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Retrograde infection of precerebellar nuclei neurons by injection of a recombinant adenovirus into the cerebellar cortex of normal and *reeler* mice*

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Summary. The *reeler* mouse is an autosomal recessive mutant mouse caused by mutation of the *reelin* gene and characterized by cerebellar ataxia. To determine whether the distribution pattern of precerebellar nuclei neurons in the brainstem of the *reeler* mouse changes, we injected a small volume of a replication-defective recombinant adenovirus carrying *E. coli* β-galactosidase (lacZ) into the cerebellar cortex of normal and *reeler* mice. Five days later, the mice were transcardially perfused by a fixative solution. X-gal staining of coronal or sagittal sections of the brainstem revealed that many origins for reticulocerebellar, cuneocerebellar, trigeminocerebellar, and pontocerebellar projections were retrogradely labeled, but only a few olivocerebellar neurons were labeled. Retrogradely labeled neurons in the lateral reticular nucleus tended to locate more laterally and be more condensed into a small compartment in the *reeler* compared with their normal counterparts. Retrogradely labeled neurons in the external cuneate nucleus were more dorsally shifted in the *reeler* mice compared with their normal counterparts. We could not find any differences between the normal and *reeler* mice in the distribution patterns of their trigeminocerebellar projection neurons. Retrogradely labeled pontocerebellar neurons in the basilar pons of the *reeler* mouse were reduced in number compared with their normal counterparts in addition to being more ventrally and laterally shifted. These findings strongly suggest that the migration of some precerebellar nuclei neurons from the rhombic lip to their final loci may be obstructed in the *reeler* mice.

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Introduction

The cerebellum receives afferents from many sources in the brainstem and spinal cord, which are collectively known as the precerebellar nuclei (Ruigrok and Cella, 1995). These precerebellar nuclei have been classified into two distinct groups. The first group consists of pontine nuclei, the reticulotegmental nucleus of the pons, trigeminal sensory nuclei, the lateral reticular nucleus, external cuneate nucleus, and dorsal nucleus of Clark. Axons arising from these nuclei neurons enter the inferior or middle cerebellar peduncle and terminate with dendrites of cerebellar granule cells as the mossy fibers. The other group consists of the inferior olivary nuclei. Axons arising from inferior olivary nuclei neurons cross at the lower medulla, enter into the inferior cerebellar peduncle, and terminate with dendritic trees of Purkinje cells as the climbing fibers. These precerebellar nuclei neurons — except for the dorsal nucleus of Clark — are developmentally derived from the rhombic lip, a germinal zone in an alar plate forming the wall of the 4th ventricle. The rhombic lip express basic helix-loop-helix transcription factor *Mouse atonal homolog 1* (*Math1*) as early as embryological day (E) 9.5 (Akazawa *et al.*, 1995).

Interestingly, mossy-fiber precrebellar nuclei require *Math1*, whereas the inferior olive and locus coeruleus do not (Ben-Arie *et al.*, 1997, 2000; Bermingham *et al.*, 2001). Recently, a series of studies using a LacZ reporter to track rhombic lip-derived cells refined the fate map of the rhombic lip-derived precerebellar nuclei neurons and their migration courses from their birthplaces to their final loci (Hoshino *et al.*, 2005; Wang *et al.*, 2005).

The Reelin protein, encoded by the *reelin* gene (gene symbol, *Reln*), is a protein of the extracellular matrix, and functions to control the migration and positioning of central nervous system cells in the embryonic period. The *reeler* mouse, caused by a disrupted *reelin* gene, is characterized by an abnormal cytoarchitecture of laminated structures such as the hippocampal formation and cerebral and cerebellar cortices (Yuasa *et al.*, 1993; D'Arcangelo *et al.*, 1995; Forster *et al.*, 2006). In this mutant, the non-laminated structures were originally considered to be normal, but Goffinet (1983, 1984) revealed that the inferior olivary nuclei and the facial nucleus are cytoarchitectually abnormal, suggesting that the long migration of neurons from the birthplaces to their final loci are generally disorganized in this mouse. The precerebellar nuclei neurons undertake a long journey from their birthplace (*i.e.*, the rhombic lip) to their final loci; therefore, it is naturally anticipated that the migration of these neurons is obstructed in the reelin-deficient mouse *reeler*.

Recently, the replication-defective recombinant adenoviruses have been recognized as highly efficient systems for gene delivery into postmitotic neurons both *in vivo* and *in vitro*, enabling the development of active gene therapies for brain diseases and injuries (Bemelmans *et al.*, 1999; Matsuoka *et al.*, 1999; Pulkkanen and Yla-Herttuala, 2005; Tyler *et al.*, 2006; Brouwer *et al*, 2007). In addition to clinical uses, the adenovirus-mediated gene transfer method has been used for retrograde labeling of projection neurons. Neurons are infected by recombinant adenovirus carrying the *E.coli* β-galactosidase gene (*lacZ*) via their axonal terminals, and adenoviruses are retrogradely transported to neuronal cell bodies (Terashima *et al.*, 1997; Tsukamoto *et al.*, 2003; Doi *et al.*, 2005, 2006). The transgene enters into the cellular nuclei of the infected neurons and produces β-galactosidase, which is accumulated in cell bodies and dendrites of infected neurons. In the present study, we injected a small volume of a replication-defective recombinant adenovirus encoding *E. coli* β-galactosidase (*lacZ*) into the cerebellar cortex of both normal and *reeler* mice to determine whether or not the migration of precerebellar nuclei neurons in the brainstem of the *reeler* mouse is obstructed.

