interface of the molecular and granule cell layers, and they elaborate apparently normal dendritic trees. In this chimera, mutant cells stained positively with the anti-CSA Mab. As shown in Figure 2A and B, CSA-positive Purkinje cells were greater in number, located in normal positions and had well-developed dendritic trees, indicating that Purkinje cells derived from the mutant can migrate to appropriate positions and achieve normal morphological differentiation. Golgi epithelial cells were also derived from the mutant, and they extend well-arranged Bergmann fibers throughout the molecular layer to the pial surface (Fig. 2C,D). Moreover, most granule cells were derived from reeler, and formed a normally-arranged granule cell layer (Fig. 2E). Similar findings indicating the normal differentiation potential of mutant cells were also noted in other *rl/rl* ↔ Balb chimeras. Some *rl/rl* ↔ Balb chimeras had several ectopic Purkinje cells in granule cell layer and white matter (Table 2). These ectopic Purkinje cells were mixed population from normal and mutant strains (data not shown). These results clearly demonstrate that the histopathological characteristics of the reeler mutant may relate to extracellular environments.

Discussion

Experimental chimeric mice have been used to study *in vivo* whether phenotypes of mutant cells are determined by cell-autonomous manner or by cellular environments (McLaren 1986). In the present study, we have clearly demonstrated in the reeler-normal chimeric mice that the morphology and position of cerebellar neurons and glial cells are controlled by extracellular environments, but not by their own genotype. According to recent reports,

the *reelin* gene contains EGF-like motifs, and is closely related to other extracellular matrix proteins such as tenascin, restrictin and integrin-β chain family (D'Arcangelo *et al.*, 1995, Hirotsune *et al.*, 1995). Most recently, the reelin gene product, Reelin, was demonstrated to be a secreted glycoprotein recognized by the CR-50 monoclonal antibody (D'Arcangelo *et al.*, 1997). Immunohistochemical study in the cerebellar histogenesis using the CR-50 antibody also revealed that Reelin is strongly expressed by the external and internal granule cells, but extracellular Reelin is only detected in the external granular and molecular layers, and that Reelin deposition was concentrated at the contacting points between granule cells and Purkinje cells (Miyata *et al.*, 1996). From their observations, Miyata *et al.* (1996) suggested that Reelin plays an important role in Purkinje cell positioning and their maturation. However, in our reeler-normal chimeric mice, even if there were only a few external and internal granule cells derived from normal mouse as shown in Figure 1, a number of Purkinje cells derived from the mutant were arranged as a single layer between the internal granular and molecular layers and had well-developed dendritic trees. From this observation we suggest that Reelin secreted from a single granule cells can affect the morphology of Purkinje cells in a wide area. The possible mechanisms of this widespread effects of Reelin are as follows: 1) diffusion of Reelin; 2) extended parallel fibers of granule cells interact with Purkinje cells in a certain area, and Reelin secretion from them has widespread effects; or 3) Reelin triggers secretion of other directly acting molecules on neuronal migration and positioning .

We also detected a number of Purkinje cells derived from both normal and mutant mice in the deep cerebellar region and along the white matter axons. It is known that Purkinje cells are born and differentiate at the ventricular zone facing the 4th ventricule, then migrate along the radial glial cells to the superficial zone during early stages of cerebellar histogenesis (Yuasa *et al.*, 1991). Therefore, those ectopic Purkinje cells in chimeric cerebella seemed to lose the scaffoldings along which they migrate to the final destination just after their differentiation or en route to their appropriate position. This situation may occur by deficiency of radial glial cells extending from ventricular zone to the pial surface as scaffoldings of migrating neurons. Although there were several reports about abnormalities of radial glial cells in the mutant (Pinto-Lord *et al.*, 1982; Mikoshiba *et al.*,1983; O'Brien *et al.*, 1987; Misson *et al.*, 1988; Steindler *et al.*, 1990; Yuasa *et al.*, 1993), the extracellular Reelin was only detected in the superficial cortical region during Purkinje cell migration, but not in the radial glial cells (Miyata *et al.*, 1996). It is considered, therefore, that deficiency of Reelin may indirectly affect the differentiation or direction of radial

