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The *reeler* Gene-Associated Antigen on Cajal-Retzius Neurons Is a Crucial Molecule for Laminar Organization of Cortical Neurons

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Summary

In the neurological mutant mouse *reeler*, the histological organization of the neocortex develops abnormally and essentially results in an inversion of the relative positions of the cortical layers. The *reeler* mutation, therefore, provides an insight into the molecular mechanisms underlying the formation of the cortical layers. We have generated a monoclonal antibody (CR-50) that probes a distinct allelic antigen present in wild-type but not in *reeler* mutant mice. CR-50 reacted specifically with Cajal-Retzius neurons, one of the first cortical neurons to differentiate in the neocortex, but whose functional role is not known. 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. These findings revealed that the selective expression of a distinct molecule on Cajal-Retzius neurons is critical for the normal lamination of cortical neurons in the mammalian neocortex.

Introduction

The mammalian neocortex is a highly ordered structure. The different classes of neurons reside in an organized radial array of six cellular layers ranging from the pial surface to the white matter. Positioning of a particular neuron at the particular cortical layer is an essential step in forming an elaborate neuronal network in the neocortex.

The pattern of sequential genesis of cortical layers is fundamental for establishing a highly ordered structure in the neocortex. Several lines of evidence indicate that the mammalian neocortex is constructed in two distinct steps (Marin-Padilla, 1971, 1978; Rickmann et al., 1977; König et al., 1977; Raedler and Raedler, 1978; Luskin and Shatz, 1985; Bayer and Altman, 1990). The first step is the formation of a primordial plexiform layer (recently called the preplate). The preplate is composed of a superficial plexus of corticopetal nerve fibers and the earliest generated neurons, including the Cajal–Retzius and prospective subplate neurons. In the second step, the preplate is split into two by the insertion of later-generated neurons that form the cortical plate proper. Therefore, the preplate is split into a superficial marginal zone, in which the Cajal–Retzius neurons differentiate, and a deep subplate, in which the subplate neurons differentiate (Marin-Padilla, 1971; Shatz et al., 1988; Allendoerfer and Shatz, 1994). The later-generated neurons at the ventricular zone migrate radially, passing through the intermediate zone and subplate along the radial glial fibers that span the width of the thickening neocortex, and reach the cortical plate. The structural maturation of the cortical plate follows an inside-out progression: the later-generated neurons are located at more superficial positions than the earlier-generated neurons (Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic, 1972, 1974).

The *reeler* mouse exhibits an autosomal recessive mutation located on chromosome 5 (Falconer, 1951; Dernoncourt et al., 1991). The mutation yields widespread morphological abnormalities in the cortical structures of the cerebrum, cerebellum, and hippocampus and in several subcortical structures, including the olfactory bulb, inferior olivary complex, and facial nucleus. However, the mutant neocortex maintains all the major morphological classes of neurons found in normal mice (for review, see Caviness et al., 1988). Light and electron microscopic studies have suggested that the initial formation of the preplate proceeds normally (Goffinet, 1979; Pinto-Lord and Caviness, 1979) and that the neurons of the forming cortical plate migrate according to the same schedule as those in normal animals (Caviness and Sidman, 1973; Derer et al., 1977; Caviness and Rakic, 1978; Stanfield and Cowan, 1979; Goffinet, 1984a; Caviness et al., 1988). However, cortical plate neurons are arranged differently, and younger neurons are placed at deeper rather than more superficial levels (Caviness and Sidman, 1973; Caviness, 1982).

Previous papers (Goffinet, 1979; Caviness, 1982) indicated that the characteristic *reeler* abnormality coincides with the onset of cortical plate formation. Thus, the *reeler* defect very likely involves some yet unspecified process associated with the initial formation of the cortical plate. We speculated that the positioning of the cortical plate may depend on its interaction with the early-generated preplate, and in the *reeler* mutant, this interaction may

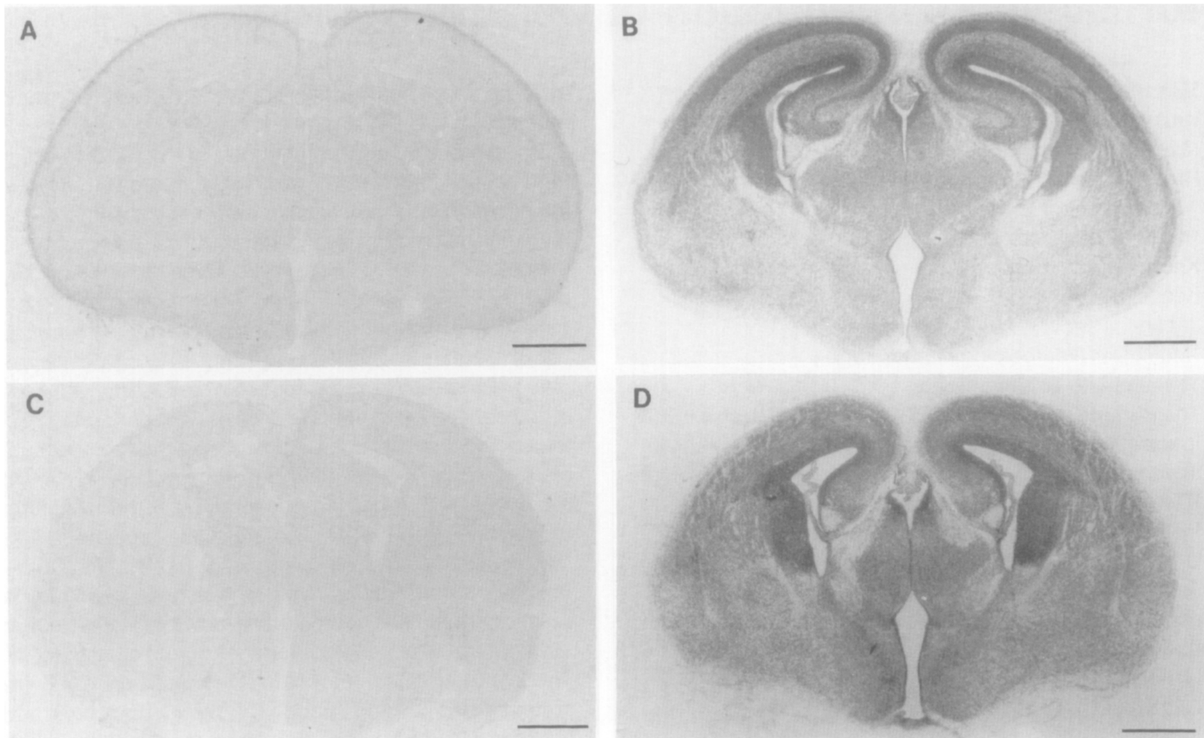


Figure 1. Differential Staining with Antiserum

Cerebral cortices at E15 from heterozygous (A) and homozygous (C) *reeler* mice were stained with antiserum (anti-CR). (B) and (D) illustrate toluidine blue staining of sections adjacent to (A) and (C), respectively, to show the histological composition of the sections. Because the mutation is recessive, the heterozygous cerebra show none of defects observed in the homozygous mutant. Note that the cells localized in the marginal zone of neocortex of the heterozygous, but not the homozygous, embryos were immunoreactive for anti-CR. Sections in (A) and (C) were processed simultaneously, and photomicrographs were made with identical exposure times. Bars, 0.5 mm.

be faulty, thereby leading to the abnormal positioning of neurons. Moreover, we hypothesized that a distinct molecule encoded by the *reeler* gene may be involved in this interaction. Thus, the presumptive interaction(s) between the preplate and the forming cortical plate in normal animals may be mediated by the *reeler* gene product; such a substance should be missing in the *reeler* mutant and be allogeneic between the normal and mutant mice, since the primary effect of mutation is to disrupt the action of a gene. In this study, we first examined whether such a presumptive substance could be detected immunologically. We succeeded in generating a monoclonal antibody (CR-50) that recognizes a distinct allelic antigen present in normal but not *reeler* mice. The antigen was expressed in the regions of normal mice in which morphological abnormalities occur in *reeler* mutant mice. The CR-50 antigen was associated specifically with the Cajal–Retzius neurons in the neocortex. The cell surface localization of the antigen and its unique pattern of expression make it likely that it plays an important role in the organization of the mammalian neocortex. We have provided evidence that the antibody disturbed the cell arrangement of cerebral cortical cells in vitro.