Materials and Methods

Animals

Mice with a C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA.) and raised in our animal facility. Adult animals of either sex at two months of age were used in the present study. All animals were housed in a temperature-controlled (22 ± 1℃) colony room with a 12 h/12 h L/D cycle in groups in acrylic cages with woodchip bedding and unlimited access to normal laboratory chow or food. All of the procedures were approved by the institutional Animal Care Ethics Committees of Kobe University School of Medicine and Tokyo Metropolitan Institute for Neuroscience

Virus preparation

In this study, we used the replication-defective recombinant adenoviral vector (AxCAlacZ), carrying the *E.coli* β-galactosidase gene (*lacZ*). The structure of this vector was previously described in detail (Terashima *et al.*, 1997; Tsukamoto *et al.*, 2003). In brief, AxCAlacZ has a construct that is human adenovirus serotype 5 (Ad5) lacking sequences in the early 1 (E1) and E3 regions. The E3 region encodes a 19kDa protein that inhibits the expression of class 1 major histocompatibility antigens on the surface of infected cells. We generated these recombinant adenoviruses by the COS-TPC method according to Miyake *et al.* (1996) and purified them by CsCl step gradients (Kanegae *et al.*, 1994). The viral suspensions were stored at −80℃.

Infection of precerebellar nuclei neurons with lacZ-carrying adenovirus

Thirteen mice at two months of age (6 normal, 7 *reeler*) were used in the present study. The animals were anesthetized with 3.5% chloral hydrate by intraperitoneal injection (0.9 m*l* per 100 g body weight) and clamped in a stereotactic apparatus (Narishige, Tokyo). Following incision of the skin overlying the occipital region, a small burr hole was made directly over the left hemisphere of the cerebellum of recipient mice using a dental drill, and a single injection of 0.2 μ*l* of the concentrated viral suspension (1.51×10^{10} plaque forming units [PFU] /m*l* dissolved in 0.1M phosphate buffer containing 0.9% NaCl (PBS) and 10% glycerol) was made into the crus 1 of the ansiform lobule of the left cerebellar cortex of the normal mouse by applying pressure through a micropipette attached to the barrel of a 1 μ*l* Hamilton microsyringe under an operating microscope. The cerebellar sulci

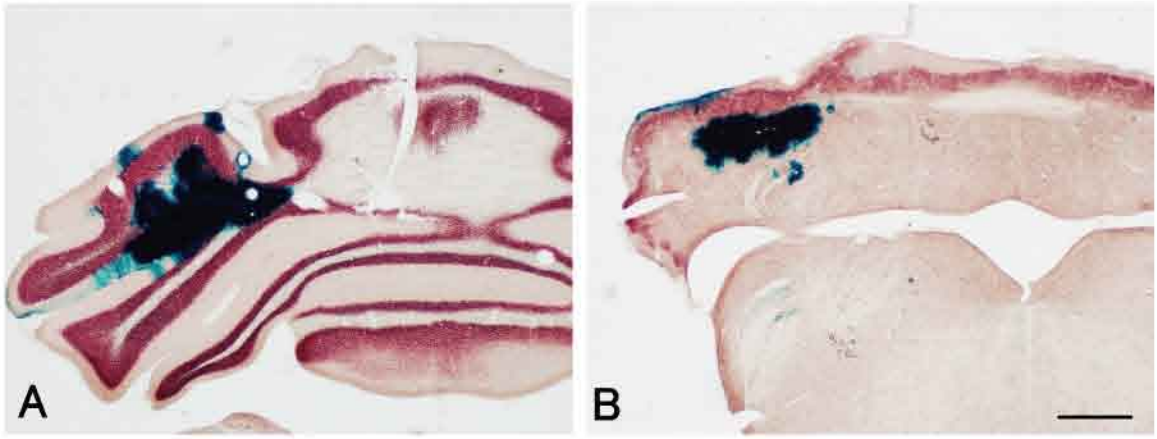

Fig. 1. β-Galactosidase histochemistry of coronal sections through the site of injection of the replication-deficient adenoviral vector (AxCALacZ) into the left cerebellar cortex of the normal (**A**) and *reeler* (**B**) mouse. Neutral Red counterstaining. Scale bar = 1 mm (for A, B)

were absent in the *reeler* mouse, so that we could not identify the area corresponding to the crus 1 in the *reeler* cerebellum. Thus, in the *reeler* mouse, we injected the adenovirus into the cortical area similar to its normal counterpart along the rostral-to-caudal and lateral-to-medial axes. The micropipette was then kept in place for 5 min. After 5 days post-infection, the animals were re-anesthetized and transcardially perfused with a solution of 4% paraformaldehyde or a mixed solution of 2% paraformaldehyde and 0.5% glutaraldehyde, buffered to pH 7.4 with a phosphate buffer (PB).