TABLE 2

SUMMARY OF CHIMERAS BETWEEN REELER AND NORMAL INBRED STRAINS

Chimera I.D. No.	Type chimera	Age (postnatal days)	Chimerism: Coat color	Chimerism: Purkinje cells	Foliation pattern	Ectopic Purkinje cells: GL	WM	DCM
3412	rl/C3H	11	rl>>C3H	rl>>C3H	(I+II),III,IV,V,(VI+VII),VIII,IX,X	+	+	+
3456	rl/C3H	30	rl>C3H	not detected	(I+II),III,IV,V,(VI+VII+VIII),IX,X	+	+	+
3457	rl/C3H	30	rl<C3H	not detected	(I+II),III,IV,V,VIa,VIb,VII,VIII,IXa,IXb,IXc,X	+	-	-
3458	rl/C3H	30	rl<C3H	not detected	(I+II),III,IV,V,VIa,VIb,VII,VIII,IXa,IXb,IXc,X	+	-	-
4538	rl/C3H	400	rl<C3H	not detected	(I+II),III,IV,V,VIa,VIb,VII,VIII,IX,X	+	+	-
4539	rl/C3H	400	rl=C3H	not detected	I,II,III,IV,V,(VI+VII),VIII,IX,X	+	+	+
4540	rl/C3H	400	rl=C3H	not detected	(I+II),III,IV,V,VI,VII,VIII,IX,X	+	+	+
4541	rl/C3H	400	rl<C3H	not detected	I,II,III,IV,V,VI,VII,VIII,IX,X	+	+	+
4542	rl/C3H	400	rl<<C3H	not detected	(I+II),III,IV,V,VI,VII,VIII,IX,X	+	+	+
4543	rl/C3H	400	rl<C3H	not detected	(I+II),IIIa,IIIb,IV,V,VIa,VIb,VII,VIII,IX,X	+	-	-
4215	rl/Balb	20	rl<<Balb	rl<<Balb	I,II,III,IV,V,VIa,VIb,VII,VIII,IXa,IXb,IXc,X	-	-	-
4216	rl/Balb	20	rl=Balb	rl=Balb	I,II,III,IV,V,VIa,VIb,VII,VIII,IXa,IXb,IXc,X	-	+	-
4217	rl/Balb	35	rl<Balb	rl<Balb	(I+II),III,IV,V,VIa,VIb,VII,VIII,IXa,IXb,IXc,X	-	+	-
4218	rl/Balb	35	rl>>Balb	rl>>Balb	I,II,III,IV,V,VIa,VIb,VII,VIII,IXa,IXb,IXc,X	+	+	-
4221	rl/Balb	40	rl=Balb	rl=Balb	I,II,III,IV,V,VIa,VIb,VII,VIII,IXa,IXb,IXc,X	-	+	-
4222	rl/Balb	40	rl>>Balb	rl>>Balb	I,II,III,IV,V,VIa,VIb,VII,VIII,IXa,IXb,IXc,X	-	-	-
Control	rl/+or+/+	30			(I+II),III,IV,V,VI,VII,Viii,IX,X	-	-	-
Control	C3H	30			I,II,III,IV,V,VIa,VIb,VII,VIII,IXa,IXb,IXc,X	-	-	-
Control	Balb	30			I,II,III,IV,V,VIa,VIb,VII,VIII,IXa,IXb,IXc,X	-	-	-

CSA staining: fibroblastic cell culture was prepared from tails of chimeric pups, and immunostained with anti-CSA to examine the existence of mosaicism.

Chimerism in coat color was visualy examined and devided into 5 groups: >> and << indicate extreme predominance (more than 90%) of one genotype.

> and < indicate apparent predominance (60-90%) of one genotype. = indicate approximately equal (50-60%) contribution of the two genotypes.

Chimerism in Purkinje cells was estimated by the ratio of the total number of Purkinje cells derived from normal and mutant strains in five sections for each chimera.

Foliation pattern: the name of each lobule was designated according to the description by Inoue and Oda (1980). The lobules in parentheses indicate the fused lobules.

GL and WM indicate the existence of ectopic Purkinje cells in the granular cell layer and white matter, respectively. DCM indicate ectopic Purkinje cells formed a cell mass in the deep cerebellar region.

glial cell processes, and disrupt the normal neuronal migration.