Results

Alloantibody Raised in *reeler* Mutant Mice

We have applied immunological procedures to isolate alloantibodies that differentially recognize distinct allelic antigens between normal (wild-type or heterozygous) and homozygous *reeler* mice. Because most cells of the preplate are destined to disappear by adulthood (Bradford et al., 1977; Luskin and Shatz, 1985; Derer and Derer, 1990; Wood et al., 1992; Soriano et al., 1994), we used the embryonic tissues as a source of antigens (see Table 1). Each cerebral cortex was excised from wild-type, heterozygous, or homozygous embryos at E14–E16 and prepared for immunization by homogenization. Adult recipient animals of wild-type, heterozygous, or homozygous genotypes were then immunized with homogenates, and the resulting antisera were screened immunohistochemically on cryostat sections of normal and mutant cerebral cortices at E15.

Systematic screening of antisera clearly showed that *reeler* recipients generate antibodies that react with cortical cells located in the marginal zone in wild-type or heterozygous mice but not in homozygous mutants (Figure 1;

Table 1. Allogeneic Antibodies in *reeler* Mice

Immunogen	Recipient	Number of Mice That Produced Alloantibodies/Total Mice Examined
Wild type	Wild type	0/8
Heterozygous	Wild type	0/17
Homozygous	Wild type	0/12
Wild type	Heterozygous	0/15
Heterozygous	Heterozygous	0/12
Homozygous	Heterozygous	0/15
Wild type	Homozygous	175/186
Heterozygous	Homozygous	89/93
Homozygous	Homozygous	0/14

Allogeneic antigenicity of antiserum was demonstrated by the presence of alloantibodies that immunostain differentially for cerebral cortices from normal (wild-type or heterozygous) and homozygous *reeler* mice, as shown in Figure 1. Only *reeler* mutant mice produced the allogeneic antibodies (anti-CR) when immunized with cerebral cortices of wild-type or heterozygous embryos.

Table 1). Without exception among the several hundred *reeler* brains tested, antisera never stained mutant sections. Surprisingly, such unique antisera were produced by most mutant mice immunized with wild-type or heterozygous cerebral cortices, and all antisera showed the same specificity of immunostaining (Table 1). We termed this polyclonal antibody anti-CR. None of the normal mice immunized with this material produced alloantibodies that differentially immunostained cortical cells of allelically different mice.

Hybridoma cell lines were produced by the polyethylene glycol–induced fusion of P3U1 mouse myeloma cells with spleen cells from immunized mutant mice. The resultant hybridoma cell lines were screened for production of a monoclonal antibody with the same specific distribution of binding to the marginal zone as polyclonal anti-CR, and the antibody secreted from 1 clone of hybridoma cells (RE-3B9) was tentatively designated CR-50 (Figure 2). Allogeneic differential binding of CR-50 to cerebrum was also demonstrated as immunoreactivity present in wild-type or heterozygous mice and absent in homozygous mice (see Figure 5F). Both CR-50 and anti-CR were found to belong to the IgG class, as tested by enzyme-linked immunosorbent assay using class-specific antibodies. Antibodies in ascites fluid harvested from hybridoma ascites tumors were used in the experiments shown in Figure 7, Figure 8, and Table 2 (see below); however, in other experiments, antibodies secreted into the culture medium by hybridoma cells were employed.

The nature of the antigen is as yet unknown. No immunoreactive bands were detectable in Western blots. Thin-layer chromatography immunostaining also failed to show any labeling of gangliosides or neutral glycolipids. Therefore, further studies are required to assess the exact nature of the antigen.

CR-50 Immunostains Cajal–Retzius Neurons

The distribution of the CR-50 antigen in neocortex was examined at various developmental stages. Sections were also labeled with a rabbit antibody against microtubule associated protein 2 (MAP2), which is an intracellular neuron–specific marker and is known to label intensely the early-generated neurons in developing neocortex (Crandall et al., 1986; Chun and Shatz, 1989).

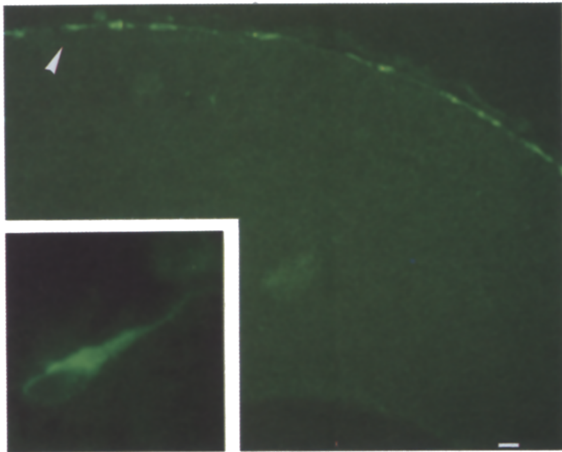
All the cells labeled with CR-50 were included in the population stained with anti-MAP2 (Figures 3A–3D). The most striking observation in these photographs is that only a single type of neocortical neuron stains for CR-50. These neurons are immunostained as early as E11, before the formation of the cortical plate (Shoukimas and Hinds, 1978), and are confined to the marginal zone at this and every subsequent embryonic stage examined. They exhibited a voluminous, horizontally oriented cytoplasm and thick cytoplasmic processes extending from the perikaryon (see Figure 2). To determine the birthdates of the CR-50-labeled cells, the thymidine analog 5-bromodeoxyuridine (BrdU) was injected intraperitoneally into pregnant dams. Many of the CR-50-labeled cells in the marginal zone of E16 cerebral cortices were found to be heavily labeled with BrdU (Figures 3E and 3F) when BrdU was injected into pregnant dams at the known birthdates of the population of preplate in the mouse, E10–E11 (Wood et al., 1992). Birthdating experiments showed that most of the CR-50-labeled cells in neocortex are born at E9.5–E10.5; however, they are not labeled with BrdU after E12 injection (data not shown). From their early birthdates, their stage of maturation, their position directly underneath the pial surface, and their morphology, the CR-50-labeled cells bear features characteristic of the Cajal–Retzius neurons described in the developing brains of various species (Duckett and Pearse, 1968; Marin-Padilla, 1971; Raedler and Sievers, 1976; Larroche, 1981; Edmunds and Parnavelas, 1982; Marin-Padilla and Marin-Padilla, 1982; Chun and Shatz, 1989; Derer and Derer, 1990; Huntley and Jones, 1990; Kostovic and Rakic, 1990; Soriano et al., 1994).

In normal animals, levels of CR-50 immunoreactivity remained high until E18 and then began to decline. The immunostaining was very weak by postnatal day 8 and was no longer detectable in the adult (data not shown). This stage-dependent change is consistent with a large proportion of the Cajal–Retzius neurons being eliminated by cell death during the immediate postnatal period (Bradford et al., 1977; Luskin and Shatz, 1985; Derer and Derer, 1990; Wood et al., 1992; Soriano et al., 1994).

Distribution of the CR-50 Antigen in the CNS

Immunohistochemical studies showed that, in normal embryos, the CR-50 antigen was enriched in the cerebrum and hippocampus and to a lesser extent in the olfactory bulb and cerebellum (Figure 4).

In the embryonic hippocampus proper, the pyramidal cell layer was not labeled, but cells in the molecular layer were costained with CR-50 and anti-MAP2. These cells are pulse-labeled with BrdU at E10–E11 (data not shown). Such early-generated molecular layer neurons have been reported previously in mouse (Angevine, 1965; Soriano et



al., 1994), rat (Schlessinger et al., 1978), cat (Wyss et al., 1983), and monkey (Rakic and Nowakowski, 1981). On the basis of morphological, calretinin-immunohistochemical, and ontogenetic similarities, these early neurons in the hippocampus are thought to be equivalent to the Cajal–Retzius neurons in the neocortex (Soriano et al., 1994).

Figure 2. CR-50-Immunoreactive Cell Bodies and Processes in Neocortex

Labeled cells are clearly distinct from unlabeled surrounding cells and are confined to the marginal zone. The inset shows a higher magnification of the cell indicated by the arrowhead. CR-50-labeled cells exhibit horizontal and thick cytoplasmic processes resembling extensions of the cell soma. The tissue section was prepared from a wild-type embryo at E15. Bar, 20 μm.

