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論文名：大脳皮質及び基底核の発達形成におけるリーラー遺伝子産物リーリンの役割

(Roles of *reeler* gene product reelin in the developmental formation of the cerebral cortex and the basal ganglia)

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Contents

1. Summary

The *reeler* gene product reelin controls neuronal migration and positioning and thereby plays a key role in brain development. Mutation of reelin leads to widespread disruption of laminar cortical regions and ectopia in some brainstem nuclei. In this issue, we demonstrate new possible roles of Reelin in the developmental formation of the cerebral cortex and the bas al ganglia.

In the embryonic striatum of normal mice, a substantial expression of reelin mRNA has been documented, however, the anomalous positioning of neurons in the basal ganglia of reeler mice remains to be studied. We provide first evidence for the potential role of the reelin in the developmental formation of the substantia nigra. In *reeler* mutant mice lacking reelin, dopaminergic neurons destined for the substantial nigra fail to migrate laterally and become anomalously clustered just lateral to the ventral tegmental area. Their axons appear to project to striatal patches forming "dopamine islands". Results from the normal mice showed that at the mid-embryonic stage, reelin identified with CR-50 is highly concentrated in the ventral mesencephalon where nigral dopaminergic neurons are in progress to migrate laterally to their eventual position of the adult brain. A combination of CR-50 labeling and anterograde axonal tracing provided evidence that embryonic striatal neurons may supply the ventral portion of the mesencephalon with reelin through their axonal projections. We hypothesize that reelin plays a role in the positioning of nigral dopaminergic neurons, and that it can act as an environmental cue at a remote site far from its birthplace via a trans-axonal delivery system.

The mammalian cerebral cortex is organized into horizontal and vertical arrays of neurons and their fiber connections that form anatomically and physiologically distinct laminar and columnar compartments. However, the dev elopmental mechanism(s)

underlying this dichotomous pattern remains mysterious. Here we provide anatomical evidence that Cajal-Retzius cells and reelin are necessary for the developmental formation of both horizontal and vertical cell structures in the mo use presubicular cortex, the unique site where the vertical columnar arrays of cortical plate neurons and their dendritic branches are identified during the early postnatal period. Our results strongly suggest that reelin plays a role in the formation of these vertical structures by acting as a stop signal for cortical plate neurons and their dendritic extensions. We hypothesize that Cajal-Retzius cells and reelin control horizontal and vertical positioning of cortical plate neurons that is indispensable to the developmental formation of laminar and vertical columnar compartments in the cerebral cortex.

2. List of published papers

1. Nishikawa S, Goto S, Hamasaki T, Yamada K, Ushio Y, Involvement of R eelin and Cajal-Retzius Cells in the Developme ntal Formation of Vertical Columnar Structures in the Cerebral Cortex: Evidence from the Study of Mouse Presubicular Cortex. Cereb Cortex 12: 1024-1030, 2002.
2. Nishikawa S, Goto S, Hamasaki T, Ushio Y, Transient and Compartmental Expression of the *Reeler* Gene Product Reelin in the Developing Rat Striatum. Brain Res 850: 244-248, 1999.
3. Nishikawa S, Goto S, Yamada K, Hamasaki T, Ushio Y, Lack of R eelin Causes Mal-positioning of Nigral Dopaminergic Neurons: Evidence from Comparison of Normal and $Reln^{rl}$ Mutant Mice. J Comp Neurol (in press).

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4. Abbreviations

AchE = acetylcholine estelase

ApoE(R) = apolipoprotein E (receptor)

BrdU = 5- bromodeoxyuridine

CNR = cadherin-related neuronal receptor

CNS = central nervous system

CP = cortical plate

CR cell = Cajal-Retzius cell

DA = dopaminergic

Dab (dab) = *Drosophilia gene disabled*

DiI = 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyamine perchlorate

E*n* = embryonic day *n*

GAD = glutamic acid decarboxylase

IG = infragranular layer

IZ = intermediate zone

MAP = microtuble-associated protein

MZ = marginal zone

NCAM-H = highly-polysialated neuronal cell adhesion molecules

PB = phosphate buffer

PBS = phosphate buffered saline

PFA = paraformaldehyde

P*n* = postntal day *n*

PP = preplate

RF = radial fiber

rl = *reeler*

SG = supragranular layer

SNc = substantia nigra pars compacta

SNr = substantia nigra pars reticulata

SVZ = subventricular zone

TF = tangential fiber

TH = tyrosine hydroxylase

VM = ventral mesencephalon

VTA = ventral tegmental area

VZ = ventricular zone

VLDL(R) = very low density lipoprotein (rece ptor)

5. Background and purpose

5-(1). *Reeler* mutant mice, reelin, and CR-50

The *reeler*, an autosomal-recessive mouse mutant (Falconer, 1951), has provided a genetic model of brain development for half a century. It leads to impaired motor coordination, tremors and ataxia, but not to seizure. As listed in table 1, the *reeler* mutant mouse displays unique and interesting anomalies of central nervous system, including the cerebral cortex (Caviness and Sidman, 1973a), hippocampal formation (Caviness and Sidman. 1973b; Stanfield and Cowan, 1979), cerebellum (Rakic, 1976; Mariani *et al*., 1977; Mikoshiba *et al*., 1980), olfactory bulb (Caviness ad Sidman, 1972; Wyss *et al*., 1980), dorsal cochlear nucleus (Martin, 1981), facial nerve nucleus (Goffinet, 1984), trigeminal nucleus (Terashima *et al*., 1994), inferior olivary nucleus (Goffinet *et al*., 1983), ambiguous nucleus (Fujimoto *et al*., 1998), and spinal automatic system (Yip *et al*., 2000).

In the developing neocortex of normal mice, postmitotic neur ons generate in the ventricular germinal zone and migrate along radial fibers. Then postmitotic neurons reach their final destination in the so-called "inside-out" manner; late-born neurons locate in the outer layer of cortex, and early-born neurons do in the inner layer of cortex. In *reeler* mutant mice, the histological organization of the neocortex develops abnormally and essentially results in an inversion of the relative positions of the cortical layers. In the developing neocortex of normal mice, pr imitive layer of early-generated neurons (i.e., the prelate) is formed underneath pial surface (Bayer and Altman, 1991; Jacobson, 1993). The preplate then splits into the two components, the marginal zone and the subplate layer, and the young postmitotic neurons that have migrated along radial glial fibers from the germinal ventricular zone (Rakic, 1995) form the cortical plate between these

components (Marin-Padilla, 1998). The preplate of *reeler* mutant mice cannot split into these two components, and then young postmitotic neurons finally position underneath the preplate. It has been thought that cell differentiation and tangential organization of *reeler* neocortex are normal despite cell mal-positioning in this mutant (Caviness, 1976).

Cerebellar hemisphere of *reeler* mice is hypoplastic, and has no foliation. At the end of their radial migration in normal mice, Purkinje cells settle in the plate where they assume a radial orientation and a regular distribution. In *reeler*, there is the defect of this Purkinje cell arrangement leading to the reduced tangential growth of the cortex and the ectopia of Purkinje cells (Goffinet, 1984a, Yuasa *et al*., 1993). Because the external granular cell layer has normal thickness despite reduced cortical surface in reeler, the (external) granular cells are reduced in *reeler* mice (Mariani *et al*., 1976, Goffinet, 1984a).

Chimera mice produced from embryos of the wild-type and the *reeler* mutant mouse by the aggregation technique displayed no abnormality in the cerebellum: the neuronal and glial subpopulations revealed no difference from those of the control (Terashima, 1986). Therefore, it was thought that *reeler* gene product, which contributes to *reeler* phenotype, is diffusible molecule. It had elucidated that the *reeler* mutation in the mouse maps to proximal chromosome 5 (Bar *et al*., 1995), but the gene contributed to *reeler* phenotype had not been identified until recently.

In 1995, D'Alcangelo *et al*. (1995) identified a strong candidate cDNA for the mouse *reeler* gene. This 5 kb transcript encodes a 99.4 kD protein consisting of 881 amino acids and possessing two EGF-like motifs. The encoded protein resembles extracellular matrix proteins involved in cell adhesion (Fig. 1). Hirotsune *et al*. (1995) assayed two independent mutant alleles--'Jackson *reeler*', which has a deletion of the entire gene, and 'Orleans *reeler*' which exhibits a 220 bp deletion in the open reading frame, including the second EGF-like motif and resulting in a frame shift. In Orleans

reeler, *reeler* gene product was identified in cell body, but not secreted out of cell body. So these studies speculated that this protein is diffusible protein, and C -terminal of this protein is responsible for its secretion. This product of *reeler* gene was named to r eelin.

During corticogenesis in normal mice, reelin is produced by preplate Cajal-Retzius cells (D'Arcangelo *et al.*; Hirotsune *et al.*; Ogawa *et al*, 1995), which are located just below the pial surface. The vertical position of cortical plate neurons is determined by the time of their origination. and then clear horizontal layering is formed in the cortex (Rakic, 1995). In *reeler* mutant mice, Cajal-Retzius cells do not produce reelin and the preplate does not split. As a consequence, the cortical plate ectopically locates underneath the subplate neurons and the characteristic inside -out layering is perturbed. This is evidence for the role of reelin in the vertical positioning of cortical neurons that is essential for the formation of horizontal laminae of the cortex. Subsequent studies demonstrated that reelin was expressed in early -generated neurons of developing stages (i.e., Cajal-Retzius cells of cerebral cortex and hippocampus, granular cell of cerebellum), and interneurons of adult stage (interne urons of cortex, and hippocampus, olfactory bulb). To date, reelin is thought to be as a crucial molecule that defines architectonic patterns by controlling neuronal migration (D'Arcangelo *et a*l., 1995; Ogawa *et al.*, 1995; Miyata *et al.*, 1997; Nakajima *et al.*, 1997; Dulabon *et al.*. 2000; Yip *et al.*, 2000), and axon growth and synaptic connectivity (Del R.o *et al.*, 1997; Ghosh, 1997; Borrell *et al.*, 1999; Rice *et al.*, 2001).

Curran (1998) suggested that reelin might be signaling the migrating neurons to insert between the marginal zone at the top of the sandwich and the so -called subplate neurons at the bottom. The other side, Nakajima (1997) proposed that reelin acts as a stop-signal for each wave of arriving cortical neurons, telling them to get off the radial fibers and develop into a layer of mature neurons just under the marginal zone. But there

are other possibilities that reelin allows insertion because it creates spaces by repelling the subplate neurons (Curran and D'Alcangelo, 1998). Until now, the actual mechanisms of reelin remain unknown.

Recently, reelin receptors and other molecules involved in the reelin signaling cascades have also been identified (Rice and Curran, 2001). Dab1 is one of the intracellular signaling components that respond to reelin (Howell *et al.*, 1997). The gene encodes a cytoplasmic protein, mDab1 p80, which is expressed and tyrosine-phosphorylated in the developing nervous system. It is an adaptor protein, docking to others through its phosphotyrosine residues and protein-interacting domain. Two autosomal recessive mutations, *scrambler* (Sweet *et al.*, 1996) and *yotari* (Yoneshima *et al.*, 1997) have been described that exhibit a phenotype identical to *reeler* (Sweet *et al.*, 1996, Yoneshima *et al.*, 1997, Goldowitz *et al.*, 1997). Sheldon *et al.* (1997) demonstrated that *scrambler* and *yotari* arise from mutation in mdab1. Both *scrambler* and *yotari* mice express mutated forms of mdab1 mRNA and little or no mDab1 protein. During development, cells expressing Dab1 are located next to those secreting reelin at critical stages of formation of the cerebral cortex, cerebellum and hippocampus, before the first abnormalities in cell position become apparent. It has been thought that Dab1 functions downstream of reelin in a signaling pathway that controls cell positioning in the developing brain (Rice *et al.* 1998). Three membrane proteins, ApoE receptor 2 (apoER2), VLDL receptor (VLDLR) and cadherin-related neuronal receptor (CNR) are identified to bind reelin (Hiesberger *et al.* 1999, Senzaki *et al.* 1999). The double knockout of apoER2 and VLDLR shows a phenotype indistinguishable from reelin deficient mice (Trommsdoff *et al.* 1999). These three classes of membrane proteins are thought to act in concert as components of the reelin receptor.