The histological abnormalities of the *rl/rl* ↔ C3H chimeras seemed to be more severe than that of *rl/rl* ↔ Balb ones (Table 2), although it is required in the further study to analyze quantitatively the ratio of each genotype in different cell types for exact comparison of the data. Goffinet (1990) introduced the reeler mutation into C57BL/6 and Balb/c strains, and demonstrated that the effect of the mutation on the cerebellar phenotype depended on the genetic background. There is a possibility that the cellular environments generated by C3H or Balb differentially influenced to the mutant cell behavior. This point should be clarified in the future.

The characteristic abnormalities of the reeler in locomotion, such as tremor, ataxia and dystonia, are considered to be due to neuronal malposition in the forebrain and the cerebellum (Caviness and Rakic, 1978). However, all the chimeras seemed to be normal in locomotion even if they had a number of ectopic Purkinje cells. This finding indicated that the ectopic neurons did not affect normal locomotion if other neurons were positioned appropriately and had normal morphology. The function of those ectopic cells is remained to be elucidated.

Materials and Methods

Animals

We used reeler mice colony originally derived from the B6C3Fe-*a/a-rl* strain (Jackson Laboratory, Bar Harbor, Maine 04609, USA). We have selected stud homozygous reeler males for production of embryos. Homozygous reeler females were treated with 5 IU pregnant mare's serum gonadotropin followed by 5 IU human chorionic gonadotropin to induce ovulation and mating. C3H/HeN (C3H) and BALB/cA (Balb) mice were used as normal embryo donors (Charles River Japan and Clea Japan, respectively). For histological analyses, rl/+ or +/+ mice from reeler breeding colony were also used as normal controls as well as C3H and Balb. The mice were maintained and bred in the animal facility of the Life Science Tsukuba Research Center, RIKEN. The day of birth was designated as postnatal day 0 (P0).

Production of chimeric mice

Chimeric mice were produced by aggregating 8-cell stage embryos between the reeler homozygotes and normal embryos. Two types of chimeras were produced, *rl/rl* ↔ C3H and *rl/rl* ↔ Balb. Procedures for making chimeric mice were described in a previous report (Yoshiki *et al.*, 1993). We used overt coat color chimeras for histological analyses in the present study.

In situ genetic marker for chimeric tissue

We used anti-C3H strain-specific antigen (CSA) antibody to detect strain origin of cells in the tissue sections (Kusakabe *et al.*, 1988). The detailed explanation about the CSA and the genotype of the mutant were described above in the Results section. As homozygous reeler mice *(rl/rl)* used in this study were heterozygous for the hsc74, three genotypes were segregating among *rl/rl* embryos as shown in Table 1. Before histological analysis of chimerism, fibroblast cell cultures were prepared from tails of overt coat color chimeras at P10 in order to determine in which chimera the cell origin could be distinguished by immunohistochemistry with the anti-CSA antibody.

Histological preparation

The detailed procedures for tissue preparation were reported in a previous paper (Yoshiki *et al.*, 1993). Briefly, tissues were fixed by microwave irradiation in 0.1 M sodium phosphate buffer (pH 7.4) at 40°C for 20 min, and were post-fixed in 95% ethanol containing 1% acetic acid overnight. The tissues were dehydrated with 100% ethanol, embedded in polyester wax and serially sectioned at 5 μm thickness.