After perfusion, the brain was removed from the skull, post-fixed in the same fixative solution for 2 h, and then immersed for 12–18 h in PB containing 15% sucrose. The brains were coronally cut on a freezing microtome at a thickness of 40 μm. The tissue sections were mounted on gelatin-coated slides, dried for 4 h, rinsed with PBS (5 min, three times), and stained by immersion in 5 mM $K_4Fe(CN)_6$, 5 mM $K_3(CN)_6$, and 2 mM $MgCl_2$ in PBS containing 0.5 mg/m*l* of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Nova) in N,N-dimethylformamide (Wako, Osaka) at 20 mg/m*l*, for 12 h at 37℃ (Terashima *et al.*, 1997). The sections were counterstained with 0.5% neutral red, dehydrated in alcohol, cleared with xylene, and coverslipped with HSR solution (Kokusai, Kobe). Histological sections were examined under an Olympus AX80 microscope. The histological images were captured from a microscope equipped with a Fujix HC-2500 Digital CCD camera connected to a Power Macintosh G3/350 computer using Photograb-2500 (ver. 1.1) software (Fujix).

Results

Injection sites

Typical injection sites of AxCALacz in the left cerebellar cortex are shown in Figure 1 (A, normal; B, *reeler*). In the normal mouse, the injection site was mainly located within the crus 1 of the ansiform lobule but spread into the underlying white matter. Golgi epithelial cells were strongly stained by β-galactosidase histochemistry. A few Purkinje cells near the injection sites were occasionally stained. In the *reeler* mouse, a similar injection was made along the rostral-to-caudal and lateral-to-medial axes. Somata of Golgi epithelial cells and their vertically arranged cellular processes (i.e., Bergmann processes) were strongly stained by β-galactosidase histochemistry. No inflammatory signs such as an accumulation of small round cells or vascular responses were observed although some tissue loss was apparent around the needle track.

Retrogradely labeled neurons in the precerebellar nuclei

After injection of the recombinant adenovirus, retrogradely labeled cells were recognized in the precerebellar nuclei including the lateral reticular nucleus, external cuneate nucleus, inferior olivary nuclei, pontine nuclei, and reticulotegmental nucleus of the pons, as described below.

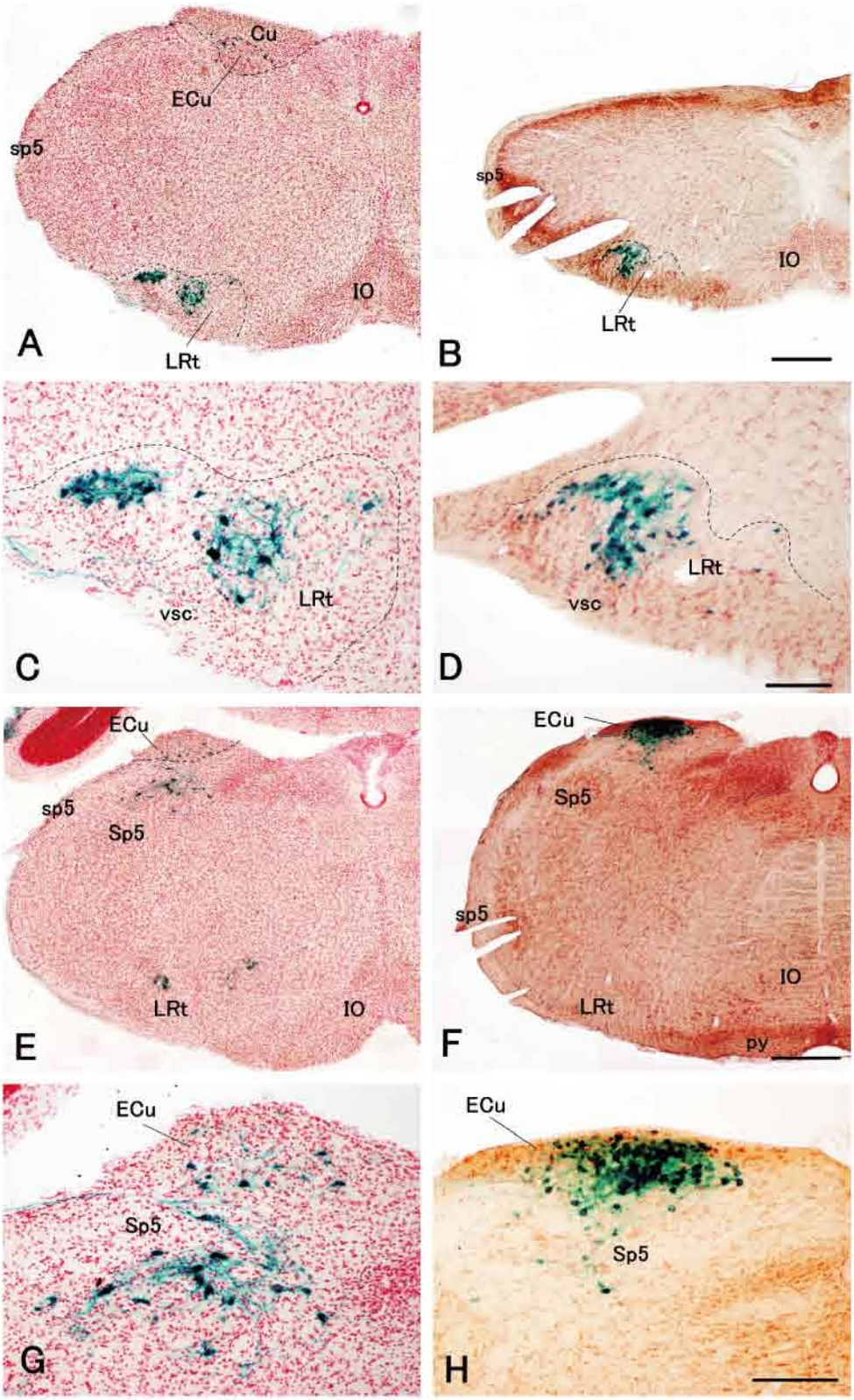

Fig. 2. Legend on the opposite page.