Ogawa *et al.* (1995) had generated a monoclonal antibody that probes a distinct allelic antigen present in wild -type but not in *reeler* mutant mice. This antibody, named CR-50, was produced by immunizing adult normal mice with homogenized *reeler* brain. Several experiments revealed that CR-50 reacts specifically with N-terminal of reelin (Fig. 1), and prevents functions of reelin in experimental environments. When dissociated cerebral cortical cells were incubated with CR -50 in reaggregation culture, the genotype-dependent histogenetic assembly of wild -type cortical cells resembled that of *reeler* mutants (Ogawa *et al.*, 1995). Nakajima *et al.* (1997) demonstrated that intraventricular injection of CR -50 at the embryonic stage disrupts the organized development of the hippocampus *in vivo*, converting it to a *reeler* pattern. Labeling experiments with 5 - bromodeoxyuridine demonstrated that the labeled cells in the stratum pyramidale of the CR -50-treated mice were distributed in a pattern similar to that of *reeler*. So, It has thought to be that N -terminal of reelin is responsible for reelin function.

5-(2). Dopaminergic system of the mammalian brain and its development

Dopaminergic neurons in the mesencephalon include those in the substantia nigra (A9 cell group) and ventral tegmental area (A10 cell group)(Dahlstom and Fuxe, 1964). They are major source for DA inputs to the striatum that plays a central role in a wide variety of motor, cognitive and emotional function. The striatum, whose development requires different types of neurons originating from distinct regions of the telencephalon (Hamasaki *et al.*, 2002), is characterized by an architecture that divided it into two distinct compartments, patches and matrix (Graybiel, 1990; Gerfen, 1992). There are reciprocal fiber connections between the striatal compartments and the mesencephalic DA neurons located in the ventral tier of the substantia nigra, which in turn send DA outputs to the striatal patches (Gerfen, 1992)(Fig. 2).

5-(3). Vertical columnar structure of cortex

Neuronal migration and positioning are essential steps in the genesis of the nervous system. The mammalian cerebral cortex is a highly ordered brain structure and its fundamental framework is similar in all mammals (Jacobson, 1993). The cerebral cortex is organized into clear laminae: the different classes of neurons reside in an organized radial array of horizontal cellular layers ranging from the pial surface to the white matter (Eccles, 1984; Mountcastle, 1997). There also are vertical columnar arrays of neurons running orthogonal to the horizontal laminae (Eccles, 1984; Mountcastle, 1997): they are particularly conspicuous in the temporal cortex of humans and other primates (Jones, 2000). This dichotomous pattern of cortical cytoarchitecture appears to be the resultant of several cellular and molecular processes that occur in spatial and temporal sequence on development. However, to date, this intriguing issue remains mysterious.

5-(4). Purpose of this study

5-(4)-i. Role of reelin in the basal ganglia development

We report the possible role of the precise positioning of their target cells, i.e., nigral DA neurons, during murine embryogenesis. We now report that in *reeler* mutant mice, the lack of reelin leads to the failure of DA neurons destined for the substantia nigra to migrate laterally, resulting in their anomalous positioning. We provide evidence that at embryonic stage, young striatal cells destined for patches neurons produce reelin, and that they transport this diffusible protein axonally to the ventral mesencephalon where reelin controls the positioning of nigral DA neurons. We hypothesized that reelin controls nigral DA neurons and that it can function as a local environmental cue not only in areas adjacent to its birthplace, but, upon axonal transport, also at remote sites distant from its place of origin.

5-(4)-ii. Role of the reelin in the cerebral cortex development

We report that vertical columnar arrays of cortical neurons and their dendritic processes can be found in the presubiculum, a multi -layered and periallocortical structure (O'Mara *et al*., 2001), of the developing mouse brain. They provide a good model for investigating the cellular and molecular cues that direct both the vertical and horizontal positioning of postmitotic neurons in the cerebral cortex. We provide anatomical evidence that reelin controls the developmental formation of the vertical structures in the presubicular cortex by acting as a stop signal for cortical plate neurons and their den dritic processes. We suggest that Cajal -Retzius cells and reelin play a role in the formation of the spatially distinct anatomical units organized horizontally and perpendicularly to the pial surface during corticogenesis.

6. Methods

6-(1). Animals

The *reeler* mouse colony was originally derived from heterozygous B6C3Fe -a/a-rl adults (The Jackson Laboratory). Timed pregnancies were induced by mating homozygous (*rl/rl*) males to heterozygous (+/*rl*) females. The morning on which vaginal plug was detected was recorded as embryonic day 0 (E0); the day of birth was recorded as postnatal day 0 (P0). Embryos and neonates of known age were kept in an in -house breeding colony. Care of the animals was in accordance with regulations promulgated by the Center for Animal Resources and Development of Kumamoto University (CARD).

6-(2). Tissue preparations

The mice received an intraperitoneal injection of a lethal dose of pentobarbital and were perfused transcardially with 0.9% (wt/vol) saline in 0.01M phosp hate buffer, pH 7.4 (PBS), followed by ice -cold 4% (wt/vol) paraformaldehyde in 0.1M phosphate buffer, pH 7.4 (PB). The brains, removed and postfixed with same fixative at 4 .C overnight and kept in 0.1M PB containing 30% (wt/vol) sucrose at 4 .C overnight for cryoprotection, were them embedded in OCT compound (Sakura Finetechnical, Japan) and frozen in dry-ice/acetone. Cryostat sections were cut and kept in PBS until use.

6-(3). Immunofluorescence stainings

CR-50, a mouse monoclonal antibody that recogniz ed an epitope in the N-terminal of reelin (D'Arcangelo *et al*, 1997) was a gift from Dr. M. Ogawa, The institute of Physical and Chemical Research (RIKEN), Japan (Ogawa *et al*, 1995, Hamasaki *et al*, 2001a). To label mesencephalic DA neurons, a rabbit polycl onal antibody to tyrosine hydroxylase (TH) was used. Monoclonal antibodies to highly -polysialated neuronal cell adhesion molecules (NCAM -H) and vimentin, microtubule -associated protein 2 (MAP2)

(Sigma, MO) were also used to visualize the tangential fiber a nd radial glial fibers in the embryonic VM, respectively. And polyclonal antibody to glutamic acid decarboxylase (GAD_{67})(Vector) was used for labeling of GABAergic neuron. The sections were blocked with 3% bovine serum albumin (BSA) -PBS for 1 h and then incubated overnight at 4 .C in 3% (wt/vol) BSA-PBS containing primary antibody. Immunoreactivity was detected by FITC- (Vector, CA) or Texas Red - (Vector) conjugated secondary antibodies. Propidium iodide (PI) staining was also used (Hamasaki et al., 2001 b). The fluorescence activities were observed and recorded under a confocal laser -scanning microscope (Fluoview, Olympus, Japan). The obtained images were printed using Pictrography 3000 (Fuji film, Japan).

6-(4). Collagen gel culture

Wister-ST rats at P0 were killed by decapitation and the brain was quickly removed. The striata were microsurgically dissected in 0.9% saline containing 0.5% trypsin and 0.25% glucose for 10 min at 37C. The dispersed striatal cells were immediately mixed with the collag en gel composed of acidic collagen solution. The acidic collagen solution was reconstructed with 0.23M NaOH and 0.14 M NaHCO3. The cell density was approximately 1 X 10^6/ml. The collagen gel containing striatal cells was placed on a 35 mm culture dish, and incubated in the culture medium containing 15.6 mg/ml of DMEM/F12, 10% fetal bovine serum, 1% N2 supplement (Gibco, BRL), 1.2 mg/ml of NaHCOH3, 10mM 2 -Mercaptometanol and 3.85 mg/ml of glucose, pH 7.20. The culture dishes were placed in an incubator fo r 3days. The striatal cell embedded in the collagen gel were fixed with 4% PFA and then processed for the histological study.

6-(5). Anterograde labeling of striatal efferent projections

To label the striatal efferent projections, we used the axonal tra cer DiI (Molecular probes. Eugene, OR). Embryonic (E15) brains were removed and fixed for 8 hr with 4%

(wt/vol) paraformaldehyde in 0.1 M PB. After removing a part of the cortical mantle, DiI crystal were transventricularly implanted into the striatal primordium using tungsten needles under a dissecting microscope. After 4 weeks, the brains were frozen, and cryostat sections were prepared. Some sections were then processed for TH - or CR-50 immunolabeling.

6-(6). Cell counting Method

Using our previously reported method (Yamada et al., 1996), the number of TH-positive neurons was counted on the light microscopic photographs from 30 -μm-thick transverse frozen sections. All DA neurons containing clear nuclei were counted in the entire VM at the level of the oculomotor nerve. The Abercrombie method was used as a correction formula (Abercrombie, 1946). In *+/rl* mice, the number of DA neurons in the VM includes those in A9 and A10, while in *rl/rl* mice that in the VM includes those in A9 and A10 and that in the ectopia. All data were evaluated on a double -blind basis by three observers in our laboratory. Data were expressed as the means ± S.D. We used two-way analysis of variance followed by the paired *t*-test to assess the statistical significance.

6-(7). Scoring of apical dendrite orientation

Apical dendrites of cortical pyramidal neurons from the presubicular cortex of heterozygous (*+/rl*) or homozygous (*rl/rl*) mice aged P14 were visualized with anti -MAP2 antibody and recorded under a confocal laser -scanning microscope (Fluoview). According to the previous report (Polleux et al., 2000), apical dendrites were scored as being directed towards the pia (45°-135°), towards the white matter (225°-315°), or horizontally (135°-225° or 45°-315°).

7. Results

7-(1). Role of reelin in migration of mesencephalic DA neurons

7-(1)-i. Anomalous positioning of nigral DA neurons in *reeler* mutant mice

Murine mesencephalic DA neurons are generated in the germinal ventricular zone around E10-12, and subsequently migrate ventrally. This leads to their accumulation in the VM around E15, thereafter, subpopulations migrate laterally and finally form the A9 and A10 cell groups (Ohyama *et al.*, 1998; Demyanenko *et al.*, 2001). Our preliminary study revealed that the developmental profile of mesencephalic DA neurons was the same in wild-type (+/+) and heterozygous (+/*rl*) mice. Therefore, to explore the potential role of reelin in the positioning of DA neurons destined for the substantia nigra (A9 cell group), we carried out comparative studies on heterozygous and homozygous mice whose genetic background is the same except for the *reeler* gene mutation.

In the E15 mesencephalon, the distribution pattern of DA neurons was very similar in homozygous and heterozygous mice (fig. 3A, B). At P0, A9 and A10 cell group normally formed in the heterozygous mice, while in homozygous mice (Fig. 3C). DA neurons had almost disappeared in the substantia nigra. Therefore, A9 cell group could not be identified, while A10 cell group appeared to be normal (Fig. 3D). Anomalous cluster of DA neurons just lateral to the VTA were present in homozygous (Fig. 3E) but not in heterozygous mice (Fig. 3F). A schematic representation of these findings is depicted in figure 4A, B. Altered distribution of DA neurons destined for the substantia nigra (A9 cell group) was also found in P28 and adult homozygous mice (not shown). Quantitative study on P0 mice disclosed that in homozygous mice there was a marked decrease in the number of DA neurons of the A9 cell group (1.8 ± 1.4; n=6) compared to heterozygous mice (59.6 ± 14.1; n=6) (Fig. 4C). The number of DA neurons in the VM of homozygous

mice (154.5 ± 14.9; n=6) was almost the same as in heterozygous mice (149.9 ± 25.0; n=6) (Fig. 4D). These observations suggest that the hypoplasia if A9 cell group in the homozygous mice resulted from a failure of DA neurons destined for the substantia nigra to migrate laterally, rather than from a lack of their generation. With respect to the currently known substrates that guide the migration of mesencephalic DA neurons, i.e. radial glial fibers (Fig. 5A, B) and tangential fibers (Fig. 5C, D), there was no difference between heterozygous and homozygous mice.