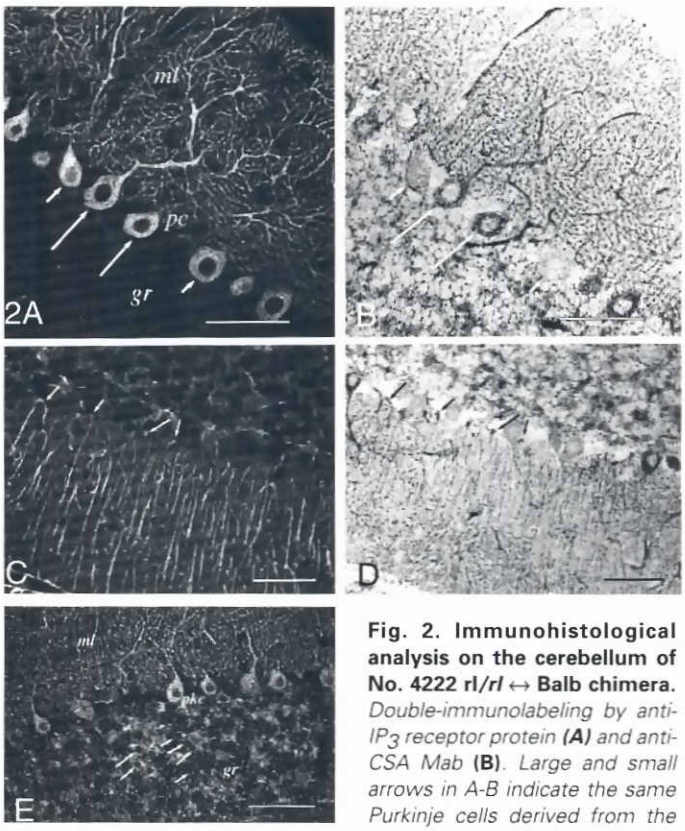

Fig. 2. Immunohistological analysis on the cerebellum of No. 4222 rl/*rl* ↔ Balb chimera. *Double-immunolabeling by anti-IP_3 receptor protein* ***(A)*** *and anti-CSA Mab* **(B)**. *Large and small arrows in A-B indicate the same Purkinje cells derived from the mutant and Balb, respectively. Double-immunolabeling by anti-GFAP* **(C)** *and anti-CSA Mab* **(D).** *Large and small arrows in C-D indicate the same Golgi epithelial cells derived from the mutant.* **(E)** *The granule cell layer of the chimera. Most granule cells (arrows) and Purkinje cells were derived from the mutant in the cerebellum of this chimera. A and C were fluorescent images. B and D were produced by transmission mode. The images in E were made by reflection of laser light by metallic silver precipitation. gr; granule cell layer; pc; Purkinje cell; ml; molecular layer. Bars, 50 μm.*

Immunohistochemistry

The sections were dewaxed with three changes of 100% ethanol, rehydrated through a graded ethanol series, and rinsed in PBS(-) containing 0.05% Tween 20 (PBS/Tween). Sections were preincubated with PBS(-) containing 5% normal goat serum and 1% BSA (blocking solution) for 30 min, and incubated with the primary antibodies described below.

Chimerism was visualized using immunohistochemistry and the anti-CSA antibody (Yoshiki *et al.*, 1993). The sections were incubated with anti-CSA antibody diluted in the blocking solution at 1:400 overnight. The sections were then rinsed in ice-cold PBS/Tween five times. Then, the sections were incubated with 1 nm colloidal gold-conjugated goat F(ab')2 anti-mouse IgG (BioCell, UK) diluted in the blocking solution at 1:200 for 1 h, washed in PBS/Tween, and washed with distilled water five times. Purkinje cells were specifically stained with rat monoclonal antibody (Mab-4C11) raised against the inositol 1,4,5-triphosphate (IP3) receptor protein (Inoue *et al.*, 1990; Maeda *et al.*, 1991). The Mab-4C11 was kind gift of Dr. Mikoshiba, Molecular Neurobiology Laboratory, RIKEN, Tsukuba, Ibaraki, Japan. After washing in PBS/Tween, sections were incubated with FITC-conjugated goat anti-rat IgG (Zymed) or 5 nm colloidal gold-conjugated anti-rat IgG (Amersham). Astroglial cells were detected with rabbit anti-glial fibrillary acidic protein (GFAP) antibody (DAKO) and FITC-conjugated anti-rabbit IgG (TAGO). Some sections were also double-immunostained with anti-CSA and Mab-4C11, or anti-CSA and anti-GFAP. The gold-labeling was visualized by silver enhancement (silver enhancement kit, BioCell).

After immunostaining the sections were mounted with Mounting Fluid (Difco), and observed under a confocal laser microscope (LSM10, Carl Zeiss, Germany). For standard analyses of cerebellar size and foliation pattern, mid-sagittal sections were stained with hematoxylin and eosin (HE). The terminology adopted for vermian lobules is according to descriptions by Inoue and Oda (1980) with minor modifications.

Estimation of chimerism in cerebellum:

Chimerism in cerebellum were estimated by the ratio of the CSA-positive and negative Purkinje cells at the interface of granular and molecular layers. We used 5 sections at 50 μm intervals from each chimeric tissue section. 0-10% contribution of mutant cells was expressed as <<, 10-40% as <, 40-60% as ≒, 60-90% as > and 90-100% as >>, respectively.