Lateral reticular nucleus

The lateral reticular nucleus represents one of the most important sources of mossy fibers to the cerebellar cortex. Both in the normal and *reeler* mice, the lateral reticular nucleus was located ventrally in the medulla oblongata, dorsolateral to the inferior olivary nuclei, and ventromedial to the spinal tract of the trigeminal nerve. It extended from a level slightly caudal to the inferior olive to a level through the rostral fourth of the inferior olive. Injection of the recombinant adenovirus into the cerebellar cortex resulted in retrograde labeling of neurons in the lateral reticular nucleus both in the normal and *reeler* mice (Fig. 2A–D). Labeled cells were bilaterally located with the ipsilateral predominance. In the normal mouse, retrogradely labeled cells were scattered within this nucleus (Fig. 2C), but in the *reeler*, they were condensed into a small area of it (Fig. 2D). The β-galactosidase-reactive axons exiting from strongly labeled cells entered the inferior cerebellar peduncle and entered into the cerebellum both in the normal and *reeler* mice.

External cuneate nucleus

In the *reeler* mice, the external cuneate nucleus was located dorsal to the cuneate nucleus, and it appeared at a level through the caudal third of the inferior olive to extend caudally at a level through the facial nucleus in a similar manner to its normal counterpart. Injection of the adenovirus into the left cerebellar cortex resulted in retrograde labeling of neurons in the external cuneate nucleus both in the normal and *reeler* mice (Fig. 2E–H). In the normal mouse, LacZ-labeled neurons were scattered in this nucleus, whereas in the *reeler*, they were densely accumulated. In addition, labeled neurons in the external cuneate nucleus were more dorsally (*i.e.*, superficially) shifted in the *reeler* mouse compared with the normal counterparts. Anterogradely labeled axons exited from the lateral margin of this nucleus and entered the inferior cerebellar peduncle both in the normal and *reeler* mice (Fig. 3B–E).

Inferior olivary nuclei

The inferior olivary nuclei of the normal mouse is composed of three large subdivisions — the principal, medial accessory and dorsal accessory olive, and four additional smaller subdivisions — the dorsal cap, the ventrolateral outgrowth, the nucleus β, and the dorsomedial cell column, as previously reported (Ruigrock and Cella, 1995). In the *reeler* mouse, these subdivisions of the inferior olivary nuclei were roughly recognized, but their boundaries were blurred. In addition, U-shaped laminae of the principal nucleus were disrupted in the *reeler* inferior olive (Goffinet, 1983). After injection of the recombinant adenovirus into the left cerebellar hemisphere, only a few labeled neurons in the inferior olivary nuclei were recognized (Fig. 3A, inset), suggesting that efficient retrograde labeling of the olivocerebellar projection neurons cannot be achieved by the present vectors. Labeled cells were exclusively contralateral to the injection site.

Pontine nuclei

The pontine nuclei were located in the basilar pons, surrounding the longitudinal fibers of the pons and extending from the trapezoid body to the interpeduncular nucleus both in the normal and *reeler* mice. The size of the *reeler* basal pons appeared to be smaller than that of the normal one. The lacZ-recombinant adenovirus injection into the left cerebellar hemisphere resulted in retrograde labeling of pontine nuclei neurons both in the normal and *reeler* mice (Fig. 4, 5). Retrogradely labeled neurons were similarly distributed in the bilateral pontine nuclei with a contralateral predominance; we described therefore the distribution pattern of the retrograde labeling on the contralateral (*i.e.*, right) side. The number of retrogradely labeled cells was reduced in number compared with normal counterparts. In the normal mouse, β-galactosidase-reactive neurons were distributed in the dorsomedial, medial, and ventral pontine nuclei (Fig. 4C). Some labeled cells were scattered in the peripeduncular

Fig. 2. The injection of AxCALacZ into the left cerebellar cortex results in the retrograde labeling of neurons in the lateral reticular nucleus (LRt) and external cuneate nucleus (ECu) of normal (left panel) and *reeler* (right panel) mice. **A–D:** Retrogradely labeled neurons appear to be more laterally shifted in the *reeler* compared with the normal counterpart.(**A**, normal; **B**, *reeler*). Labeled neurons in the LRt shown in **A** and **B** are enlarged into **C** and **D**, respectively. **E–H:** Retrogradely labeled neurons in the ECu are more dorsally (*i.e.*, superficially) distributed in the *reeler* compared with the normal couterpart (**E**, normal; **F**, *reeler*). Both in the normal and *reeler* mice, β-galactosidase reactive neurons are recognized in the spinal trigeminal nucleus (Sp5). Retrogradely labeled neurons in the ECu in **E** and **F** are enlarged in **G** and **H**, respectively. Other abbreviations: Cu, cuneate nucleus; IO, inferior olivary complex; sp5, spinal trigeminal tract; vsc, ventral spinocerebellar tract; py, pyramidal tract. Neutral Red counterstaining. Scale bars = 500 μm (A, B, E, F), 200 μm (C, D, G, H)