7-(1)-ii. Preserved formation of the "dopamine islands" in *reeler* mutant mice

Since axonal projections from nigral DA neurons preferentially innervate striatal patches, patch compartments transiently show heightened TH-staining and are called "dopamine islands". These are observed during the perinatal period before the arrival of massive DA projections from the VTA neurons. To see whether theses patch -directed innervations are formed in *reeler*, we carried out TH staining of the P0 striatum. We found that both heterozygous and homozygous mice manifested discrete patches visualized by TH-staining (i.e. dopamine islands)(Fig. 6A, B). The percentage of the striatum occupied by patch compartments was almos t the same in heterozygous and homozygous mice (12.5±4.3; n=8 vs. 11.9±3.9; n=8)(Fig. 6C). Furthermore, there was no apparent difference between heterozygous and homozygous mice with respect to the number of TH-immunoreactive dots in 100mm x 100mm squares of striatal patches (347±84; n=8 vs. 350±70; n=8)(Fig. 6D). These observations suggest that despite their mal-positioning, the nigral DA neurons of homozygous mice can sent their axonal projections to the striatum and form dopamine islands.

7-(1)-iii. Reelin expression in the mesencephalon and its origin in normal mice

Using CR-50 labeling, we next examine the distribution profile of reelin expression in the mesencephalon at E15 when nigral DA neurons proceed to migrate laterally to their

final positions. Robust CR-50 labeling was seen in the VM at E15 (Fig. 7A) and also at P0 (Fig. 7B), and the distributional correspondence between reelin expression and DA neurons was evident. Confocal microscope images showed that DA neurons were surrounded by numerous granular CR-50-labeled dots (Fig. 7C-D) reminiscent of axon terminal staining. Faint CR-50 immunoreactivity was also diffusely distributed in other extracellular areas. Confocal images using z -series analysis (Kornack and Rakic, 2001) disclosed that throughout the VM there were no cells of any type, including DA neurons, whose perikarya exhibited CR -50 labeling. Svhiffmann *et al*. (1997) and Alcántara *et al*., (1998) used *in situ* hybridization to show that at this embryonic period, reelin mRNA signals are detectable in the striatum but not in the VM. We posited the striatal primordium as the origin of the mesencephalic reelin expression we detect, because young striatal cells destined for patch neurons are known to sent their axons to form the early projections to the mesencephalon during embryogenesis (Fishell and van der Kooy, 1987).

To test our hypothesis, we first examined the localization of reelin in the E15 striatum. As shown in figure 8A, there was substantial CR -50 immunolabeling in the postmitotic region of the striatal primordium, but not in the subventricular and ventricular zones. Under the microscope, young neurons whose perikarya were labeled with CR -50 comprised this striatal postmitotic region (Fig. 8B). This observation suggested most of these neurons were fated to patch neurons since they are generated first around E13, while matrix neurons are late -born after E15 (Hamasaki *et al*., 2001b). This assumption is supported by the findings that in the P0 striatum, CR -50 labeling was differentially concentrated between patches and matrix showing a mosaic pattern: the patch neurons showed heightened CR-50 labeling (Fig. 8C) as well as TH -staining (Fig. 8D). To determine whether striatal efferent projections to the mesencephalon were formed at E15,

we directly injected DiI as an anterograde axonal tracer into the post -fixed E15 striatal primordium (Fig. 10A). There was substantial fluorescence activity in the VM, and the fluorescence distribution profile was similar to that noted in DA neurons and similar to CR-50 labeling (Fig. 7A). Under the confocal microscope, there were numerous dot -like DiI-labeled puncta (probably axon terminals) surrounding DA neurons (Fig. 10C -E). Furthermore, the DiI -labeled granular dots corresponded almost perfectl y with CR-50-labeled dots (Fig. 10F -H, I-K). Thus, taken together, these observations raise the possibility that at the embryonic stage, reelin is produced by young striatal patch neurons and is axonally transported from the striatal primordium to VM. Th is hypothesis is supported by the fact that GABAergic neurons, projection neuron of striatum, express reelin in developing striatum of rat (Fig. 9).

7-(2). Role of reelin in the developmental formation of vertical columnar structures

7-(2)-i. Vertical co lumnar arrays of neuronal cell bodies in the presubicular cortex

At age P3 and P4, vertical columnar arrays of neuronal cell bodies running orthogonal to the horizontal laminae were clearly present in the mouse presubicular cortex. Vertical columns were v isible even to the untutored eye in PI - (Fig. 11*A*) or Nissl- (Fig. 11*B,C*) stained preparations. They were conspicuous in the upper half of the cortical plate that corresponds to the supragranular layer (i.e., layers II and III), and had a periodicity of approximately 55. m (55.2±12.3. m) in the horizontal extent. Each vertical column was composed of a cluster of young postmitotic neurons; these columns were segregated a narrow inter -columnar space poor in neurons (Fig. 11 *B,C*).

7-(2)-ii. Relationship between reelin and v ertical columnar arrays of cortical plate neurons

At birth (P0), when neurons in layers IV -VI have arrived at their final position while neurons in layers II and III are still migrating toward their final destination (Jacobson,

1993). At this stage, the superficial marginal zone contained Cajal -Retzius cells strongly labeled with CR-50, a monoclonal antibody against reelin (Fig. 12A, B). Young postmitotic neurons stopped to migrate when they encountered the reelin -containing extracellular matrix of the m arginal zone and then formed the cortical plate beneath the marginal zone. At P0, we found no definite vertical structures in either the cortical plate or the marginal zone.

At P2-4, late-born cortical plate neurons have migrates from the germinal ventricular zone and are approaching to their final position to form the supragranular layer (Jacobson, 1993). At P2, PI staining showed an indication of vertical columnar arrays of neurons in the upper half of the cortical plate (i.e. the future supragranular layer; Fig. 12C). In the marginal zone (Fig.12D), CR -50 labeling was found in both the neuronal perikarya and neuropils of Cajal -Retzius cells, and in the surrounding extracellular area, probably due to the diffusible nature of reelin (D 'Arcangelo *et al*. 1997). Cajal-Retzius cells appeared to be proceeding to form clusters and CR -50 labeling was inhomogeneously distributed in the marginal zone (Fig. 12D -F). It seemed that cortical plate neurons preferentially accumulated underneath the marginal zone are a poor in Cajal -Retzius cells to form the supragranular layer (Fig. 12E -H). At P3 - 4, vertical columns composed of cortical plate neurons were clearly present in the supragranular layer (Fig. 12I -K), as described above. Interestingly, clusters of Cajal -Retzius cells formed well -delineated territories with a specific topography and CR -50 labeling showed a periodic modulation in the horizontal dimension tangential to the cortical lamination (Fig. 12J, K). Reelin -rich zones appeared to descend into the in ter-columnar spaces in the cortical plate (Fig. 12*J,K*); they contained the perpendicularly -oriented processes of Cajal-Retzius cells. Sections cut parallel to the cortical surface revealed a mosaic -like pattern of regularly spaced spots with low CR-50 immunoreactivity in the marginal zone (Fig. 12 *L*).

Comparison with adjacent PI-stained sections at the border between the marginal zone and the supragranular layer (Fig. 12 *M-O*) showed that the distribution of cortical plate neurons and CR-50 labeling was almost complementary; regions with poor CR-50 immunoreactivity contained clusters of cortical plate neurons. These observations suggest that cortical plate neurons arrange in a columnar fashion due to being stopped by reelin, and the horizontal positioning of cortical plate neurons seemed to be stereotypically defined by the specific territorial distribution of reelin secreted by Cajal-Retzius cells in the marginal zone.

7-(2)-iii. Relationship between reelin and dendritic clusters in the marginal zone

By P14, all cortical plate neurons had settled in their final positions and were engaged in the formation of fiber connections (Jacobson, 1993). The apical dendrites of cortical pyramidal cells of layers II, III and V form clusters that ascend through the cortical plate and send their terminal arrays to the marginal zone (Fleischhauer *et al.*, 1972; Peters and Walsh, 1972) (also see Fig. 15A). At this stage, the columnar arrays of neuronal cell bodies were no longer visible in the cortical plate (Fig. 13 *A,C*) although CR-50 labeling continued to show periodic modulation in the marginal zone (Fig. 13 *B,C*). Interestingly, reelin-poor zones contained columnar tufts of dendritic processes of cortical pyramidal neurons positive for MAP2 (Fig. 13D,E). Sections cut parallel to the cortical surface revealed a mosaic-like pattern of irregularly-spaced spots poor in CR-50 labeling (Fig. 13F) or enriched in MAP2 labeling (Fig. 3G) in the marginal zone. Comparison with adjacent sections in the marginal zone showed that the distribution of CR-50 labeling and MAP2-immunoreactive dendrites was almost complementary: regions with poor CR-50 immunoreactivity exhibited clusters of dendrites of cortical pyramidal neurons (Fig. 13H,I). Thus, the dendrites of cortical pyramidal neurons clustered by avoiding areas of reelin in the marginal zone, suggesting that reelin may act as a barrier to their dendritic

extensions.

In P28 and adult mice, Cajal-Retzius cells labeled with CR-50 were scarcely seen in the marginal zone (Fig. 13J). At this stage, columnar tufts of dendritic branches were no longer apparent in the marginal zone (Fig. 13K). Also, vertical columnar arrays of neurons were not clearly identifiable in the cortical plate (fig. 13L).

7-(2)-iv. Disruption of the vertical structures in *reeler* mutant mice

Compared to wild-type (*rl/rl*) or heterozygous (*+/rl*) mice (Fig. 14*A*), in homozygous (*rl/rl*) P3 mice there was no detectable vertical columnar arraying or horizontal layering of neuronal cell bodies in the cortical plate (Fig. 14*B*). At P14, clusters of dendritic branches of cortical pyramidal neurons in the marginal zone were clearly evident in wild-type and heterozygous littermates (Fig. 15A), but not in homozygous mice (Fig. 15B). Furthermore, the orientation and positioning of cortical plate neurons varied in homozygous mice (Caviness, 1976; Landrieu and Goffinet, 1981). Although the apical dendrites of cortical pyramidal neurons were oriented almost orthogonal to the pial surface in wild-type or heterozygous mice (Fig. 5A), they appeared to be often oblique, to run horizontally, or to be inverted in homozygous mice (Fig. 15B). Our semi-quantitative study (Fig. 15C) showed that most apical dendrites were towards the pial surface in the heterozygous (77.5 ±3.5%; n=200), but not in the homozygous (28.5 ±2.6%; n=200) mice. These observations indicate that Reelin function is required for the developmental formation of both horizontal lamination and the vertically-orientated cytoarchitectures in the mouse presubicular cortex.

8. Discussion

8-(1). Role of reelin in migration of mesencephalic DA neurons

The *reeler* gene mutation results in the mal-positioning of neurons, leading to disruption of architectonic patterning in many parts of the central nervous system. The ectopic cluster of nigral DA neurons we document is the first evidence that altered neuronal cell migration occurs in the *reeler* basal ganglia circuit. Our data also show that striatal "dopamine islands" that in normal mice are composed of axon terminals from nigral DA neurons, appeared to be normally formed in the homozygous mice. Projection neurons can send their axons, whose presynaptic terminals will accurately and efficiently target matching postsyanaptic targets, even if the neurons themselves are malpositioned (Jacobson, 1991). Therefore, we suggested that malpositioned nigral DA neurons in reeler mice could form the nigrostriatal pathway.