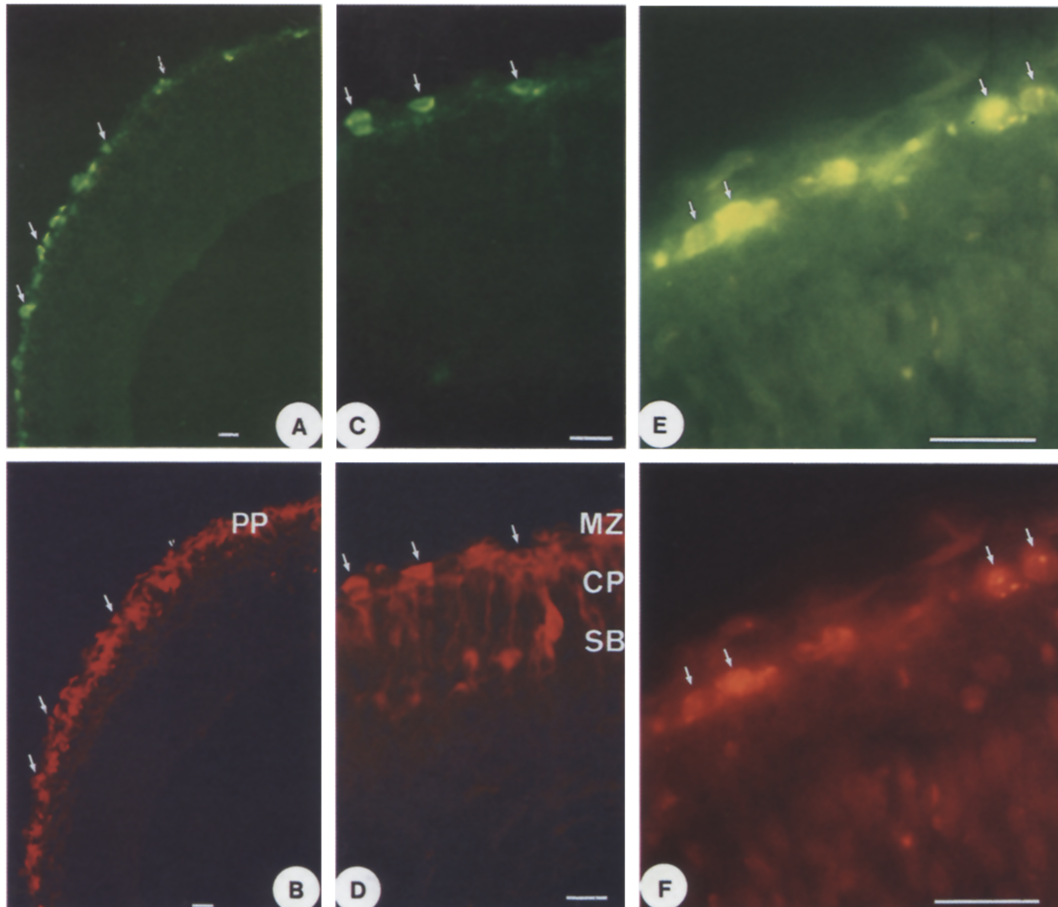


Figure 3. CR-50 Antigen in Developing Neocortex

(A and B) A cryostat section of dorsolateral neocortex at E12 was double-stained with CR-50 plus FITC-conjugated secondary antibody (A) and anti-MAP2 antibody plus rhodamine-conjugated secondary antibody (B). In the neocortex of the mouse at E12, the earliest-generated neurons accumulate just beneath the pia and form the preplate (PP). The CR-50-stained cells are located most superficially among the entire population of MAP2-labeled preplate neurons. Arrows indicate corresponding cells in the two microphotographs of each field.

(C and D) Dorsolateral neocortex at E14 was double-stained with CR-50 plus FITC-conjugated secondary antibody (C) and anti-MAP2 antibody plus rhodamine-conjugated secondary antibody (D). The strong MAP2 immunoreactivity in both the marginal (MZ) and subplate (SB) zones reflects the presence of the Cajal–Retzius neurons in MZ and the subplate neurons in SB, respectively. Both cells are early-generated and differentiate prior to the development of the cortical plate neurons. The cortical plate (CP) develops between them.

(E and F) A section from an E16 embryo that had received BrdU at E10 was double-stained with CR-50 plus FITC-conjugated secondary antibody (E) and anti-BrdU plus rhodamine-conjugated secondary antibody (F). Note that many of the CR-50-labeled cells have strong BrdU staining in their nuclei (arrows).

Bars, 20 μm.

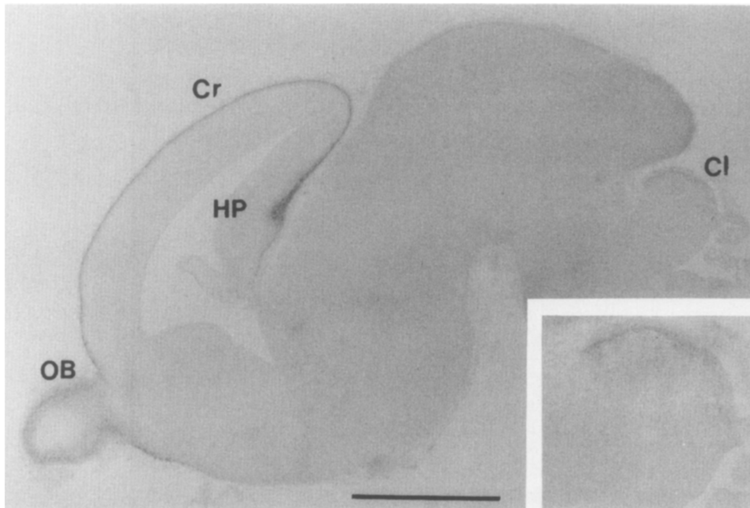


Figure 4. CR-50 Immunostaining in the CNS

In wild-type embryos at E16, CR-50 antigen was enriched in the cerebrum (Cr) and hippocampus (HP) and to a lesser extent in the olfactory bulb (OB) and cerebellum (Cl). The anatomical abnormalities are known to occur in these tissues in *reeler* mutant mice. The inset shows higher magnification of the forming cerebellum. Bar, 1 mm.

The onset of expression of the CR-50 antigen in the cerebellum is consistent with the timing of appearance of the external granule layer. CR-50 antigen was expressed in a fraction of granule neurons located in the deep external granule layer (Altman, 1972; Ryder and Cepko, 1994), before their migration to the internal granule layer. In the cerebellum at postnatal day 7, a number of cells in the internal granule layer exhibited CR-50 immunoreactivity; however, there was a marked reduction in the number of CR-50$^+$ cells in the internal granule layer after 2–3 weeks, with no labeled cells in the adult cerebellum (data not shown). We do not know for certain whether the same population of granule cells differentiate to CR-50$^-$ cells at later stages.

CR-50 also stained the inferior olivary complex, dorsal cochlear nucleus, and facial nucleus in wild-type animals (data not shown). In *reeler* mutant mice, these subcortical structures were not immunostained with CR-50, and anatomical abnormalities in cell groupings have been documented in these structures (Wyss et al., 1980; Martin, 1981; Goffinet, 1984a, 1984b).

The similarity in the distribution of CR-50 immunostaining in normal mice and regions of morphological abnormality in mutants suggests the possibility that the antigenic determinant is common in all of these regions and that it plays an essential role in neuronal alignment in each region.

Lack of CR-50 Antigen on the Cajal–Retzius Neuron and Abnormal Positioning of the Cortical Plate in *reeler* Neocortex

To investigate the morphological differences in neocortices from wild-type and *reeler* mice, serial sections of dorsolateral E16 cerebrum were comparatively examined by means of immunohistochemistry. Pregnant dams were also administered BrdU at E10 to follow the position of cells belonging to the preplate. To identify the Cajal–Retzius neurons in mutants, we stained the sections for another known Cajal–Retzius neuron marker, calretinin, a calcium-binding protein confined to the Cajal–Retzius neurons in the embryonic neocortex (Soriano et al., 1994).

In both wild-type and *reeler* embryos at E16, calretinin immunoreactivity occurred only in superficial cells in close apposition to the pial surface (Figures 5B and 5E). These cells were observed to localize in the marginal zone (MZ) in the adjacent sections stained with anti-MAP2 (Figures 5A and 5D). Furthermore, many of the calretinin-labeled cells were heavily labeled with BrdU following injection at E10 (Figures 5G and 5H [wild type]; Figures 5K and 5L [mutant]). These observations indicate that the calretinin-labeled cells are Cajal–Retzius neurons. The calretinin-labeled cells in wild-type embryos, as expected, were costained with CR-50 (Figure 5C). (The expression of calretinin is confined exclusively to the Cajal–Retzius neurons in the embryonic neocortex, whereas the population of calretinin$^+$ cells is generally segregated from the population of CR-50-labeled cells in other areas.) In contrast, in *reeler* neocortex, while there are Cajal–Retzius neurons (see Derer, 1985), they lack antigenicity against CR-50 (Figure 5F). This distinguishing feature also indicates that the CR-50 antigen is not essential for cell viability.