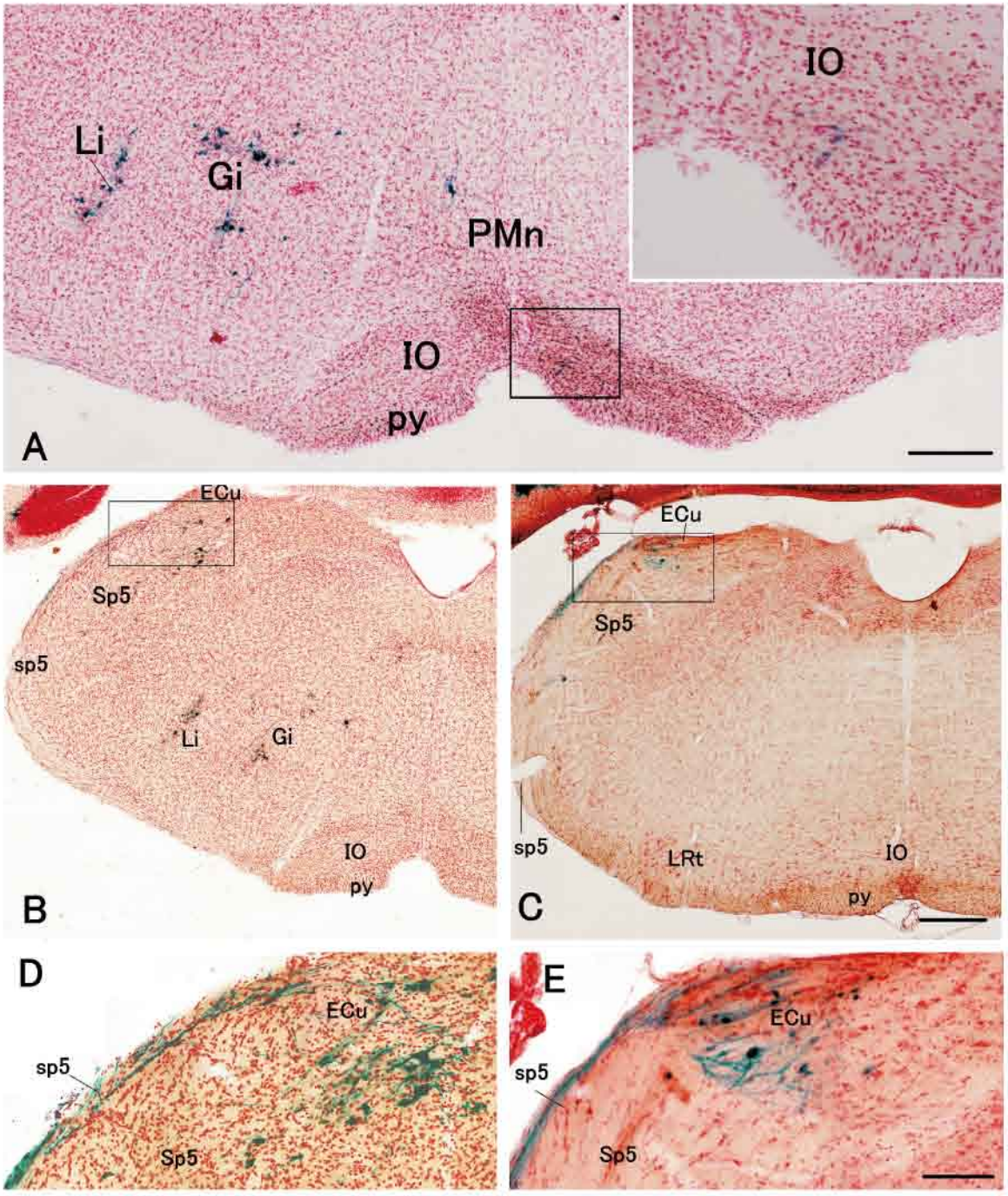

Fig. 3. The injection of AxCALacZ into the left cerebellar cortex results in the retrograde labeling of neurons in the inferior olivar y complex (IO), gigantocellular reticular nucleus (Gi), linear nucelus of the medulla (Li), and external cuneate nucleus (ECu) of normal (**A**, **B**, **D**) and *reeler* (**C**, **E**) mice. **A:** In the normal mouse, labeled neurons are recognized in the contralateral inferior olivary complex (IO), the ipsilateral paramedian reticular nucleus (PMn), gigantocellular reticular nucleus (Gi), and linear nucleus of the medulla (Li). β-galactosidase reactive IO neurons are shown in the inset. **B**, **C:** LacZ-positive neurons in the external cuneate nucleus (ECu) are more dorsally or superficially shifted in the *reeler* mouse than those in a normal one (**B**, normal; **C**, *reeler*). The areas shown in the rectangles in **B** and **C** are enlarged in **D** and **E**. Anterogradely labeled axons arising from retrogradely labeled ECu neurons are recognized in the inferior cerebellar peduncle of the normal (**D**) and *reeler* (**E**) mouse. Neutral Red counterstaining. Scal bars = 200 μm (A–C), 100 μm (D, E)