Based on the model proposed by Ohyama *et al.* (1998) of migration of mesencephalic DA neurons, we posit that nigral DA neuron s migrate to their given positions in the adult brain in three sequential phase (Fig. 16): first, they migrate toward the ventromedial mesencephalon along radial glial fibers with the DA neurons destined for the VTA; second, they migrate laterally in the b asal part of the mesencephalon along tangentially arranged nerve fibers with VTA neurons; and finally, by migrating further laterally, they separate from VTA. Our results shows that in *reeler* mice, the VTA (A10 cell group) appears to develop normally altho ugh the nigral DA neurons are anomalously positioned just lateral to the VTA. Therefore, we suggest that reelin function may be involved in the further lateral migration of nigral DA neurons leaving from the VTA and/or the tangential fibers.

Divergent hypothesis have been offered to explain reelin function in the CNS

development. For instance, reelin has been suggested to function as an inhibitory or stop signal for neuronal migration (Ogawa *et al.*, 1995, D'Arcangelo *et al.*, 1997; Curran and D'Arcangelo, 1998; Frotcher, 1998; Pearlman *et al.*, 1998; Dulabon *et al.*, 2000; Walsh and Goffinet, 2000; Yip *et al.*, 2000). On the other hand, Gilmore and Herrup (2000) suggested that reelin plays a role as a chemoattractant for migrating Purkinje cells. Challenge these propositions, a recent study by Magdaleno *et al.* (2002) found that reelin functions in concert with other positional cue to promote cell -cell interactions. The molecular process(es) by which reelin acts on the migration of nigral DA neurons remains an intriguing issue that awaits clarification.

Our study provides evidence that embryonic striatal neurons may supply the VM with reelin through their striato -mesencephalic projections. This observation gives rise to our hypothesis that reelin can function at sites remote from its origination through axonal transport. Until now, the action of reelin has been questioned in some brain structures because its distribution was thought to occur by simple diffusion into areas adjacent to its origin. We can now address reelin function in target structures of projection neurons that produce reelin (e.g., young striatal patch neurons). The lack of a correlation between the expression of reelin mRNA in some brain areas and the morphological abnormalities present in *reeler* mice has been noted (Schiffmann *et al.*, 1997; Alcántara *et al.*, 1998). For instance, there is prominent reelin mRNA expression in the striatum, septum, and hypothalamus, regions in which previous studies failed to detect cytoarchitectonic alternations in *reeler* mice (Alcántara *et al.*, 1998). As shown here, in the relationship between the striatum and target nucleus (i.e., the substantia nigra), due to axonal transport, reelin may play roles not in the nuclei of its origin, but in their target structures.

8-(2). Role of reelin in the developmental formation of vertical columnar structures

In addition to the horizontal layering, the microcolumn (or minicolumn) that consists of vertical arrays of neurons and their fiber connections functions as a fundamental anatomical processing unit of the cerebral cortex (Eccles, 1984; Mountcastle, 1997). While the microcolumns are thought to be produced by the iterative division of a small cluster of progenitor cells and to form a series of repeating units acros s the horizontal extent of the cortex (Mountcastle, 1997; Jones, 2000), vertical chains of cells are morphologically conspicuous only in the primate temporal cortex but not in other areas, nor in the cortex of non -primates.

In this study we showed the pr esence of two transient vertical structures, identified with simple anatomical techniques in the developing presubicular cortex (Fig. 17). One structure consists of the vertical columnar arrays of young cortical plate neurons in the supragranular layer. These occur most conspicuously at P3 -P4, when the late-born cortical neurons ascend in linear arrays along a scaffold of radial fibers of astroglia or are processing to their final positions (Jacobson, 1993). The other structure is comprised of columnar tufts of dendritic processes of cortical pyramidal neurons in the marginal zone that are most remarkably at P14 when dendritic deployment proceeds and the fiber connections are being established (Jacobson, 1993): it seems to be a prototype of the dendritic clusters that group cortical neurons into modules of microcolumnar size (Mountcastle, 1997). In P28 mice as well as in adult mice, these structures can no longer be clearly discerned because they are veiled by tightly-packed cell bodies and dendritic bran ches of cortical neurons. In this stage, there is only a small population of Cajal-Retzius cells in the marginal zone, as previously suggested (Vogt Weisenhorn *et al*, 1994; Del Río *et al*., 1995, 1996; Spreafico *et al*., 1995; Marin-Padilla, 1998). Our present result lead us to posit that the structures represent micro -anatomical units that

underlie the development of the territorial organization that results in the positioning of cortical neurons and their formation of fiber connections in the horizontal d imension. According to the radial unit hypothesis (Rakic, 1988), the horizontal coordinates of cortical neurons are determined by the relative position of their precursor cells in the germinal ventricular zone. It has been suggested that the germinal ven tricular zone contains a mosaic of ontogenetic units that are composed of the neuronal precursors for a cortical column; alternatively, the mosaicism of the germinal ventricular zone is reproducible in the cortical plate. It is presently unknown whether o r how the cellular microcolumns shown here are anatomically and functionally related to the microcolumns originating from the clonal modality. We are currently investigating the possible role of these microcolumns as functional units of the cerebral corte x in cortical activities related to intracortical fiber connections.

It has been suggested that reelin functions as an inhibitory or stop signal for neuronal migration that defines architectonic patterning of CNS (Ogawa *et al.*, 1995; D'Arcangelo *et al.*, 1997; Curran and D'Arcangelo, 1998; Frotscher, 1998; Pearlman *et al.*, 1998; Dulabon *et al.*, 2000; Walsh and Goffinet, 2000; Yip *et al.*, 2000). This hypothesis is strongly supported by our results. We found that at the early postnatal period, CR -50 labeling in the marginal zone appears to be organized in a mosaic pattern with periodic modulation in the horizontal dimension. This mosaicism of reelin statement resulting from the strategic location of Cajal-Retzius cells is almost complementary to that of vertical columnar arrays of cortical plate neurons or their dendritic tufts, which are both preferentially located in the reelin -poor zones. Thus, we suggest that reelin plays a role in the formation of the vertical columns shown here by acting as a stop si gnal for cortical plate neurons and their dendritic extensions.

Finally, we hypothesize that Cajal -Retzius cells and reelin in the marginal zone

may play a role in the formation of not only horizontal laminations, but also vertical columnar structures in the developing cerebral cortex. This is supported by the present finding that the vertical columnar structures shown here are totally disorganized in *reeler* mutant mice. Our hypothesis coincidates with suggestions that Cajal -Retzius cells may coordinate positional information essential for the early areal and columnar specification of the underlying cortex (Galuske and Singer, 1996; Schmidt *et al*., 1996; Schwartz *et al*., 1998; Soria and Fairén, 2000; Hevner *et al*., 2001; Zecevic and Rakic, 2001). However , our hypothesis is based on developmental and anatomical evidences found in a specialized region of the mouse cortex, i.e., the presubicular cortex. Further studies are needed to determine whether our hypothesis applies to other cortical areas of rodents or those of different species as a fundamental developmental mechanism of the cerebral cortex.

9. Conclusion

We conclude that reelin plays a role in the positioning of nigral dopaminergic neurons, and that it can act as an environmental cue at a remote site far from its birthplace via a trans-axonal delivery system. Cajal-Retzius cells and reelin control horizontal and vertical positioning of cortical plate neurons that is indispensable to the developmental formation of laminar and vertical columnar compartments in the cerebral cortex.

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Cerebral cortex	Disruption of cortical layering
hippocampus	Disruption of layering Reduction of dentate granular cells
Cerebellum	Malpositioning of Purkinje cells Reduction of granular cells
Olfactory bulb	Reduction of granular cells
Dorsal cochlear nuclei	Absence of AChE- positive fibers in the dorsal nucleus Reduction of granular cells
Trigeminal nerve nucleus	More widely scattering jaw-opening motoneurons
Facial nerve nucleus	Anomalous location and disorganization
Inferior olivary nuclei	Anomalous shape of principle olive
Ambiguus nucleus	Scattered arraignment
Spinal autonomic system	Anomalous positioning of sympathetic preganglionic neurons

Table 1. Anatomical abnormalities of *reeler* mutant mice

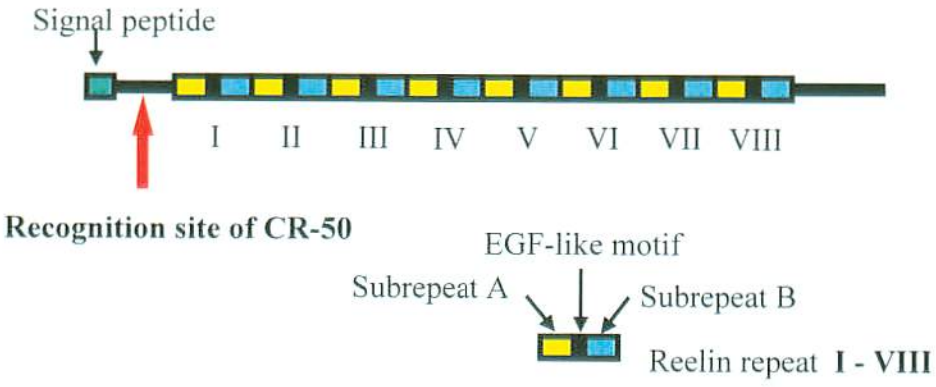

Fig. 1. Schematic representation of the Reelin structure.

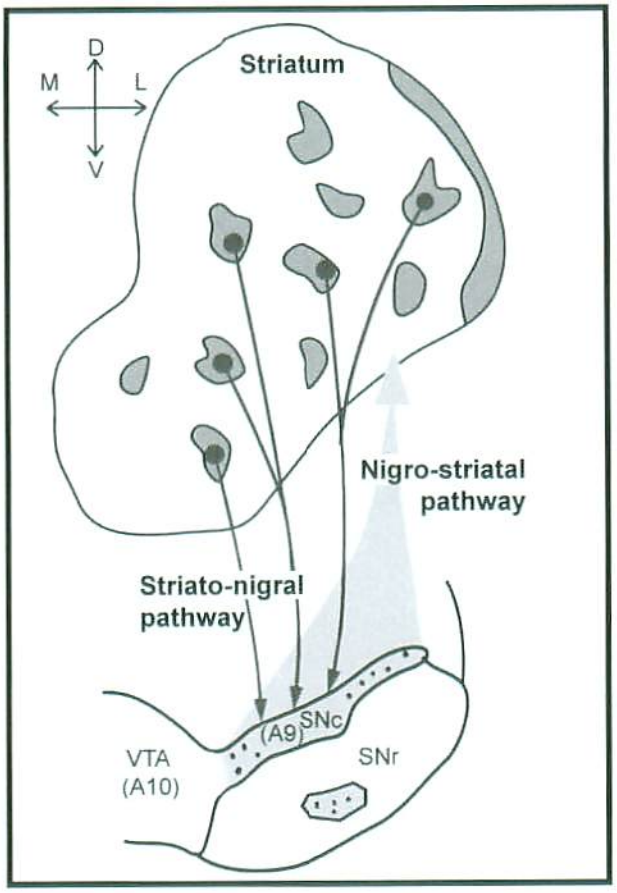

Fig. 2. Schematic representation of reciprocal connections between the striatum and substantia nigra pars compacta. The striatum is divided into two distinct compartments, patches (gray islands) and matrix. Striatal patch neurons project their efferent axons principally to the nigral dopaminergic neurons constituting the striato-nigral pathway. Nigral dopaminergic cells send their projections to the striatum forming the nigro-striatal pathway.