At E16, the *reeler* abnormality in neocortex is comparatively recognizable in MAP2-stained sections. In wild-type neocortex, the population of strongly MAP2-labeled cells is clearly visible in the subplate area (SB; Figure 5A, arrows), immediately beneath the cortical plate (CP) and above the intermediate zone (IMZ). Many of these neurons are colabeled heavily with BrdU following injection at E10 (Figures 5I and 5J). By their position, their characteristic MAP2 labeling, and their birthdates, these cells are recognizable as subplate neurons, according to similar observations in mouse (Crandall et al., 1986; Wood et al., 1992) and cat (Luskin and Shatz, 1985; Chun and Shatz, 1989). In *reeler* neocortex, while a distinct cortical plate is evident, histological abnormalities can be seen in both the deep and superficial borders of the cortical plate. The zone corresponding to the subplate is absent in the mutant, so there is a gradual transition between the cortical plate and

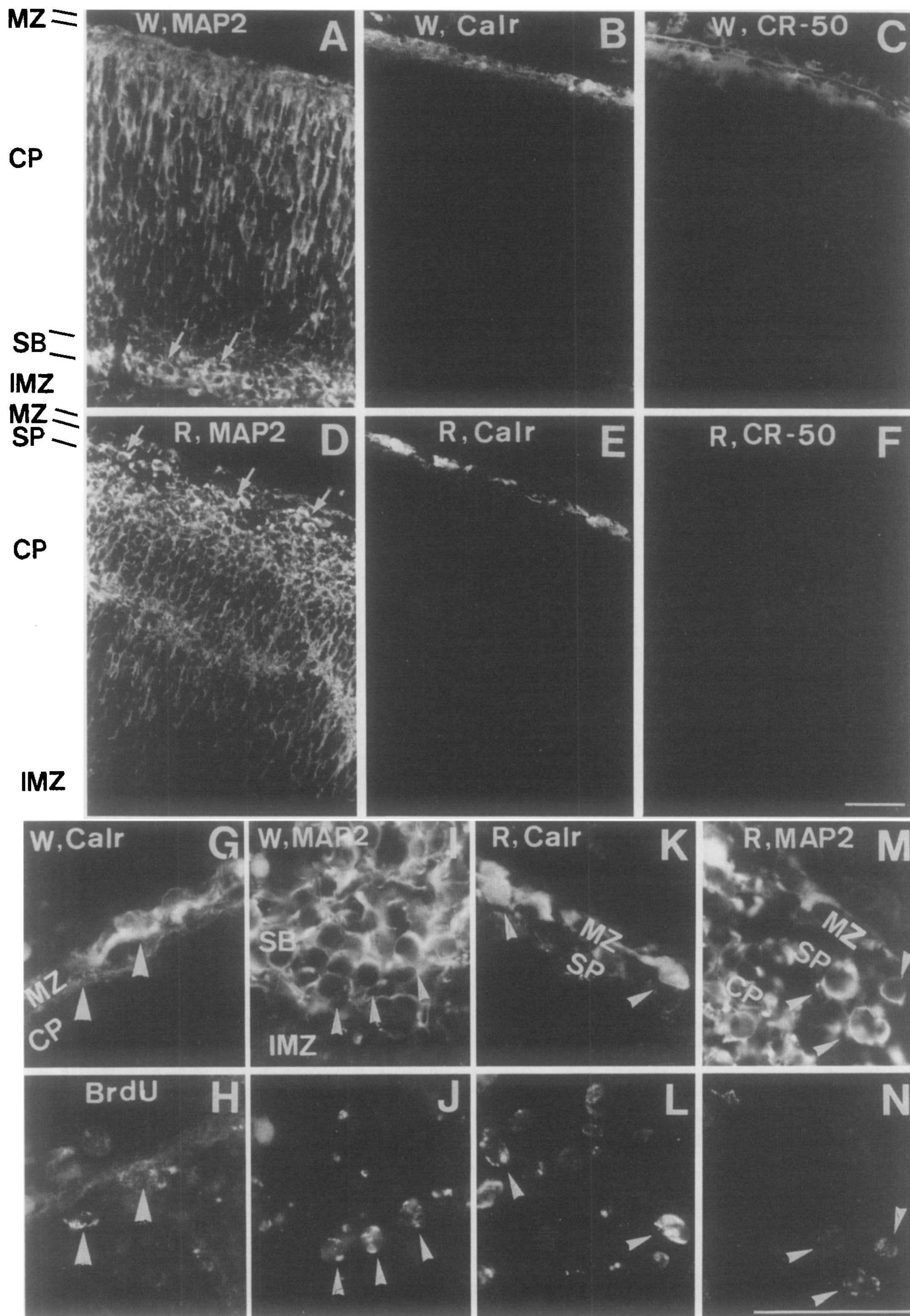


Figure 5. Abnormal Positioning of the Population of the Cortical Plate in *reeler* Neocortex

The immunohistology of dorsolateral neocortices in wild-type (A–C and G–J) and *reeler* (D–F and K–N) mice at E16 following BrdU administration at E10. Cryostat sections were single-stained with anti-MAP2 (A and D), anti-calretinin (B and E), or CR-50 (C and F) or double-stained with anti-calretinin/BrdU (G, H, K, and L) or anti-MAP2/BrdU (I, J, M, and N).

(A) The relationship of MAP2 immunostaining to the histological organization of the neocortex in wild-type mouse. A strong MAP2-immunoreactive

the intermediate zone (Figure 5D). A dense array of MAP2-labeled cells is visible in the superficial border of the cortical plate (Figure 5D, arrows), but not in the deep border. This superficial zone has been referred to as the superplate (SP) because of its similarity to the subplate of the normal animals (Caviness, 1982). Many if not all of the MAP2$^+$/calretinin$^-$ cells within the superplate are heavily labeled with BrdU (Figures 5M and 5N). The cell corresponding to the subplate neuron, therefore, appears to have retained its normal morphological characteristics and birthdate, but lies in an abnormal position relative to neighboring structures in mutant.

These observations in wild-type and *reeler* mutant neocortices at E16 indicate that the primary preplate has already been divided by the insertion of the forming cortical plate. The Cajal–Retzius and subplate neurons are arranged above and below the forming cortical plate in a parallel fashion in wild type; however, in the mutant, the population of the preplate primarily localizes superficially, and the cortical plate forms below it. Conversely, it suggests that the later-generated neurons pass by the early subplate neurons en route to the cortical plate in wild-type but not in mutant mice. Thus, the ultimate positioning of the cortical plate neurons may depend on whether they are aligned within the preplate. The following observations will suggest that the CR-50 antigen acts as a specific substratum that promotes alignment of the cortical plate within the preplate.

Extracellular Binding of CR-50

To analyze the subcellular distribution of the CR-50 antigen, we examined an immunolabeling pattern of CR-50 to cerebrum before and after fixation. Living cerebrum of wild-type mice at E14 was isolated under sterile conditions and incubated with CR-50 for 1 hr prior to fixation with 4% paraformaldehyde. Cryostat sections of specimen were further stained with anti-calretinin to identify the cell bodies of Cajal–Retzius neurons.

The living cerebral cortex exhibited only punctate immunoreactivity for CR-50 in the superficial zone of neocortex (Figure 6A; cf. Figure 2), as if it surrounded the calretinin-labeled cells (Figure 6B). The concentration of CR-50 antigen in the cell somata of the Cajal–Retzius neurons (see Figure 2 and Figure 3) was not detected. The double-exposed fluorescent photomicrograph illustrated in Figure 6C shows that the punctate staining initiated at the cell somata of the Cajal–Retzius neurons and extended horizontally, parallel to the cortical surface and to some extent downward into the cortical plate. When living cerebra are exposed to anti-calretinin antibody prior to fixation, Cajal–Retzius neurons are not labeled (data not shown), indicating that the intact plasma membrane prevents access of antibodies to intracellular antigens. The Cajal–Retzius neuron has horizontally oriented main processes and a few short descending processes that end within the cortical plate (Shoukimas and Hinds, 1978; Derer and Derer, 1990). Accordingly, the epitope recognized by CR-50 in living cerebrum is likely on the outer surface of Cajal–Retzius neurons. In fixed sections of cerebrum, such surface reactivity of CR-50 was reduced as compared with that of living cerebrum, which may have resulted from loss of antigen during the labeling procedures. This finding may indicate a loose association of the CR-50 antigen on cell surfaces of Cajal–Retzius neurons.