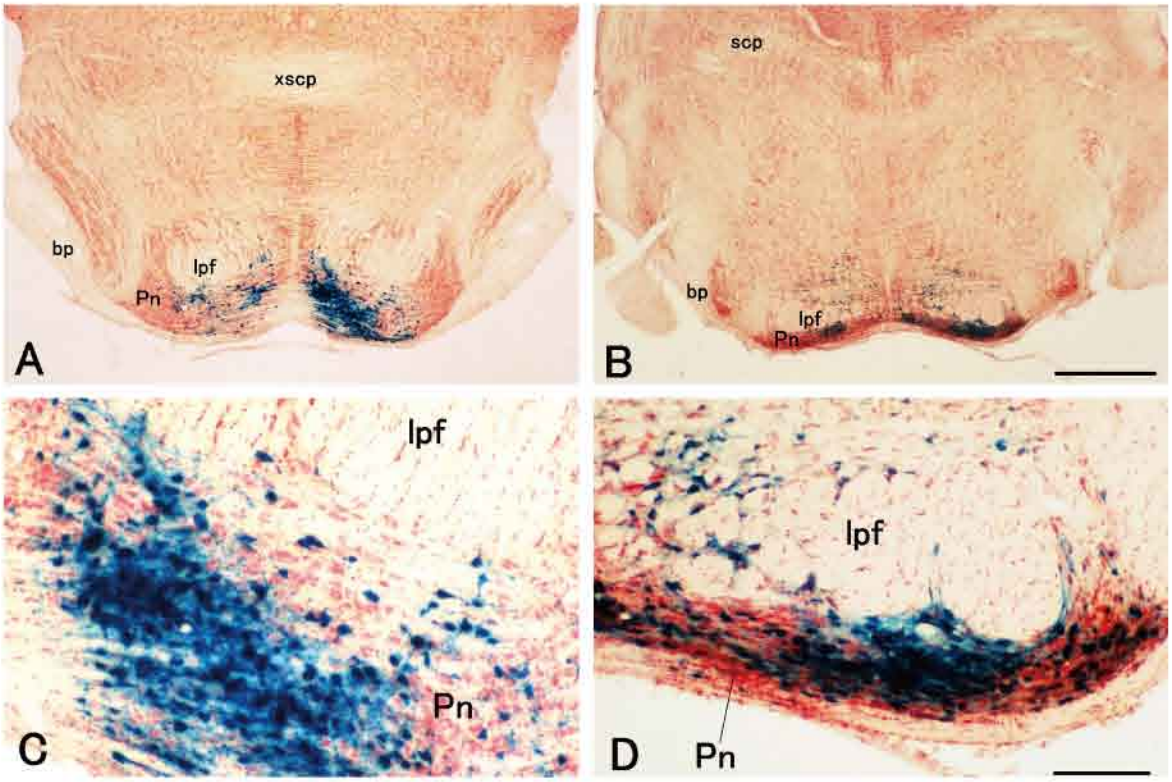

Fig. 4. The injection of AxCALacZ into the left cerebellar cortex results in the retrograde labeling of neurons in the pontine nuclei (Pn) of normal (**A**,**C**) and *reeler* (**B**, **D**) mice. In the *reeler*, retrogradely labeled neurons are reduced in number and more ventrally (i.e., superficially) shifted than those in the normal mouse. LacZ-positive Pn neurons in **A** and **B** are enlarged in **C** and **D**, respectively. Other abbreviations: bp,brachium pontis; lpf, longitudinal fibers of the pons, scp, superior cerebellar peduncle; xscp, decussation of superior cerebellar peduncle. Scale bars = 200 μm (A, B), 50 μm (C, D)

pontine nuclei. In the *reeler* mouse, a similar injection resulted in retrograde labeling of neurons in the medial, ventral and lateral pontine nuclei (Fig. 4D). A few β-galactosidase-reactive neurons surrounded the longitudinal fibers of the pons. It should be noted that retrogradely labeled cells in pontine nuclei of the *reeler* mouse tended to be more ventrally and laterally shifted compared with their normal counterparts.

Reticulotegmental nucleus of pons

The reticulotegmental nucleus of the pons was located dorsal to the pontine nuclei both in the normal and *reeler* mice. In addition to the pontine nuclei neurons, many neurons in the reticulotegmental nucleus of the pons were retrogradely labeled both in the normal and *reeler* mice (Fig. 5E–H). Again, retrogradely labeled neurons tended to be more ventrally shifted in the *reeler* in comparison with their normal counterparts.

Miscellaneous

A few labeled neurons were also recognized in the spinal trigeminal nucleus, gigantocelluar reticular nucleus, paramedian reticular nucleus, and linear nucleus of the medulla both in the normal and *reeler* mice (Fig. 2, 3). We could not find any differences in their distribution patterns between the normal and *reeler* mice.

Discussion

The present study has demonstrated that precerebellar nuclei — including the lateral reticular nucleus, external cuneate nucleus, inferior olivary nuclei, pontine nuclei, and reticulotegmental nucleus of the pons of the *reeler* mouse — are cytoarchitectually disrupted although the abnormalities recognized both in the external cuneate nucleus and the lateral reticular nucleus are very subtle.