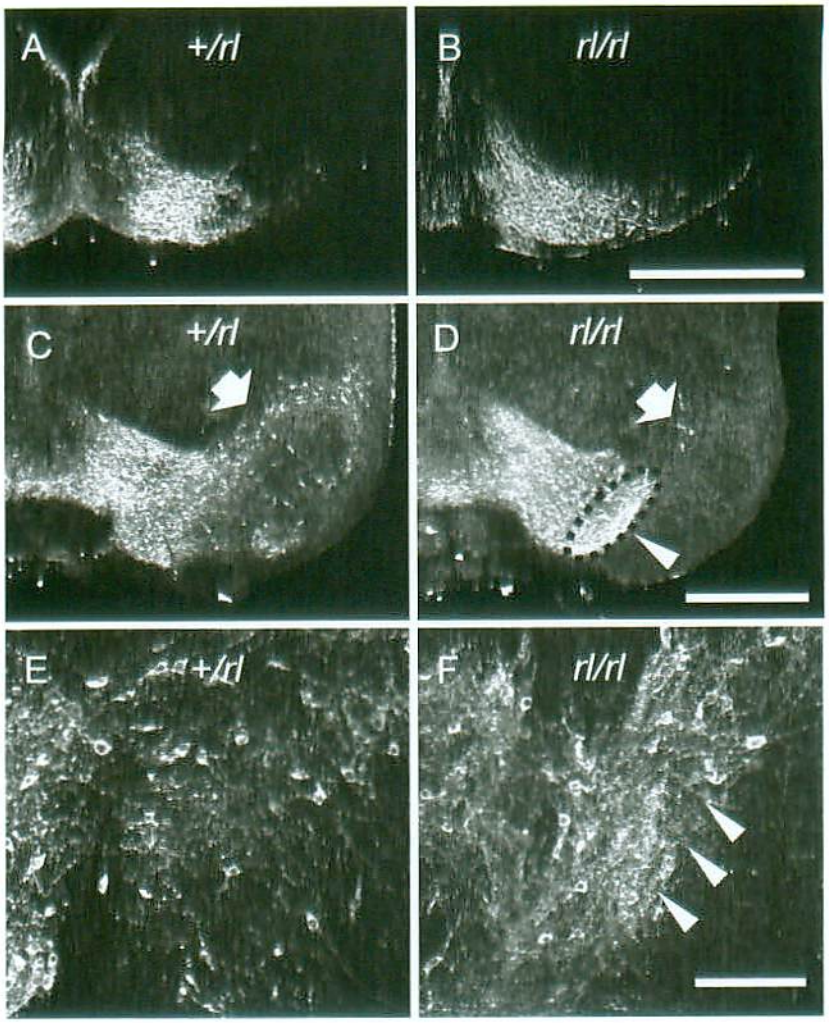

Fig. 3. Comparison of heterozygous (+/*rl*) and homozygous (*rl/rl*) mice in the distribution profile of mesencephalic DA neurons.

A, B: Frontal sections of the mesencephalon from +/*rl* (A) and *rl/rl* (B) mice at E15 are stained for TH. **C-F**: Frontal sections of mesencephalon from +/*rl* (C, E) and *rl/rl* (D, F) mice at P0 are stained for TH. Noted that the substantia nigra pars compacta (A9 cell group)(arrow) is normally formed in +/*rl* mice (C) but not in *rl/rl* mice (D), and that DA neurons (arrowhead) are anomalously clustered just lateral to the VTA in *rl/rl* mice (D). Higher magnification show that, compared to +/*rl* mice (E), *rl/rl* mice display an ectopic cluster of DA neurons (arrowheads) just lateral to the VTA.

Scale bars: A, B, 1mm; C, D,100μm; E, F

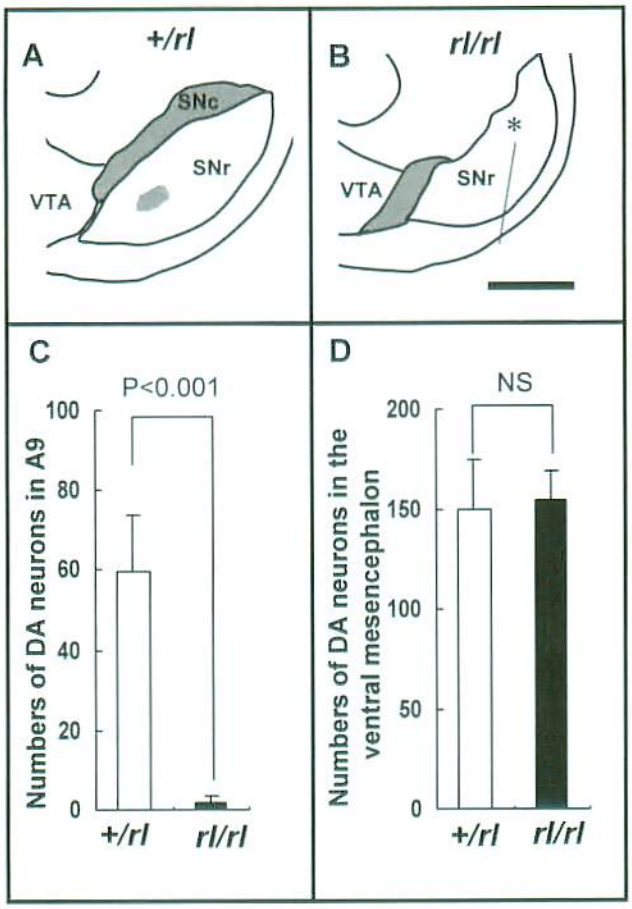

Fig. 4. **A, B**: Schematic drawing of nigral DA neurons in *+/rl* mice (A) and their anomalous clustering (arrow) in *rl/rl* mice (B). **C**: Quantitative results of neurons of DA neurons in A9 area of *+/rl* and *rl/rl* mice. **D**: Quantitative results of total numbers of DA neurons in the ventral mesencephalon of *+/rl* and *rl/rl* mice.

Scale bars: 800μm

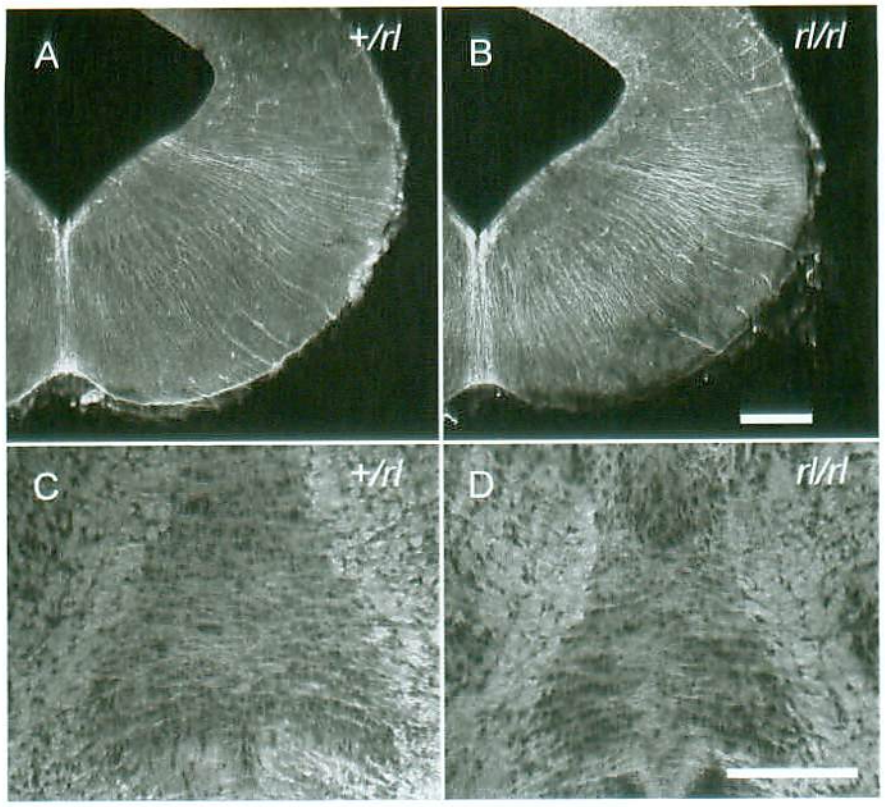

Fig. 5. Radial glial fibers and tangential fibers in the frontal sections of the mesencephalon of *+/rl* and *rl/rl* mice at E15.
A, B: There is no apparent difference in the distribution profile of radial fibers stained with anti-vimentin antibody between *+/rl* (A) and *rl/rl* (B) mice.
C, D: As in *+/rl* mice (C), tangential fibers stained with anti-NCAM-H antibody are well formed in *rl/rl* mice (D).
Scale bars: A, B, 400μm; C, D, 100μm

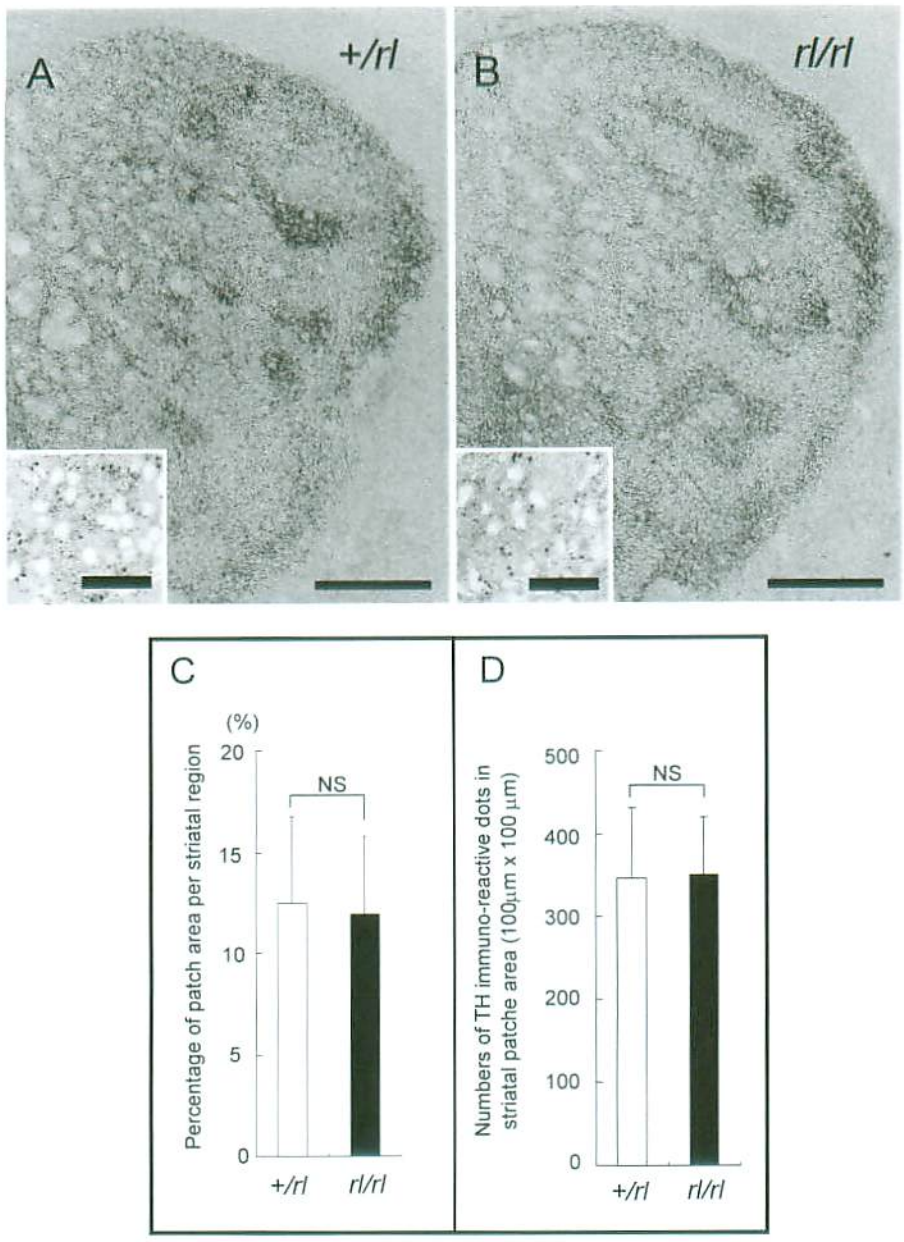

Fig. 6. "Dopamine islands" in the *reeler* striatum at P0.
A, B: The striatum from *+/rl* (A) and *rl/rl* (B) mice is stained for TH. Discrete striatal patches are found in both *+/rl* and *rl/rl* mice. **C**: Percentages of patch areas per total striatal area in *+/rl* and *rl/rl* mice are quantified. **D**: Numbers of TH-immunoreactive dots in the square area (100μm x 100μm) are analyzed in the striatal patches of *+/rl* and *rl/rl* mice.
Scale bars: A, B, 1mm