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References

BENJELLOUN-TOUIMI, S., JACQUE, C. M., DERER, P., DE VITRY, F., MAUNOURY, R. and DUPOUEY, P. (1985). Evidence that the mouse astrocytes may be derived from the radial glia. An immunohistochemical study of the cerebellum in the normal and reeler mouse. *J. Neuroimmunol. 9:* 87-97.

CAVINESS, V.S., Jr. and RAKIC, P. (1978). Mechanisms of cortical development: A view from mutations in mice. *Annu. Rev. Neurosci. 1:* 297-326.

CAVINESS, V.S., Jr. (1982). Neocortical histogenesis in normal and reeler mice: a developmental study based upon [3H] thymidine autoradiography. *Dev. Brain Res. 4:* 293-302.

D'ARCANGELO, G., MIAO, G.G., CHEN, S-C., SOARES, H.D., MORGAN, J.I. and CURRAN, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant *reeler. Nature 374:* 719-723.

D'ARCANGELO, G., NAKAJIMA, K., MIYATA, T., OGAWA, M., MIKOSHIBA, K. and CURRAN, T. (1997). Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. *J. Neurosci. 17:* 23-31.

DEL RIO, A.J., HEIMRICH, B., BORRELL, V., FORSTER, E., DRAKEW, A., ALKANTARA, S., NAKAJIMA, K., MIYATA, T., OGAWA, M., MIKOSHIBA, K., DERER, P., FROTSHER, M. and SORIANO, E. (1997). A role for Cajal-Retzius cells and reelin in the development of hippocampal connections. *Nature 385:* 70-74.

DVORAK, P., DVORAKOVA, D., YOSHIKI, A., OHASHI, T., KITAMURA, K. and KUSAKABE, M. (1995). Expression of paternal and maternal mitochondrial HSP70 family, hsc74, in preimplantation mouse embryos. *Int. J. Dev. Biol. 39:* 511-517.

FALCONER, D.S. (1951). Two new mutants, trembler and reeler, with neurological actions in the house mouse (Mus musculus L.). *J. Genet. 50:* 195-201.

FALCONER, D.S. (1952). Location of reeler in linkage group III of the mouse. *Hereditary 6:* 255-258.

GHANDOUR, M.S., DERER, P., LABOURDETTE, G., DELAUNOY, J.P. and LANGLEY, O.K. (1981). Glial cell markers in the reeler mutant mouse: a biochemical and immunohistological study. *J. Neurochem. 36:* 195-200.

GOFFINET, A.M. (1984). Events governing organization of postmigratory neurons: studies on brain development in normal and reeler mice. *Brain Res. 319:* 261-296.

GOFFINET, A.M. (1990). Cerebellar phenotype of two alleles of the "reeler" mutation on similar backgrounds. *Brain Res. 519:* 355-357.

HIROTSUNE, S., TAKAHARA, T., SASAKI, N., HIROSE, K., YOSHIKI, A., OHASHI, T., KUSAKABE, M., MURAKAMI, Y., MURAMATSU, M., WATANABE, S., NAKAO, K., KATSUKI, M. and HAYASHIZAKI, Y. (1995). Reeler gene encodes a protein with EGF-like motif expressed by pioneer neurons. *Nature Genet. 10:* 77-83.

INOUE, Y., MAEDA, N., KOKUBUN, T., TAKAYAMA, C., INOUE, K., TERASHIMA, T. and MIKOSHIBA K. (1990). Architecture of Purkinje cells of the reeler mutant mouse observed by immunohistochemistry for the inositol 1,4,5-triphosphate receptor protein P_{400}. *Neurosci. Res. 8:* 189-201.

INOUE, M. and ODA, S-I. (1980). Strain-specific variations in the folial pattern of the mouse cerebellum. *J. Comp. Neurol. 190:* 357-362.

KUSAKABE, M., YOKOYAMA, M., SAKAKURA, T., NOMURA, T., HOSICK, H.L. and NISHIZUKA, Y. (1988). A novel methodology for analysis of cell distribution in chimeric mouse organs using a strain specific antibody. *J. Cell Biol. 107:* 257-265.

MCLAREN A. (1986). *Mammalian chimaeras.* Cambridge University Press. London.

MAEDA, N., NIINOBE, M. and MIKOSHIBA, K. (1991). Immunohistochemical localization of an inositol 1,4,5-triphosphate receptor, P_{400} protein in neural tissue: studies in developing and adult mouse brain. *J. Neurosci. 11:* 2075-2086.