Cell surface staining of living Cajal–Retzius neurons was also examined with dissociated cortical cells in culture. Cortical cells dissociated from E14 wild-type embryos were cultured for 1 day and then exposed to CR-50 for 1 hr. There were no signs of cytotoxicity after treatment with CR-50. The culture was then fixed and labeled further with anti-calretinin antibody. The surface expression of CR-50 antigen (Figure 6D) was confined exclusively to the calretinin-labeled cells (Figure 6E). Figure 6F shows that CR-50 stains surfaces of calretinin-labeled Cajal–Retzius neurons.

CR-50 Disturbs the Histotypic Organization of Cortical Cells In Vitro

The cell surface localization of the CR-50 antigen raises the possibility that the antigen is involved in cell–cell interactions within the cortex. Previous reaggregation experiments using dissociated cerebral cortical cells isolated from wild-type and *reeler* mutant embryos have shown that the patterns of reaggregation of these cells are strikingly reminiscent of those seen in vivo for normal and mutant cortices, respectively (DeLong, 1970; DeLong and Sid-

layer can be seen in the subplate area (SB), immediately beneath the less immunostained cortical plate (CP) and above the MAP2$^-$ intermediate zone (IMZ).

(B) Adjacent section stained with anti-calretinin shows the location of Cajal–Retzius neurons in the marginal zone (MZ).

(C) Another section adjacent to that in (B) was stained with CR-50. The staining pattern of CR-50 almost overlaps with that of calretinin.

(D) The relationship of MAP2 immunostaining to the histological organization of the neocortex in *reeler* mutant mouse. There is no strong MAP2-immunoreactive layer beneath the cortical plate, but one can see a gradual transition in immunoreactivity between the cortical plate and the intermediate zone. Those cells, of comparable size and with the same MAP2 immunostaining characteristics, corresponding to the subplate neurons could be seen in the superplate (SP) zone (also see [I] and [M]).

(E and F) Calretinin-labeled cells are also observed directly underneath the pial surface in *reeler* mutants (E). Whereas the calretinin-labeled Cajal–Retzius neurons are present in the marginal zone in *reeler*, they are never labeled with CR-50 (F).

(G and N) Enlarged views demonstrate that some of the calretinin-labeled cells in the marginal zone (G) and strongly MAP2-labeled neurons in the subplate (I) in wild-type embryos are costained heavily with BrdU (H and J, respectively). In *reeler* mutant embryos, the early-generated neurons, which should be heavily labeled with BrdU after injection at E10, are observed only superficially and not below the cortical plate. Some calretinin-labeled cells (K) and MAP2-labeled cells in the superplate (M) are heavily labeled with BrdU (L and N, respectively). Arrowheads indicate corresponding cells in the paired microphotographs of each field.

Bars, 50 μm.

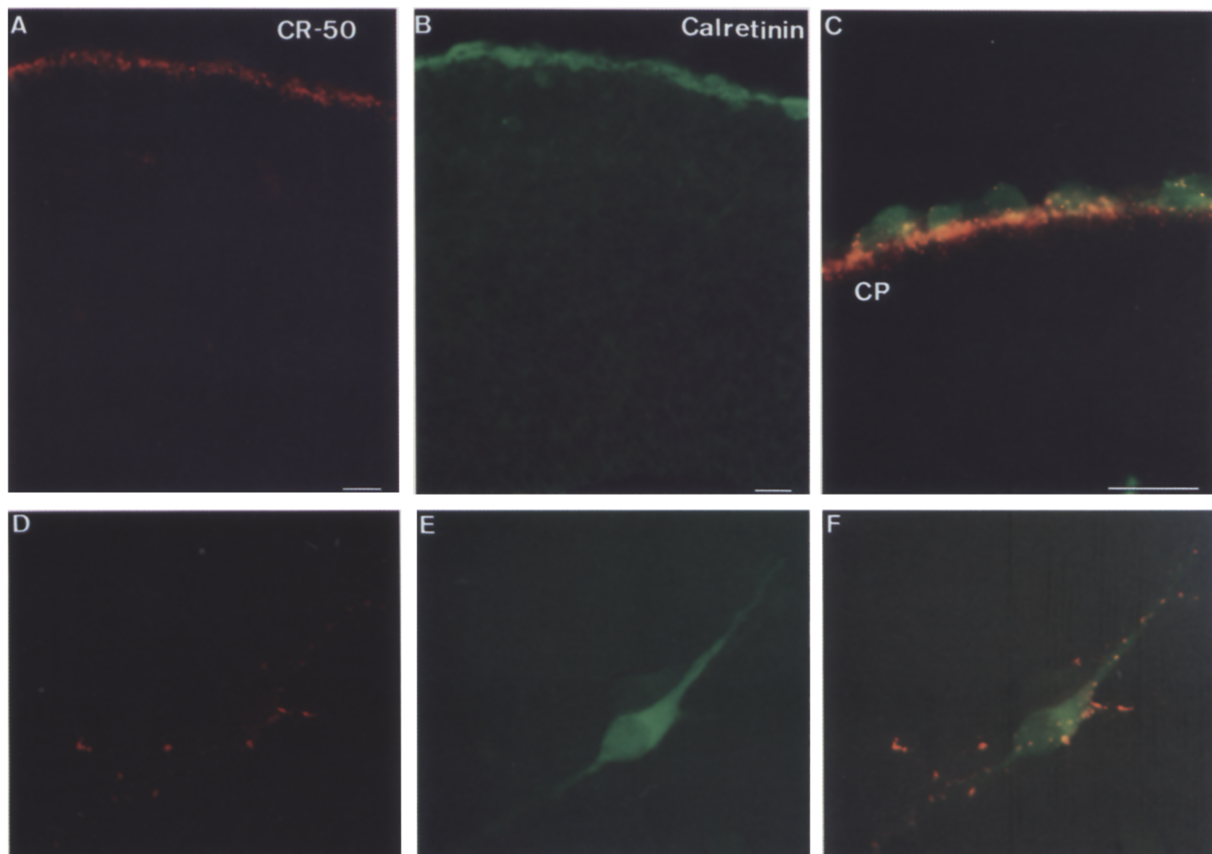


Figure 6. Expression of CR-50 Antigen on Cell Surfaces of Living Cajal–Retzius Neurons in Cerebrum and in Culture

(A–C) Living cerebrum isolated from a wild-type embryo at E14 was incubated with CR-50 prior to fixation with paraformaldehyde (A). After fixation, the cryostat section was further incubated with anti-calretinin antibody (B). The red and green signals correspond to CR-50 and calretinin immunofluorescence, respectively. The enlarged, double-exposed fluorescent photomicrograph (C) illustrates that the punctate staining initiates at the cell soma of the calretinin-labeled Cajal–Retzius neurons and extends horizontally, parallel to the cortical surface and downward to some extent into the cortical plate (CP). (D–F) Dissociated wild-type cortical cells in culture were double-labeled with CR-50 before fixation (D) and with anti-calretinin after fixation (E). The surface expression of CR-50 antigen was confined exclusively to the calretinin-labeled cells (F).
Bars, 20 μm.

man, 1970). With the aid of this culture system, we examined the physiological effects of CR-50 on the histogenetic assembly of cortical cells isolated from E12 embryos in vitro.

When dissociated cortical cells from E12 cerebrum were placed in a tube and rotated, they rapidly formed a single spherical aggregate that gradually became larger (1000–1300 μm in diameter after 5 days). The aggregate was fixed after 5 days of incubation, and cryostat sections were immunostained with anti-MAP2 antibody to elucidate the neuronal arrangement within the aggregate. The aggregates formed with wild-type cortical cells (wild-type aggregates) exhibited a consistent pattern of organization into two concentric layers (Figures 7A and 7F). MAP2-labeled neurons were uniformly concentrated and aligned symmetrically in the outer 1/3 zone of the aggregate. This result is consistent with previous observations using Golgi stain (Garber et al., 1980), which showed that most cortical neurons have the tendency to align radially and parallel to each other in aggregates. The CR-50$^+$/calretinin$^+$ Cajal–Retzius neurons were found to lie superficially and horizontally in the aggregates (Figure 7K and 7L).