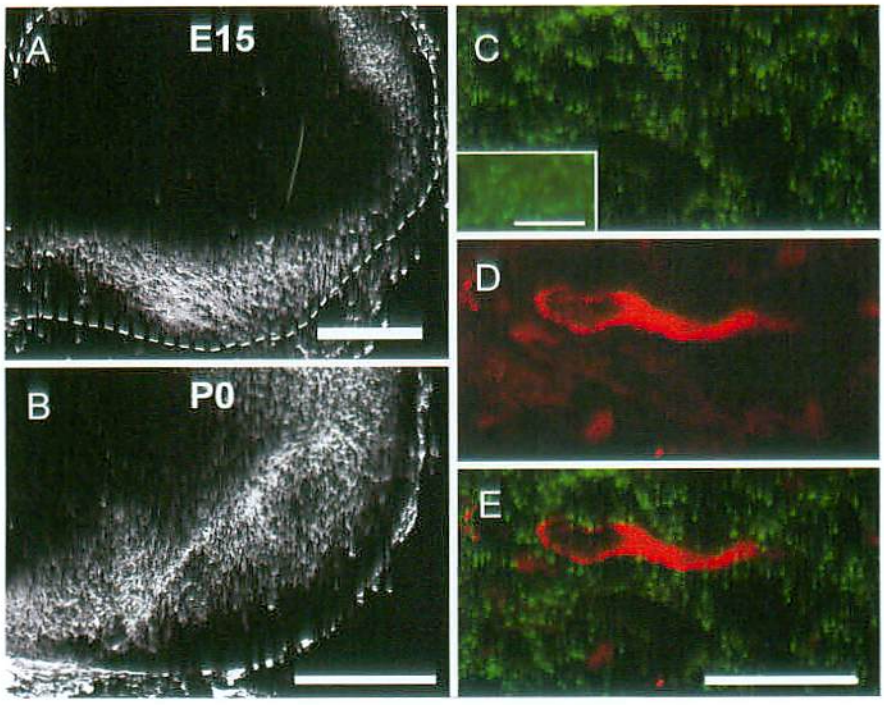

Fig. 7. Reelin localization determined by CR-50 in the mesencephalon of normal mice.

A: CR-50 labeling is highly concentrated in the E15 ventral mesencephalon where migrating DA neurons are also accumulated. **B**: The distribution profile of CR-50 labeling in the P0 substantia nigra is similar to that of DA neurons. **C-E**: Double labeling for reelin (C) and TH (D). The merged image is shown in E. DA cells positive for TH are surrounded by neurons dots labeled with CR-50. There are no cells that exhibit CR-50 labeling in their perikarya.

Scale bars: A, 500μm; B, I, 1mm; C-E, G, 20μm; F, 450μm

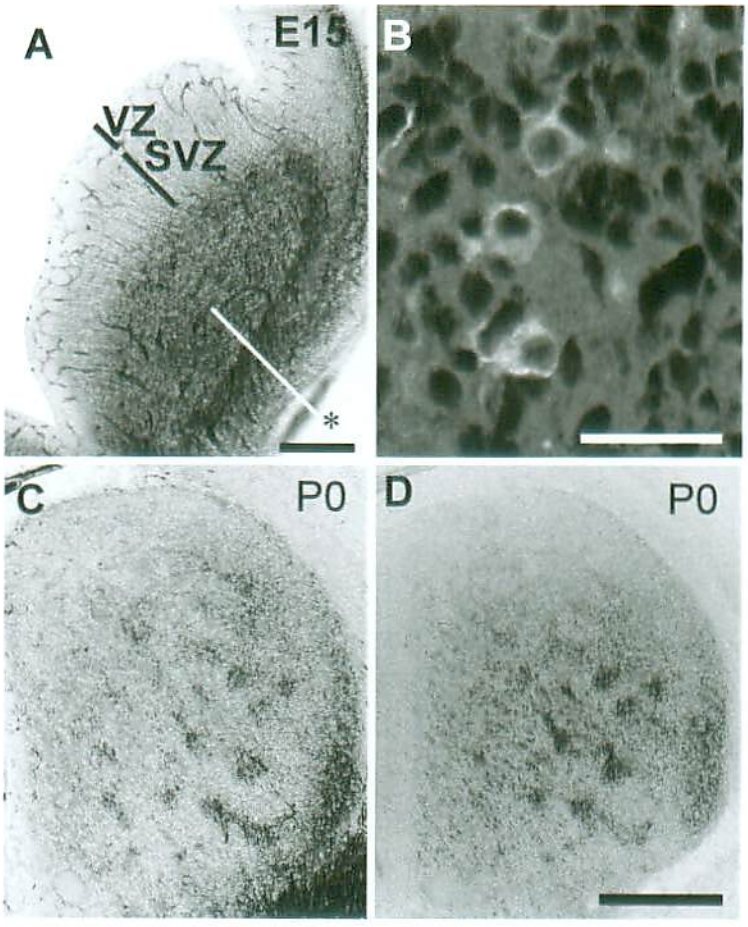

Fig. 8. Reelin localization determined by CR-50 in the striatum of normal mice.
A: The lateral ganglionic eminence is stained with CR-50. Substantial CR-50 labeling is seen in the striatal primordium (asterisk) but in the ventricular zone and subventricular zone. **B**: Higher magnification of the striatal primordium stained with CR-50. **C, D**: A frontal section from the P0 striatum is simultaneously labeled for CR-50 (C) and TH (D). Note that the distribution of striatal patches with heightened CR-50 labeling (C) matches that of TH-positive dopamine islands (D).
Scale bars: A, 50μm; B, 20μm; C, D, 500μm

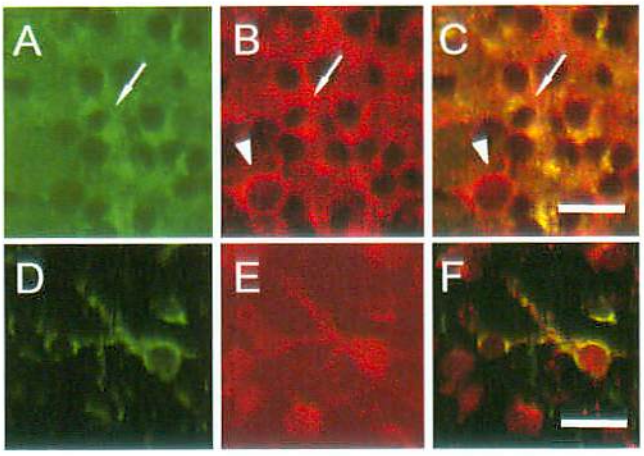

Fig. 9. CR-50- and GAD_{67}-immunostainings of the P3 striatum and of the dispersed striatal cell embedded in the collagen gel. CR-50-stainig (A, D), GAD_{67}-staining (B, E), and merged image (C, F).

A-C: In the striatum at P3, many neurons that exhibit strong CR-50-immunolabelings in their perikarya are also immunopositive for GAD_{67} (an example indicated by arrows), while some GAD_{67}-positive cells exhibited faint CR-50 immunoreactivity (an example indicated by arrowhead). **D-F**: CR-50- and GAD_{67}-immunolabelings are almost found in the cultured striatal neurons at 3days in vitro.

Scale bars: 20μm

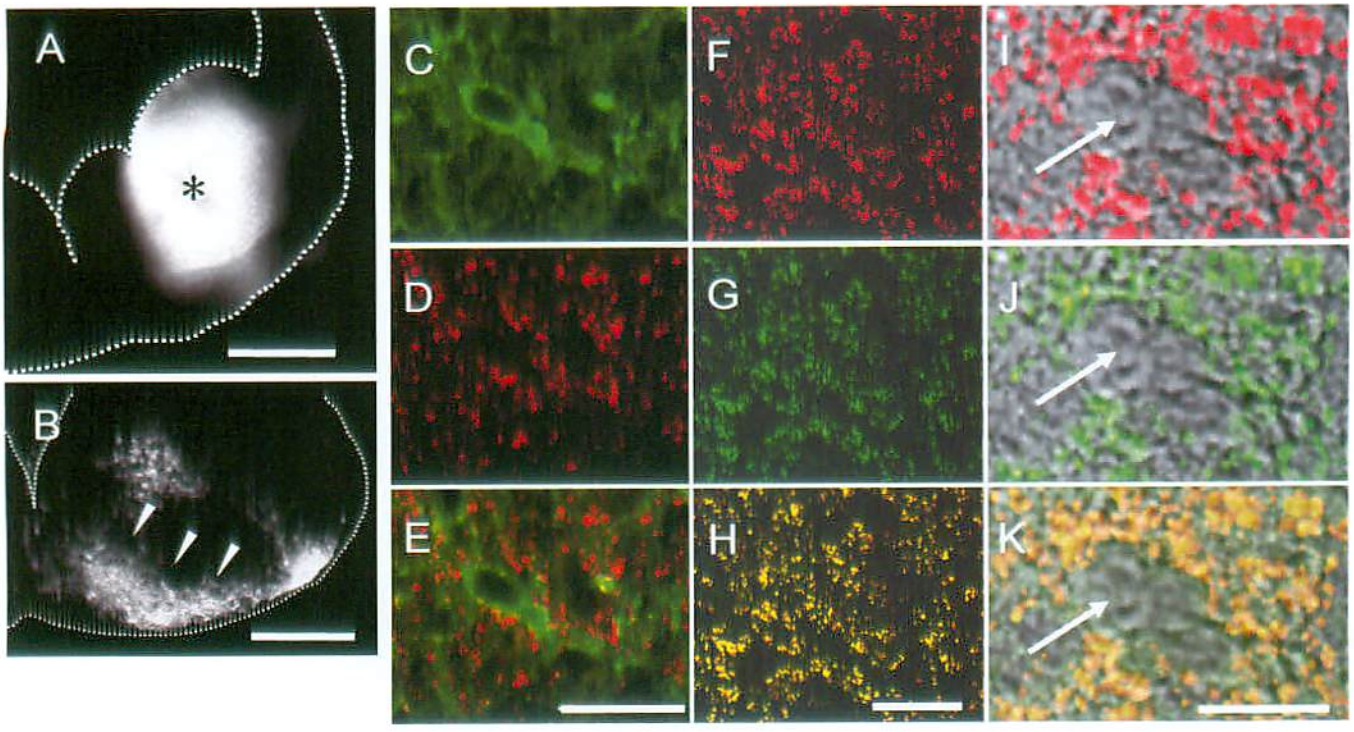

Fig. 10. Colocalization of CR-50 labelings and DiI fluorescence activities transported anterogradely from the striatal primordium in the ventral mesencephalon at E15.
A. B: Four weeks after localized injection of DiI (asterisk) into the post-fixed striatal primordium at E15 (A), there is robust DiI fluorescence activity transported anterograde-axonally from the striatal primordium in the ventral mesencephalon (B, arrowheads). **C-E**: A DA neuron stained for TH (C) and numerous dot-like structures labeled with DiI are present in the same microscopic field. The merged image is shown in E. **F-H**: Dot-like material labeled with DiI (F) almost perfectly correspond with CR-50-labeled materials (G). The merged image is shown in H. **I-K**: Nomarski optics shows that a mesencephalic neuron (arrow) is surrounded by DiI-labeled dots (I) that were simultaneously stained with CR-50 (J). The merged image is shown in K. Scale bars: A, 1mm; B; 600μm; C-H, 20μm; I, K, 10μm