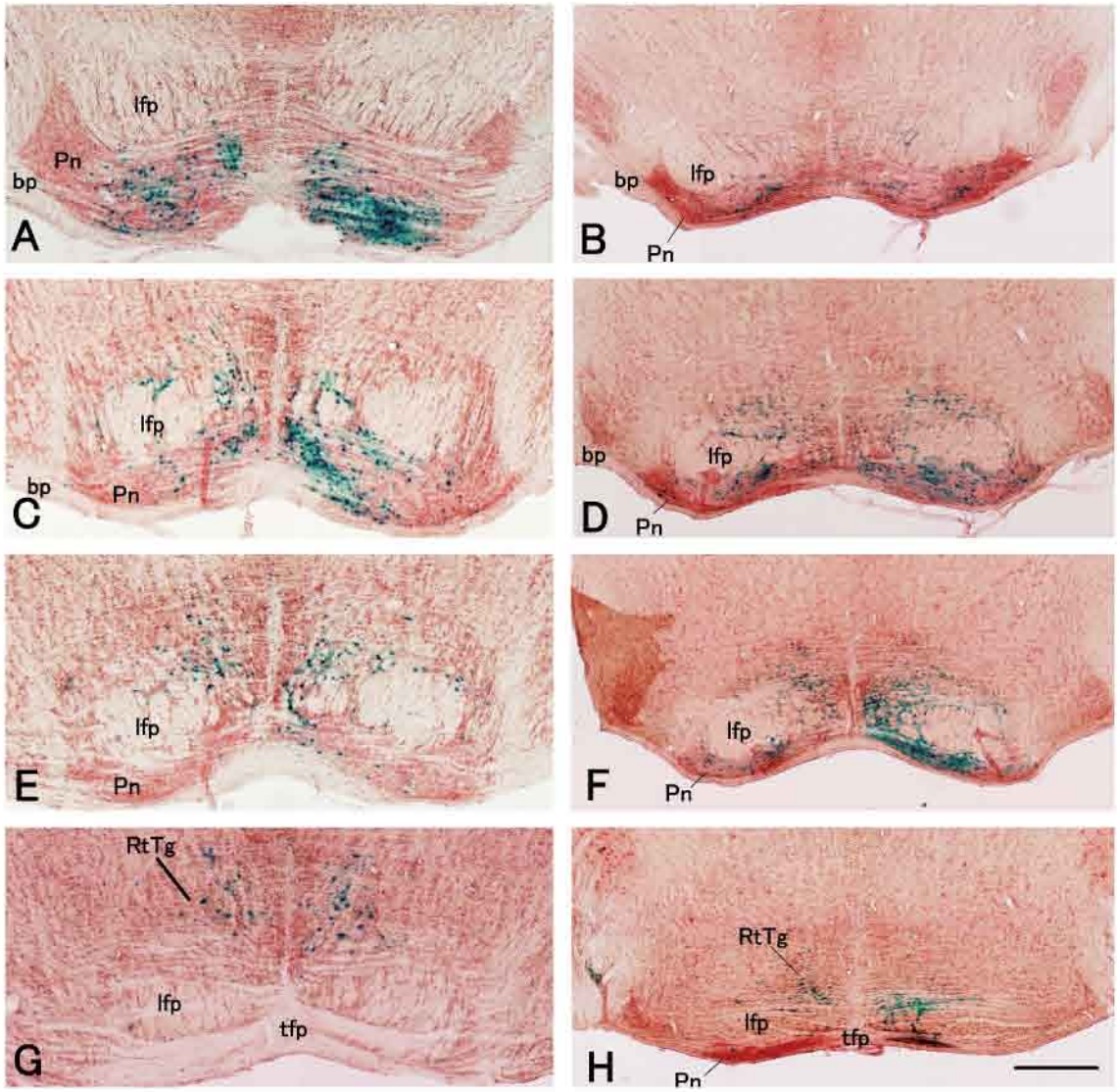

Fig. 5. Four pairs of coronal sections arranged in rostocaudal sequence show retrogradely labeled neurons in the pontine nuclei (Pn) and reticulotegmental nucleus of the pons (RtTg) after injection of the recombinant adenovirus into the left cerebellar cortex. LacZ-labeled Pn and RtTg neurons are reduced in number and more ventrally shifted in the reeler mouse (**B**, **D**, **F**, **H**) than those in a normal control (**A**, **C**, **E**, **G**). See other abbreviations in Figure 4. Neutral Red counterstaining. Scale bar = 200 μm (A–H)

While non-laminated structures in the brainstem of this mutant mouse had been long considered to be normal, Goffinet (1983, 1984) demonstrated an abnormal cytoarchitecture in the inferior olivary nuclei and the facial nucleus. More recently, other branchiogenic motor nuclei of the cranial nerves — including the motor trigeminal nucleus and ambiguus nucleus — have also been found cytoarchitectually disorganized in *reelin*-deficient animals, *reeler* and SRK (Terashima *et al.*, 1994; Fujimoto *et al.*, 1998; Setsu *et al.*, 2001; Ohshima *et al.*, 2002). The present study has confirmed that the Reelin protein exerts widespread effects on the central nervous system both in cortical and non-cortical structures.

Over a century ago, His (1891) identified the region

along the dorsal edge of the fourth ventricle of human embryos as the rhombic lip. The precerebellar nuclei neurons are born in the rhombic lip, and undertake a long journey to settle in the final loci in the brainstem (Wang *et al.*, 2005). The rhombic lip is a germinal zone in the alar plate forming the wall of the 4th ventricle (Jacobson, 1991). The most rostral part of the rhombic lip gives rise to the external granular layer of the cerebellar cortex, the more caudal parts of which give rise to cells that migrate to form the inferior olivary nuclei, cochlear nuclei, and pontine nuclei (Harkmark, 1954; Taber Pierce, 1966, 1967, 1973). The basic helix-loop-helix transcription factor *Mouse atonal homolog 1* (*Math1*) is required for development of the cerebellar granule neurons and mossy-fiber nuclei but not for the inferior olivary nuclei (Ben-Arie *et al.*, 1997, 2000; Bermingham *et al.*, 2001).