The *reeler* cortical cells gave aggregates (*reeler* aggregates) matched in size and spherical shape to those seen in wild type. However, the internal organization of *reeler* neurons was different from the pattern in the wild type. Several clusters of strongly MAP2-stained neurons that had segregated from the lightly stained neurons were observed in the outer 1/3 zone of the aggregate, and the inner border of the MAP2-stained band was ill defined (Figures 7B and 7G). The calretinin$^+$ Cajal–Retzius neurons (although CR-50$^-$) were also observed to lie superficially and horizontally, as in the wild type (Figures 7M and 7N). Thus, the aggregates showed distinct morphological features depending on the genotype of the cells. For convenience, we refer to aggregates in which neurons were uniformly and symmetrically concentrated in the outer zone as type A, and to those showing patches of clustered neurons within the outer zone as type B. None of the wild-type cortical cells produced type B aggregates (Table 2).

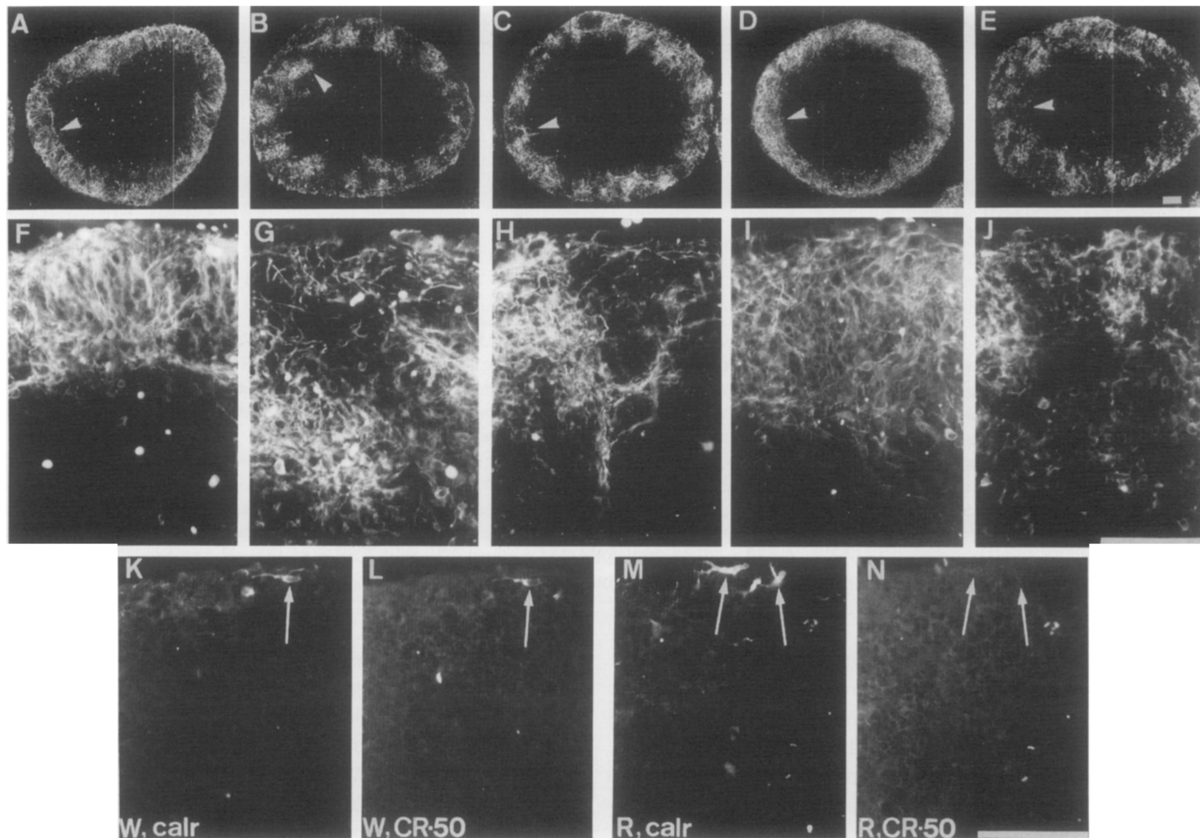


Figure 7. Disturbance of Histotypic Organization of Cerebral Cortical Cells in Response to CR-50 In Vitro

(A–J) Cortical cells, dissociated from wild-type (A) or mutant (B) embryos at E12, were cultured for 5 days in rotating tubes. They reaggregated, and the MAP2-labeled neurons were arranged depending on their genotypes. In the presence of 200 μg/ml CR-50 (C), wild-type cortical cells showed a distorted neuronal arrangement resembling that of the mutant pattern. This activity was abolished when the starting antibody was preabsorbed with wild-type cortical cells (D), but not with *reeler* cortical cells (E). Arrowheads in (A)–(E) indicate areas that are enlarged in (F)–(J), respectively.

(K–N) Pairs of immunofluorescent photomicrographs of wild-type (K and L) and *reeler* (M and N) aggregates double-stained with anti-calretinin (K and M) and CR-50 (L and N). Calretinin$^+$ Cajal–Retzius neurons (arrows) were observed to lie superficially in both aggregates; however, those of *reeler* were not stained with CR-50.

Bars, 100 μm.

In a minor population of aggregates, neurons were loosely arrayed, and the inner border of the MAP2-labeled layer was vague. These aggregates were classified as type C. Results with the different conditions are summarized in Table 2.

When wild-type cortical cells were exposed to CR-50 (200 μg/ml) from initial cultivation, 83% of the aggregates (10 of 12) displayed type B (Figures 7C and 7H; Table 2). This effect of CR-50 on the histotypic pattern of cortical cell arrangement within the wild-type aggregates was abolished when CR-50 was preabsorbed to wild-type cortices (Figures 7D and 7I; Table 2) but was not removed with absorption to mutant cortices (Figures 7E and 7J; Table 2). The presence in the culture medium of either nonimmunized *reeler* IgG or boiled CR-50 (200 μg/ml) had no effect on the observed reaggregation pattern of wild-type cortical cells (Table 2). Rabbit anti-calretinin antiserum (10% in culture medium) also showed no effect.

Effect of CR-50 on Cell Sorting in Cortical Cell Aggregates

The functional assay shown above, along with its cell surface localization on the Cajal–Retzius neurons, suggests a role for the CR-50 antigen in the histotypic organization of normal cortical neurons in vitro. We comparatively analyzed the distribution of the early- (labeled with BrdU at E10–E10.5 in vivo) and later-generated (labeled with BrdU at 2 days in vitro) neurons within the aggregates and examined the possibility that CR-50 modulates their distribution. The distribution patterns of the early neurons in wild-type and *reeler* aggregates are shown in Figures 8A–8F. In wild-type aggregates, many BrdU-labeled cells were observed to localize in the upper and lower borders of the MAP2-labeled neuronal layer (Figures 8A and 8D). On the other hand, many of them were localized relatively superficially among the clustered neurons in the *reeler* aggregates (Figures 8B and 8E).

Table 2. Effects of CR-50 on the Histotypic Organization of Cortical Neurons in Aggregates Formed in Culture

Culture Condition	Number of Aggregates	Frequency of Aggregates		
		Type A	Type B	Type C
Wild type	19	15	0	4
reeler	30	0	24	6
Wild type plus CR-50 (20 μg/ml)[a]	6	1	3	2
Wild type plus CR-50 (200 μg/ml)	12	0	10	2
Wild type plus CR-50 Fab (1 mg/ml)[b]	6	0	5	1
Wild type plus non-immunized *reeler* IgG (1 mg/ml)[c]	8	6	0	2
Wild type plus CR-50 (absorbed with wild-type cortices)[d]	4	3	0	1
Wild type plus CR-50 (absorbed with *reeler* cortices)[d]	4	0	2	2

Aggregates were classified into three types according to the morphology revealed with MAP2 staining. Type A had MAP2-labeled neurons uniformly and symmetrically concentrated in the outer ⅓ zone of the spherical aggregates (e.g., see Figures 7A and 7D). Type B aggregates contained several patches of clustered MAP2-labeled neurons that were formed in the outer ⅓ zone of the spherical aggregates (e.g., see Figures 7B, 7C, and 7E). MAP2-labeled neurons of type C were parallel but rather loosely arrayed, and the inner border of the MAP2-labeled layer was vague.

[a] CR-50 was purified from ascites fluid by protein A–Sepharose affinity chromatography (MAPs kit, Bio-Rad).

[b] Fab fragments of CR-50 were prepared according to the manufacturer's instructions (ImmunoPure Fab Preparation Kit, Pierce Chemical Company).