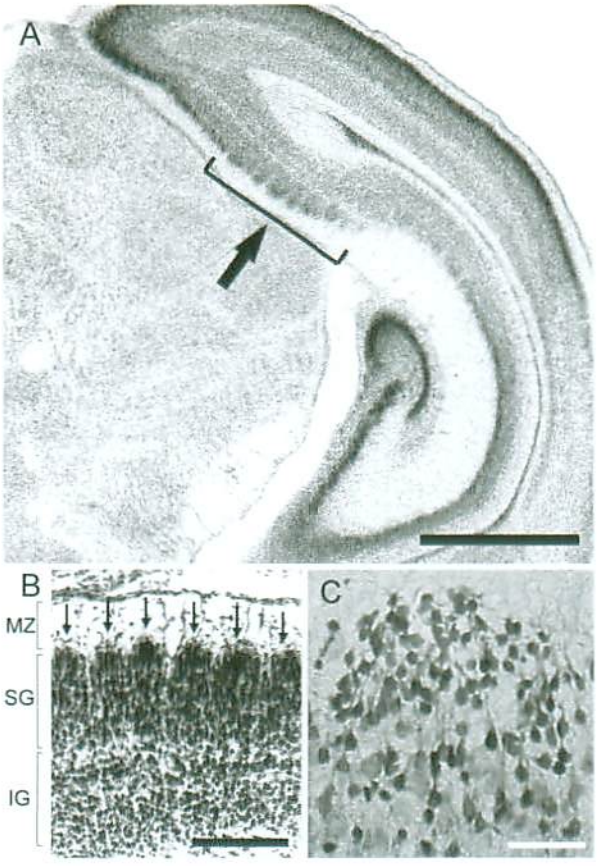

Fig. 11. Vertical columnar arrays of neurons in the presubicular cortex of P3 mice. **A**: PI-staining of a coronal section at the level of the hippocampus. The presubicular cortex is indicated by the arrow. **B**: Nissl-staining of the presubicular region. Note the columnar arrays of cortical neurons in the upper half of the cortical plate (i.e. supragranular layer) that are perpendicular to the pial surface. They show a periodic distribution in the horizontal extension. **C**: Nissl-staining of one vertical column at the just below the marginal zone, in which young post-mitotic cortical neurons appear to be clustered.
Scale bars: A, 500μm; B; 100μm; C, 10μm

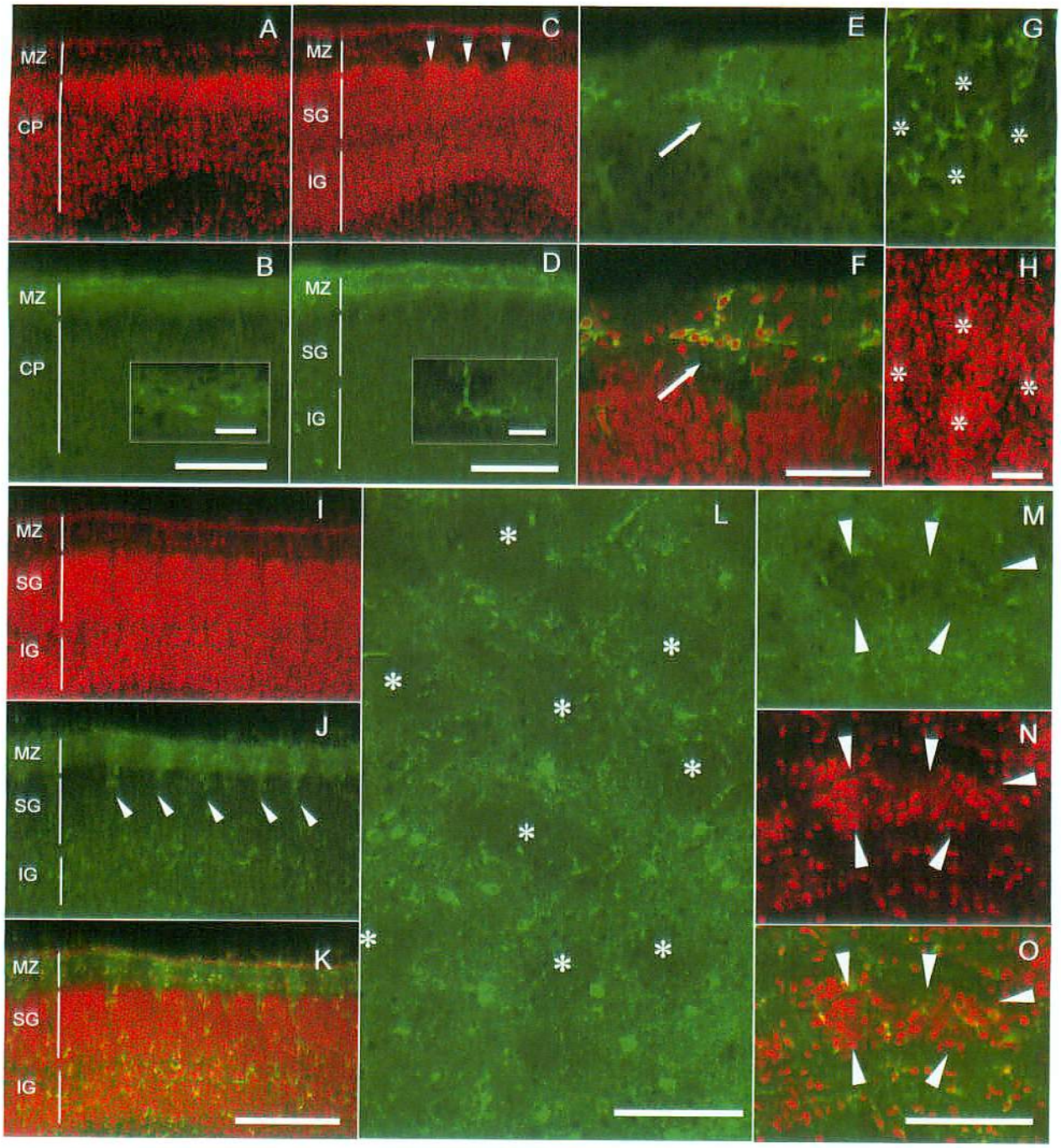
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Fig. 12. Relationship between the reelin statement and vertical columns of cortical plate neurons.

A, B: A coronal section from the P0 presubicular cortex is stained with PI (A) and CR-50 (B). The inset in B shows Cajal-Retzius cells labeled with CR-50. The marginal zone is enriched in reelin produced and secreted by Cajal-Retzius cells. The cortical plate neurons are settled beneath the marginal zone and show no definite columnar arrangement. **C-F**: A coronal section from the P2 presubicular cortex is stained red with PI (C, F) and green with CR-50 (D-F). The inset in (D) shows Cajal-Retzius cells labeled with CR-50. There is an indication of vertical columns of cortical plate neurons (arrowhead in C). Cajal-Retzius cells are also inhomogeneously distributed (D) and clusters of these cells are occasionally found in the marginal zone (E, F). **G, H**: A sections at P2, cut parallel to the cortical surface is stained with CR-50 (G) and PI (H). It is likely that cortical plate neurons preferentially accumulated underneath the marginal zone area that is poor in Cajal-Retzius cells (asterisk). **I-K**: A coronal section at P3 is stained red with PI (I) and green with CR-50 (J). The merged image is shown in (K). Columnar arrays of cortical neurons are identified in the upper half of the cortical plate corresponding to the supragranular layer (I). In the marginal zone, there is a periodic distribution of CR-50 labeling in the horizontal dimension (J, K). The reelin-rich zones contain clusters of Cajal-Retzius cells labeled with CR-50; their downward processes protrude into the inter-columnar spaces in the cortical plate (for examples see the arrowheads). **L**: A section from the P3 presubicular cortex, cut parallel to the cortical surface, shows a mosaic-like pattern of regularly spaced spots with low CR-50 labeling (for examples see the asterisks) in the marginal zone. **M-O**: Adjacent sections from P3 presubicular cortex, cut parallel to the cortical surface at the border between the marginal zone and the supragranular layer, are stained green with CR-50 (M) or red with PI (N). The merged image is shown in O. The distribution of cortical plate neurons and CR-50 labeling is almost complementary: regions with poor CR-50 immunoreactivity exhibit clusters of cortical plate neurons. For examples, see the arrowheads.

Scale bars: A-D, 200 μm; Inset in B, D, 10 μm; E,F, 25 μm; G, E, 50 μm; I-K, 200 μm; L-O, 100 μm

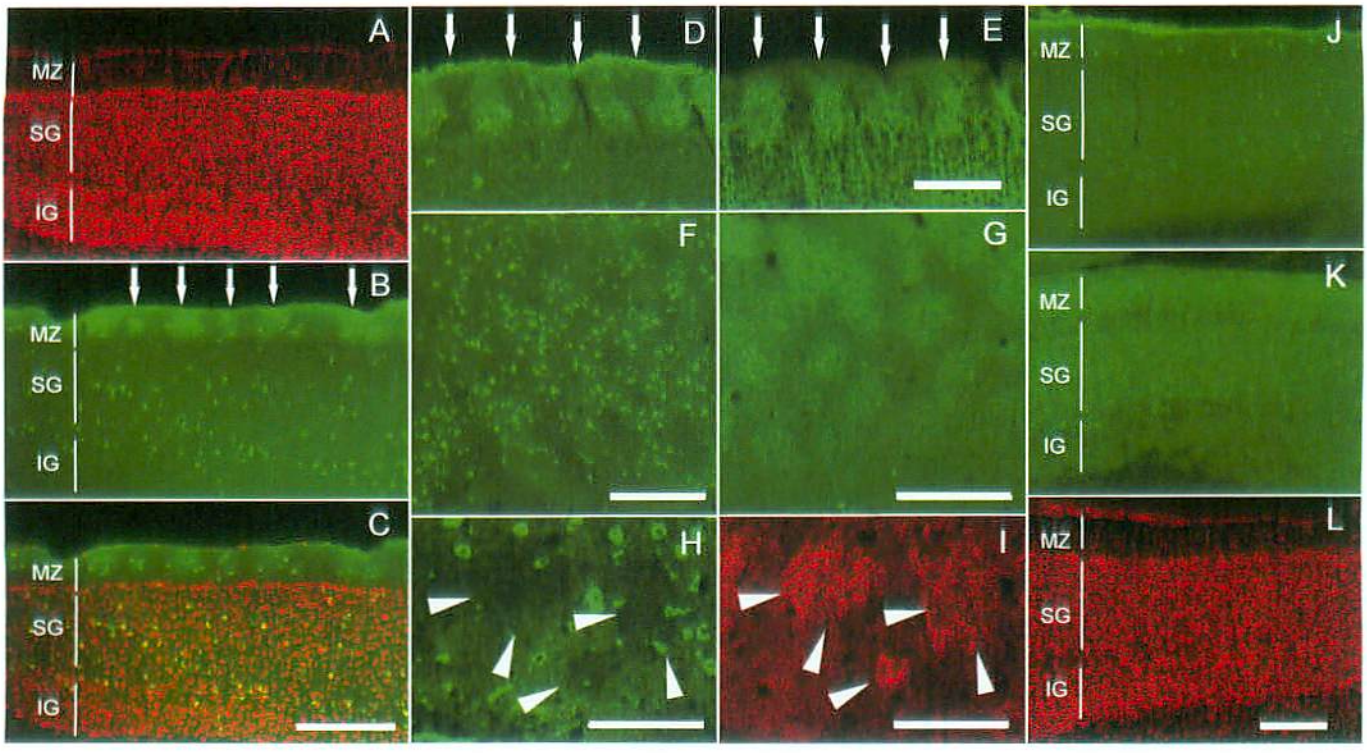

Fig. 13. Relationship between the reelin statement and dendritic clusters in the marginal layer of the P14 and P28 presubicular cortex.