MARIANI, J., CREPEL, F., MIKOSHIBA, K., CHANGEUX, J.P. and SOTELO, C. (1977). Anatomical, physiological and biochemical studies of the cerebellum from reeler mutant mouse. *Philos. Trans. R. Soc. Lond. [Biol.] 281:* 1-28.

MIKOSHIBA, K., NAGAIKE, K., KOHSAKA, S., TAKAMATSU, K., AOKI, E. and TSUKADA, Y. (1980a). Developmental studies on the cerebellum from reeler mutant mouse *in vivo* and *in vitro. Dev. Biol. 79:* 64-80.

MIKOSHIBA, K., KOHSAKA, S., TAKAMATSU, K., AOKI, E. and TSUKADA, Y. (1980b). Morphological and biochemical studies on the cerebral cortex from reeler mutant mice: Development of cortical layers and metabolic mapping by the deoxyglucose method. *J. Neurochem. 34:* 835-844.

MIKOSHIBA, K., NISHIMURA, Y. and TSUKADA, Y. (1983). Absence of the bundle structure in the neocortex of the reeler mouse at the embryonic stage. *Dev. Neurosci. 6:* 18-25.

MISSON, J.P., EDWARDS, M.A., YAMAMOTO, M. and CAVINESS, V.S., Jr. (1988). Identification of radial glial cells within the developing murine central nervous system: studies based upon a new immunohistochemical marker. *Dev. Brain Res. 44:* 95-108.

MIYATA, T., NAKAJIMA, K., ARUGA, J., TAKAHASHI, S., IKENAKA, K., MIKOSHIBA, K. and OGAWA, M. (1996). Distribution of a reeler gene-related antigen in the developing cerebellum: an immunohistochemical study with an allogeneic antibody CR-50 on normal and reeler mice. *J. Comp. Neurol. 372:* 215-228.

O'BRIEN, T.F., STEINDLER, D.A. and COOPER, N.G. (1987). Abnormal glial and glycoconjugate dispositions in the somatosensory cortical barrel field of the early postnatal reeler mutant mouse. *Brain Res. 429:* 309-317.

OGAWA, M., MIYATA, T., NAKAJIMA, K., YAGYU, K., SEIKE, M., IKENAKA, K., YAMAMOTO, H. and MIKOSHIBA, K. (1995). The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. *Neuron 14:* 899-912.

PINTO-LORD, M.C., EVRARD, P. and CAVINESS, V.S., Jr. (1982). Obstructed neuronal migration along radial glial fibers in the neocortex of the reeler mouse: a Golgi-EM analysis. *Brain Res. 256:* 379-393.

RAKIC, P. (1988). Specification of cerebral cortical areas. *Science 241:* 170-176.

STEINDLER, D.A., O'BRIEN, T.F., LAYWELL, E., HARRINGTON, K., FAISSNER, A. and SCHACHNER, M. (1990). Boundaries during normal and abnormal brain development: *in vivo* and *in vitro* studies of glia and glycoconjugates. *Exp. Neurol. 109:* 35-56.

TERASHIMA, T., INOUE, K., INOUE, Y., MIKOSHOBA, K. and TSUKADA, Y. (1985). Observations on Golgi epithelial cells and granule cells in the cerebellum of the reeler mutant mouse. *Dev. Brain Res. 18:* 103-112.

TERASHIMA, T., TERASHIMA-T; INOUE, K., INOUE, Y., YOKOYAMA, M. and MIKOSHIBA, K. (1986). Observations on the cerebellum of normal-reeler mutant mouse chimera. *J. Comp. Neurol. 252:* 264-278.

YOSHIKI, A., SAKAKURA, T. and KUSAKABE, M. (1993). The mouse chimera during intrauterine stages: immunohistological analysis with the C3H strain-specific antibody. *J. Histochem. Cyotochem. 41:* 1583-1590.

YUASA, S., KAWAMURA, K., ONO, K., YAMAKUNI, T. and TAKAHASHI Y. (1991). Development and migration of Purkinje cells in the mouse cerebellar primordium. *Anat. Embryol. 184:* 195-212.

Yuasa, S., Kitoh, J., Oda, S. and KAWAMURA, K. (1993). Obstructed migration of Purkinje cells in the developing cerebellum of the reeler mutant mouse. *Anat Embryol. 188:* 317-329.

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