[c] *reeler* IgG from nonimmunized animals was prepared using protein A–Sepharose affinity chromatography.

[d] Cerebral cortices were dissected out from E14 embryos and suspended into culture medium. Lyophilized CR-50 was dissolved in culture medium at 400 μg/ml and mixed with the above solution at 1:1 (v/v). After trituration with a Pasteur pipette, the cell suspension was incubated for 1 hr at 37° and centrifuged in an Eppendorf microfuge for 10 min to pellet particulate material. We confirmed immunohistochemically that the original immunoreactivity of CR-50 was lost with the absorption with wild-type but not mutant cerebrum.

The relative depth from the surface of the aggregate for BrdU-labeled neurons was measured by subdividing the aggregate into 20 bins parallel to the surface (Figure 8G; see Experimental Procedures), and the results were summarized in a set of histograms (Figures 8H–8J). Each histogram represents the mean distribution of the early- and later-generated neurons within the aggregates. In the wild-type aggregates, the distribution of the early neurons made dual peaks (Figure 8H), just beneath the aggregate surface and at the inner border of the MAP2-labeled layer, and the majority of later neurons accumulated among these early-generated neurons. On the other hand, in the *reeler* aggregates, most of the early neurons were distributed superficially, and the later-generated neurons piled up beneath them (Figure 8I). Thus, the cell arrangement of the early- and later-generated neurons within the aggregates showed distinct features reflecting the fact that the preplate-derived neurons sandwich the later-generated cortical plate neurons in wild-type animals; in contrast, in *reeler* animals, the preplate-derived neurons localize superficially, and those of the cortical plate pile up broadly beneath the preplate.

In response to CR-50, the distribution of the early-generated neurons in wild-type aggregates exhibited a marked positional sorting and resulted in a pattern similar to that observed in *reeler* aggregates (Figures 8C and 8F), and the later-generated neurons in vitro were also distributed similarly to those in *reeler* aggregates (Figure 8J).

Discussion

Allogeneic Antigen Related to *reeler* Gene

We have generated a monoclonal antibody (CR-50) that probes a distinct allelic antigen present in normal but not in *reeler* mutant mice. The following observations indicate that the CR-50 antigen is strongly related to the *reeler* gene product. The anti-CR was generated only when *reeler* mutant mice were immunized with brains of normal animals. CR-50 immunoreactivity was observed in tissue sections of normal mouse but not in those of *reeler* mutants. The CR-50 antigen in normal mouse was distributed in the regions where morphological abnormalities are known to occur in *reeler* mutant mice. Living Cajal–Retzius neurons expressed the CR-50 antigen on their cell surface in vivo and in vitro, and the histogenetic assembly of dissociated normal cortical cells in reaggregation culture was converted to a *reeler*-like pattern by the presence of CR-50. It is also conceivable that the Cajal–Retzius neuron is the cellular site of action of the wild-type allele at the *reeler* locus.

Arrangement of Cortical Plate Neurons within the Preplate Associated with the CR-50 Antigen

In normal animals, the cells of the cortical plate are generated at the ventricular zone and migrate outward, using radial glial fibers as substrates for migration (Rakic, 1972). Once within the preplate, they terminate their migration and then align at the correct location. Positioning of neurons within the cortical plate is therefore a part of migratory events, but the ultimate location is thought to be regulated independently of the preceding process (Caviness and Rakic, 1978; see also McConnell, 1992). In *reeler* mutant mice, the cells of the cortical plate migrate according to the schedules and pathways of the normal mouse (Caviness et al., 1988); however, most of them are not aligned within the preplate, but instead pile up beneath it (see Figure 5). Thus, the ultimate positioning of migrating neurons depends on their ability to align within the preplate in normal animals.

Based on results from reaggregation experiments, DeLong and Sidman (1970) suggested that cerebral cortical cells bear the surface determinants that allow cells to align and orient themselves properly and that the *reeler* mutation alters this ability. The results of disturbing cortical cell reaggregation with CR-50 make it evident that the CR-50 antigen is such a surface determinant and that it

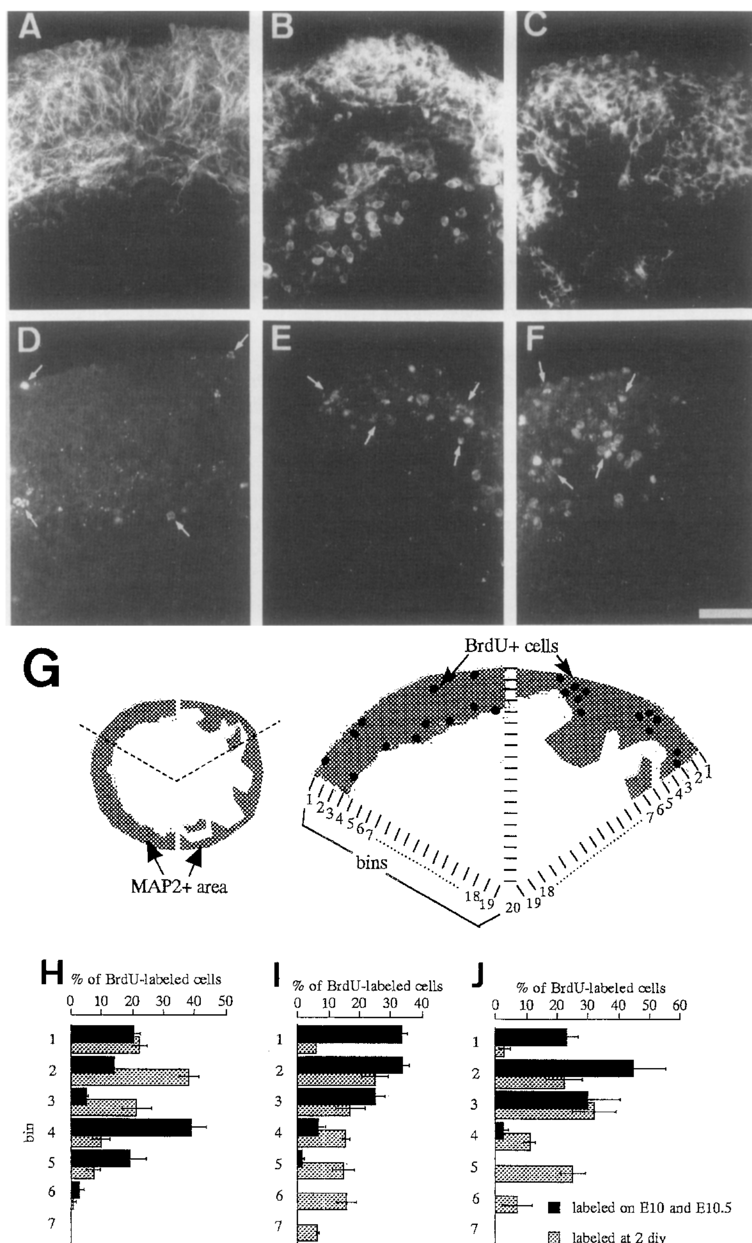


Figure 8. Alteration of Positioning of the Early- and Later-Generated Neurons within the Aggregates in Response to CR-50

(A–F) E12 cortical cells were cultured for 5 days as described in Figure 7. Embryos that had received BrdU at E10 and E10.5, for labeling most cells belonging to the preplate, were used to analyze the distribution of the early-generated neurons within the aggregates. Sections of each aggregate were double-stained with anti-MAP2 (A–C) and BrdU (D–F), and the paired immunofluorescent photomicrographs were arranged in tiers: (A and D) wild type; (B and E) *reeler*; (C and F) wild-type aggregates in the presence of CR-50 at 200 μg/ml. Bar, 50 μm.

(G) The relative depth of BrdU-labeled neurons was measured by subdividing the aggregate into 20 bins parallel to the surface (see Experimental Procedure).