A-C: A coronal section at P14 is stained with PI (A) and CR-50 (B). The merged image is shown in C. Note that no vertical columnar arrays of cortical plate neurons can be identified (A). However, CR-50 labeling continues to show periodic modulation (arrows) in the marginal zone (B). **D, E**: Adjacent coronal sections at P14 are stained with CR-50 (D) or anti-MAP2 antibody (E). Note that the reelin-poor zones (arrows) contain columnar tufts of cortical neuron dendrites. **F, G**: Sections cut parallel to the cortical surface at P14 are stained with CR-50 (F) or anti-MAP2 antibody (G). A mosaic-like pattern of irregularly-spaced spots poor in CR-50 labeling (F) or of areas enriched in MAP2 labeling (G) is shown. **H, I**: Adjacent sections cut parallel to the cortical surface are stained with CR-50 (H) or anti-MAP2 antibody (I). Note that the distribution of CR-50 labeling and of MAP2-positive dendritic tufts is almost complementary; regions with poor CR-50 labeling contain clusters of dendrites. For examples, see arrowheads. **J-L**: Frontal sections at P28 are stained with CR-50 (J), anti-MAP-2 antibody (K), and PI (L). Note that there is only a small population of Cajal-Retzius cells stained with CR-50 in the marginal zone; no vertical columnar structures are clearly identified in the cortex.

Scale bars: A-C, 200 μm; D, E, 100 μm; F, G, 200 μm; H,I, 50 μm; J-L, 200 μm

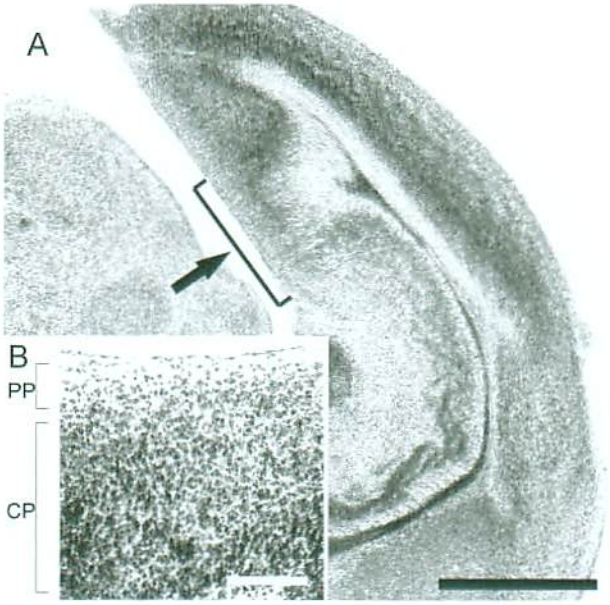

Fig. 14. Disruption of vertical columnar arrays of neurons in the presubicular cortex of P3 *reeler* mice.

A: Nissle staining of coronal brain section at the level of the hippocampus. The presubicular region is indicated by an arrow. **B**: A higher maginification image of the presubicular. Note the virtual absence of detectable vertical columnar arrays and horizontal layering of cortical neurons.

Scale bars: A, 500μm; B, 100μm

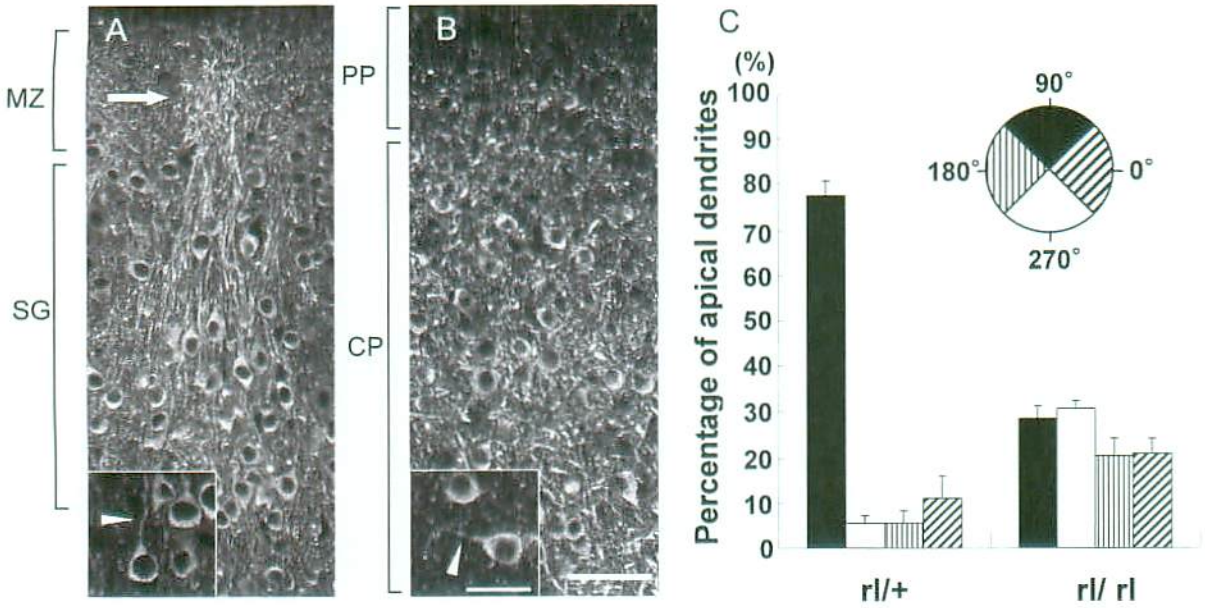

Fig. 15. Disruption of the vertical structures in the presubicular cortex of P14 *reeler* mice. **A, B**: Coronal sections from a heterozygous (A) and homozygous (B) mouse are stained with anti-MAP-2 antibody. In the heterozygous (A) mouse, the apical dendrites of cortical pyramidal neurons are oriented almost orthogonal to the pial surface; their distal dendritic branches are clustered in the marginal zone (arrow in A). The inset in (A) shows a pyramidal neuron that projects its apical dendrite (arrowhead) perpendicularly to the pial surface. By contrast, the homozygous mouse (B) displays varied orientation and positioning of cortical neurons, and no clustering of dendritic branches in the superficial layer. The inset in (B) shows a pyramidal neuron whose apical dendrite is horizontally oriented. C: The apical dendrite orientation histogram shows that most apical dendrites are directed towards the pial surface in heterozygous but not in homozygous mice.
Scale bars: A, B, 500μm; Inset in A, B, 20μm

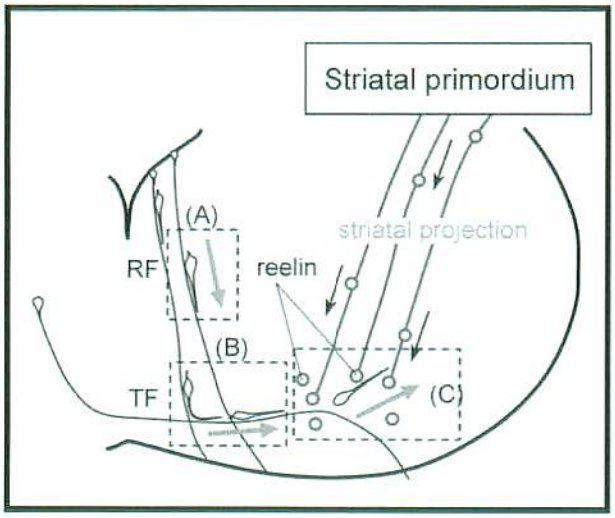

Fig. 16. Schematic diagram of the hypotherical role of reelin in the lateral migration of nigral dopaminergic neurons. Based on a proposed model of the migrating of mesencephalic DA neurons, it appears that nigral DA neurons migrate to their given positions in the adult brain in three sequential phase; first [*phase* (A)], they migrate toward the ventral mesencephalon along radial fibers (RF) with the DA neurons destined for the VTA; second [*phase* (B)], they migrate laterally in the basal part of the mesencephalon along tangentially arranged nerve fibers (TF) with the VTA neurons; and finally [*phase* (C)], they migrate further laterally, leaving from the TF or VTA cell group. Since the VTA is almost normally formed but the nigral DA neurons are anomalously clustered just lateral to the VTA in *rl/rl* mice, we posit that reelin derived from the striatal primordium plays a role in the further lateral migration of nigral DA neurons found in *phase* (C).

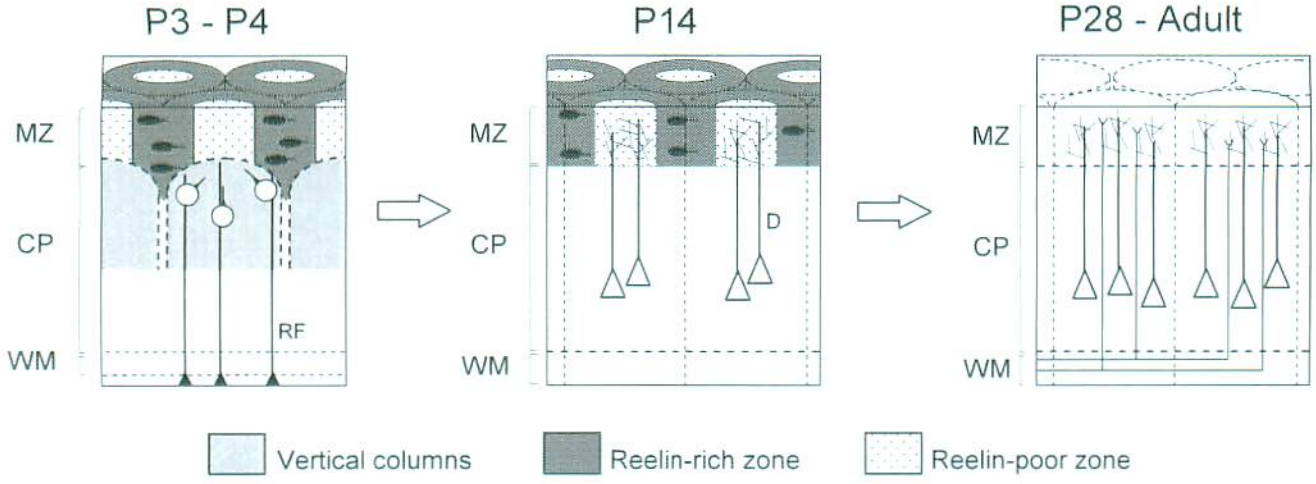

Fig. 17. Schematic diagram of the hypothetical role of Cajal-Retzius cells and reelin in the formation of the vertical columnar structures. At P3-P4, the distribution of Reelin produced by Cajal-Retzius cells (horizontal dark cells) forms well-delineated territories with a precise topography in the marginal zone. Late-born cortical plate neurons (white round cells) migrate along radial glial fibers (RF) until they reach the marginal zone where they are stopped by reelin and take up their vertical positions according to the rule of inside-out layering. Simultaneously, they take up horizontal positions in the cortical plate avoiding the reelin-rich zone that results from the strategic location of Cajal-Retzius cells in the marginal zone. Consequently, they accumulate underneath the reelin-poor zone and form vertical columns in the supragranular layer. At P14, the distribution of reelin continues to show an inhomogeneous pattern in the marginal zone. Cortical pyramidal neurons (white triangle cells) send their dendritic processes (D) towards the pial surface. However, being stopped by reelin, their distal dendritic branches do not enter into the reelin-rich area in the marginal zone. Consequently, columnar tufts of dendritic branches of cortical pyramidal neurons are formed in the reelin-poor zones. In P28 and adult mice, no vertical columnar structures are identified in either the cortical plate or the marginal zone, and Cajal-Retzius cells containing reelin are scarcely found in the marginal zone.