A recent study using *Math1*-heterologous *and Math1-null* mice that carry the lacZ gene in the *Mash1*-open reading frame have demonstrated four migratory streams from the Mash1-positive rhombic lip, *i.e.*, the rostral rhombic lip migratory stream, cochlear extramural migratory stream, anterior extramural migratory stream, and posterior extramural migratory stream (Wang *et al.*, 2005). The rostral rhombic lip migratory stream generates some neurons of the parabrachial, lateral lemniscal, and deep cerebellar nuclei, in addition to cerebellar granule neurons. The cochlear migratory stream generates the ventral cochlear nucleus and cochlear granule neurons. The anterior extramural migratory stream produces neurons in the pontine nuclei and reticulotegmental nucleus of the pons. The posterior extramural migratory stream generates neurons in the external cuneate nucleus and the lateral reticular nucleus. Among these migratory streams, neurons migrating along the rostral rhombic lip migratory stream and those migrating along the cochlear migratory stream are known to be ectopically distributed in the *reeler* mouse (Martin, 1981; Yuasa *et al.*, 1993; Takaoka *et al.*, 2005, Englund *et al.*, 2006).

The present study has confirmed that neurons in the pontine nuclei and the reticulotegmental nucleus of the pons, which migrate along the anterior extramural migration stream, were ventrolaterally shifted in the *reeler* mouse compared with those in the normal counterparts. It has further demonstrated that neurons in the external cuneate nucleus and lateral reticular nucleus, which migrate along the posterior extramural migratory stream, show very subtle abnormalities in their final positioning in the *reeler*. Finally, it is well known that migration of Mash1-negative inferior olivary nuclei neurons is disrupted in the *reeler* mouse (Blatt and Eisenman, 1985; 1988; Goffinet *et al.*, 1983, Ohshima *et al.*, 2002). Taken together with the present and previous studies, these findings confirm that this migration from the rhombic lip to the final loci in the brainstem is generally disrupted in the *reeler* mouse.

Although the mechanisms to cause disorders in the migration and final settlement of these precerebellar nuclei neurons reminds obscure, it should be noted that pontine nuclei neurons of the mouse embryo express *reelin* mRNA or the Reelin protein (Ikeda and Terashima, 1997; Schiffmann *et al*, 1997; Martinez-Cerdeno *et al.*, 2003) during embryonic and early postnatal days, suggesting that the Reelin protein may serve to guide the migration of pontine nuclei neurons along the anterior extramural migration stream. Although there are no data to show the transient expression of *reelin* mRNA by neurons in the lateral reticular nucleus or those in the external cuneate nucleus, cerebellar granule cells and cochlear granule cells express reelin mRNA and Reelin protein during embryological periods (Ikeda and Terashima, 1997; Takaoka *et al.*, 2005), the migration and final positioning of rhombic-lip-derived neurons may be regulated by the Reelin protein. X-irradiation of pregnant rats during the period of generation and migration of pontine nuclei neurons has been shown to kill proliferating neurons and induce migration defects (D'Amato and Hicks, 1980; Jensen and Killackey, 1984; O'Leary *et al.*, 1990). In this case, basilar pontine neurons are ectopically scattered in the brainstem along the course of the anterior rhombic lip migratory stream. However, in the case of the *reeler* mouse, pontine nuclei neurons as well as other precerebellar nuclei neurons almost succeed in the migration except for the final stage, *i.e.*, positioning, and therefore, the cytoarchitectual anomalies recognized in precerebellar nuclei are very subtle compared with those in the X-irradiated pups, suggesting that the Reelin protein may exert some influence at the final stage of migration.

Recently, Kawauchi *et al.* (2006) succeeded in the direct visualization of precerebellar nuclei migration using *exo utero* electroporation with an enhanced yellow fluorescent protein (EYFP) gene and found that EYFP-labeled precerebellar nuclei neurons migrate tangentially along the extramural migratory streams, and then radially towards the ventricle along the radial fibers. In the *reeler* mouse, neurons both in the pontine nuclei and external cuneate nucleus appear to be more superficially distributed compared with their normal counterparts, suggesting that the second phase of migration, *i.e.*, ventricle-directed, radial fiber-associated migration, may be disrupted in this mutant. In the *reeler*, the timely detachment of migrating neurons from radial (glial) fibers is obstructed, as has been repeatedly reported (Pinto-Lord *et al.*, 1982; Goffinet, 1986; Sanada *et al.*, 2004; Zhao

et al., 2004). Obstructed ventricle-directed migration of precerebellar nuclei neurons along radial fibers may result in a more superficial distribution of pontocerebellar and cuneocerebellar projection neurons in this mutant. Further studies employing time-lapse anatomical investigations and genetic studies on Reelin shall have to clarify whether or not the multi-phase migration (*i.e.*, the first-phase tangential migration and the second-phase ventricle-directed radial migration) is normal in this mutant mouse.

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