(H–J) Histograms show the relative position of the early- and later-generated neurons within wild-type aggregates (H), *reeler* aggregates (I), and wild-type aggregates in the presence of 200 μg/ml CR-50 (J). For labeling neurons generated in aggregates (later-generated neurons), 10 μM BrdU was added into the culture medium for 1 hr at 2 days in culture (stippled bars). After washing, the aggregates were further incubated in renewed medium for 3 days. The frequency of $BrdU^+/MAP2^+$ cells in each bin was calculated as a percentage of the total number of $BrdU^+/MAP2^+$ cells (all $BrdU^+/MAP2^+$ cells were observed within 1–7 bins) in each aggregate. The data are expressed as the mean ± SEM from 4 aggregates each of two independent experiments performed on separate cell isolations. The average numbers of neurons labeled with BrdU in vivo and in vitro were 96 ± 7 and 160 ± 9 (H), 119 ± 1 and 220 ± 8 (I), and 95 ± 7 and 206 ± 11 (J), respectively.

mediates the cell–cell interactions of cortical neurons. The expression of the allogeneic CR-50 antigen in the preplate occurred concurrently with the formation of the cortical plate within the preplate. Moreover, the CR-50 antigen is confined exclusively to the surfaces of living Cajal–Retzius neurons. The Cajal–Retzius neurons localize superficially before and during the formation of the cortical plate and extend their processes into the cortical plate (Marin-Padilla, 1971; Shoukimas and Hinds, 1978; Derer and Derer, 1990). It seems likely, therefore, that each of the migrating young neurons may contact or interact with the Cajal–Retzius neurons at the end of its migration, and then receive the positional information for aligning within the preplate through association with the CR-50 antigen. Alternatively, it is also conceivable that the CR-50 antigen actively promotes the separation of the subplate neurons from the Cajal–Retzius neurons and makes room for the postmigratory neurons to align between them. In *reeler*, the postmigratory neurons are not able to meet with the CR-50 antigen, so they cannot be aligned within the preplate and may localize abnormally. Although the mechanism by which the CR-50 antigen exerts its effect on cell–cell interactions between the Cajal–Retzius neurons and other types of neurons in early neocortex requires further investigation, our observations implicate the Cajal–Retzius neurons in providing a framework for the successive lamination of cortical plate neurons within the preplate in normal animals.

Experimental Procedures

Animals
Homozygous *reeler* mice were bred from heterozygous B6C3Fe-a/a-rl adults (Jackson laboratory, Bar Harbor, ME). Homozygous embryos were obtained by mating homozygous males with homozygous females. Heterozygous embryos were obtained by mating homozygous males with wild-type females. The day at which a vaginal plug was detected was designated as E0.

Immunization
Immunogens of wild-type, heterozygous, and homozygous animals were prepared by homogenizing E14–E17 cerebral cortices of each with phosphate-buffered saline (PBS) by a single stroke with a Teflon-glass homogenizer. The preparation was then emulsified in Freund's complete adjuvant (Boehringer) for the initial injection, followed by the same preparation without emulsification for subsequent injections. The adult wild-type, heterozygous, and homozygous mice were immunized intraperitoneally at approximately biweekly intervals more than six times. Each recipient received an injection of a hemisphere of cerebrum at each step. All antisera were immunohistochemically screened on cryostat sections of both wild-type and homozygous cerebral cortices. Allogeneic antigenicity of antiserum was examined by the presence of alloantibodies that stain selectively for cerebral cortical cells of allelically different animals.

Tissue Sections
Pregnant mice were killed by an overdose of ether. Embryos were placed on ice for anesthesia and then perfused intracardially with 4% paraformaldehyde fixative in 0.1 M PBS (pH 7.4). Brains were removed, postfixed in the same solution for 4–6 hr at 4°C, and transferred to 20% sucrose in PBS. After sinking, the brains were embedded in OCT compound (Miles) and frozen in liquid nitrogen. Cryostat sections (10–20 μm) were mounted on gelatin-coated microscope slides and air dried.

CR-50
Hybridoma cell lines were produced by the polyethylene glycol–induced fusion of P3U1 mouse myeloma cells with spleen cells from immunized homozygous *reeler* mice. The resulting hybridoma cell lines were screened for production of a monoclonal antibody with the same specific distribution of binding to the marginal zone as polyclonal anti-CR. Positive cell lines were then cloned by limited-dilution methods (1 cell per well) and rescreened. Of 5647 hybridoma cell lines obtained from 22 mice, 6 were found to synthesize antibodies. The antibody secreted from 1 clone of hybridoma cells (RE-3B9) was designated CR-50.

Immunohistochemistry
The cryostat sections were preincubated with 5% normal horse serum–PBS (blocking solution) for 30 min and then incubated with primary antibodies diluted in blocking solution for 1–2 hr at room temperature or overnight at 4°C. Dilutions of the primary antibodies were as follows: mouse antiserum (anti-CR), 1:100; mouse monoclonal CR-50, culture supernatant of hybridoma (RE-3B9); rabbit polyclonal anti-MAP2, 1:500 (provided by N. Niinobe; Niinobe et al., 1988); rabbit polyclonal anti-calretinin (Chemicon International, Inc.), 1:500. After incubation with the primary antibodies, the sections were washed with PBS and reacted with Vector Elite immunoperoxidase kits for mouse or rabbit antibodies (Vector) followed by the chromogen diaminobenzidine. Alternatively, they were detected with fluorescein isothiocyanate (FITC)– or rhodamine-conjugated secondary antibodies (Vector; diluted as 1:100). Double labeling with CR-50/anti-MAP2 or CR-50/anti-calretinin antibodies was accomplished by the simultaneous application of both primary antibodies followed by rinses and the application of both fluorescent secondary antibodies. Double labeling with anti-BrdU/anti-MAP2 or anti-BrdU/anti-calretinin antibodies was conducted as follows. Cryostat sections were first labeled with polyclonal anti-MAP2 or anti-calretinin antibodies and then treated with 2 N HCl for 30 min. After neutralization with 0.1 M sodium borate buffer (pH 8.5), the sections were incubated with a blocking solution for 30 min, followed by mouse monoclonal anti-BrdU antibody (Becton Dickinson; diluted as 1:20). After the primary antibody reaction was completed, the sections were incubated for 1 hr at room temperature with fluorescent secondary antibodies. Double labeling with CR-50/anti-BrdU antibodies was done using the method of Watanabe and Raff (1990). The section initially labeled for CR-50 was treated with 2 N HCl for 30 min, neutralized, and washed in PBS, after which the specimen was incubated with FITC-conjugated secondary antibody, washed in PBS, incubated with anti-BrdU antibody, washed in PBS, and then incubated with rhodamine-conjugated secondary antibody.

BrdU Labeling
Pregnant mice were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU, Sigma; 5 mg/ml in saline solution) at 50 μg per gram of body weight.

Dissociated Cell Culture
Cerebral cortices from E14 wild-type embryos were isolated, and the meninges were pulled off. The neocortical region from the caudatopallial angle to the dorsal boundary of the lateral ventricle was dissected and digested in 0.25% trypsin. Single-cell suspensions were produced by trituration. Cells were centrifuged at 200 × g and resuspended in culture medium twice, then seeded onto poly-L-lysine-coated plastic dishes at 5×10^3 cells/cm^2. Cultures were grown at 37°C in a humidified atmosphere of 5% CO_2 in a medium of N2 (Bottenstein, 1985) supplemented with 10% fetal calf serum.

Reaggregation Culture
Reaggregation culture of cortical cells was principally done using the method of DeLong (1970). Dissociated cerebral cortices from E12 embryos were prepared as described above. After centrifugation, cells were suspended at a density of 3×10^5 cells per milliliter of N2 medium supplemented with 10% fetal calf serum in a tube (9 mm inner diameter; PYREX TCT-SCR). The tubes were incubated on the rotating holder in an atmosphere of 5% CO_2 with saturated humidity at 37°C and rotated at 5 rpm at an angle of 30°. After 5 days of incubation, cell aggregates were fixed in 4% paraformaldehyde and cryosectioned. Immunostaining was conducted similarly with tissue sections, but rat monoclonal antibody against BrdU (BIOSYS, France) was used with aggregate sections.

Construction of Histograms Representing the Distribution of BrdU-Labeled Cells in Aggregates
To determine the distribution of BrdU-labeled cells within each aggregate, we measured the relative depth of the labeled cell within the aggregate as follows. First, a section of an aggregate was double-stained with BrdU and anti-MAP2, and a pair of fluorescent photomicrographs of BrdU- and MAP2-labeled cells was made (e.g., see Figures 8A–8F). Next, on the MAP2-immunostained photomicrograph, the section was subdivided into 20 bins, parallel to the surface of the aggregate, and the bins were numbered from the surface inward (e.g., see Figure 8D). Then, each BrdU-labeled cell within the aggregate was assigned to a bin location on the MAP2-immunostained photomicrograph. Finally, the frequency of $BrdU^+/MAP2^+$ cells in each bin was calculated as a percentage of the total number of double-labeled cells.

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Note Added in Proof

Recently, Tom Curran's group has succeeded in cloning a *reeler* candidate gene (Nature *374*, 719–723, 1995).