Development 128, 561-569 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV1642

Role of cadherins in maintaining the compartment boundary between the cortex and striatum during development

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Accepted 29 November 2000; published on WWW 23 January 2001

SUMMARY

In ventricular cells of the mouse telencephalon, differential expression of cadherin cell adhesion molecules defines neighbouring regions; R-cadherin delineates the future cerebral cortex, while cadherin-6 delineates the lateral ganglionic eminence. By using cell labelling analyses in the whole embryo culture system, we demonstrated that the interface between R-cadherin and cadherin-6 expression is a boundary for cell lineage restriction at embryonic day 10.5. Interestingly, when a group of cells with exogenous cadherin-6 were generated to straddle the cortico-straital boundary by electroporation at embryonic day 11.0, ectopic cadherin-6-expressing cortical cells were sorted into the striatal compartment, and the reverse was the trend for ectopic R-cadherin-expressing striatal cells. Although *cadherin-6* gene knockout mice engineered in this study showed no obvious phenotype in telencephalic compartmentalisation, the preferential sorting of ectopic cadherin-6-expressing cells was abolished in this mutant background. Thus, the differential expression pattern of cadherins in the embryonic telencephalon is responsible for maintaining the cortico-striatal compartment boundary.

Key words: Cadherins, Cell lineage restriction, Compartment, Electroporation, Mammalian whole embryo culture, Neuromeres, Telencephalon, Cortico-striatal boundary, Mouse

INTRODUCTION

Compartmentalisation is one of the conserved morphogenetic strategies of multicellular organisms to pattern and maintain their body structures (for review, see Dahmann and Basler, 1999). In the development of the vertebrate central nervous system, for example, the neural plate/tube is gradually subdivided into several longitudinal units called ‘neuromeres’ (or ‘rhombomeres’, specifically for hindbrain subdivisions). The results of several studies have clearly indicated that movement of neuroepithelial cells during development is confined within some of these units (Fraser et al., 1990; Figdor and Stern, 1993; Fishell et al., 1993). Such cell lineage restricted compartment units must be significant in maintaining embryonic brain organisation since boundaries prevent cells, once patterned, from random intermingling during development. Neuromeres may also provide a basic framework for complex neuronal circuits as the neuromere boundaries often match the territories between functional brain subdivisions and with initial axonal tracts (Lumsden, 1990; Figdor and Stern, 1993).

The molecular mechanisms underlying brain compartmentalisation have been the major subject of early studies. For instance, expression of several transcription factors or secretary molecules is known to delineate unique sets of neuromeres (for reviews, see Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Lumsden and Krumlauf, 1996), suggesting differential chemoaffinities between compartment units. Eph receptor tyrosine kinases and their ligands are expressed in odd- and even-numbered rhombomeres in the zebrafish embryo (Xu et al., 1999), and bi-directional signalling at the rhombomere boundaries has been demonstrated to inhibit intermixing of neuroepithelial cells (Mellizer et al., 1999), thereby playing a crucial role in compartmentalisation of the hindbrain (for review, see Lumsden, 1999). Another category of cell surface molecules, cell adhesion molecules, have also been suggested to sort or maintain cells within a compartment unit and/or at the border regions through differential cellular affinities (Garcia-Bellido, 1966; Götz et al., 1996; Wizenmann and Lumsden, 1997), although the evidence is still indirect.

Classic types of cadherin cell adhesion molecules are grouped into more than 20 subclasses, each of which endows cells with selective adhesiveness; cells expressing particular

cadherin(s) prefer to bind those expressing similar cadherin(s) in a Ca^{2+}-dependent manner (Nose et al., 1988; for reviews, see Takeichi, 1991, 1995). Provided compartment units are differentially defined by cadherin expression, their characteristic binding specificity could prevent cells from random intermixing. In support of this scenario, combinatorial expression patterns of the cadherin subclasses is known to subdivide the embryonic brain into functional regions (for reviews, see Redies and Takeichi, 1996; Redies, 2000). For example, we have shown that cadherin-6 (cad6, Cdh6) and R-cadherin (Rcad, Cdh4) delineate neighbouring brain subdivisions, the lateral ganglionic eminence (lge) and the future cerebral cortex (ctx), respectively, in ventricular cells of the embryonic day (E) 12.5 mouse telencephalon (Matsunami and Takeichi, 1995; Inoue et al., 1997). Notably, dissociated cells from the ctx and lge of E14 rat embryos (roughly corresponding to E12 mouse embryos) segregate in a Ca^{2+}-dependent manner in suspension cultures (Götz et al., 1996). In addition, the ctx and lge are defined as cell lineage-restricted compartments, based on studies using slice preparations from E15 mouse embryos (Fishell et al., 1993). Rcad, the expression of which is Pax6-dependent in the ctx, is also considered as a candidate molecule for such selective affinity between the ctx and lge (Stoykova et al., 1997). In the *Xenopus* neural tube, F-cadherin has already been shown to function in positioning of neuroepithelial cells at the sulcus limitans (Espeseth et al., 1998).

Based on these early studies, we investigated whether more than one cadherin subclass, cad6 and Rcad, play important roles in formation/maintenance of the cortico-striatal (ctx/lge) boundary in the mouse embryo. We first confirmed that the expression of Rcad and cad6 commences in the ctx and lge of the mouse telencephalon by E10.5. Then we found that restriction of ctx and lge cell mixing occurred in cultured embryos at E10.5. We next established *cad6* gene knockout mice, whose ctx/lge boundary was found to be morphologically normal and had normal cell lineage restrictions, mainly because of redundant cadherins in the embryonic telencephalon. However, when various cadherin subclasses were electroporated into the area straddling the ctx/lge boundary of cultured embryos, cells expressing cad6 or Rcad were sorted into the lge or ctx, respectively, in response to the endogenous expression patterns of these cadherins. The preferential sorting by ectopic cad6 was totally abolished in the *cad6* mutant background. These results provide the first in vivo evidence that a differential expression of Rcad and cad6 is required in the telencephalon to maintain the ctx/lge boundary.

MATERIALS AND METHODS

Immunostaining

Monoclonal antibodies for cad6 (C42720, Transduction Laboratories) and Rcad (Matsunami and Takeichi, 1995) were used at 1:150 and 1:10 dilutions, respectively. The polyclonal antibody for HA Tag (Y-11; Santa Cruz, CA) was used at 1:50 dilution. For detection of these specific antibodies, secondary antibodies against mouse, rat, and rabbit IgG conjugated with horseradish peroxidase were used at 1:200 dilutions (Chemicon International Inc.). Whole-mount immunostaining was performed as described previously (Matsunami and Takeichi, 1995). It should be stressed that the microwave process in the boiling buffer (10 mM sodium citrate, pH 6.0) prior to fixation is necessary to obtain intense signals with these antibodies.

Whole embryo culture and cell labelling method

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of National Institute of Neuroscience. ICR mice were mated to obtain the embryos and the day of appearance of the vaginal plug was taken as E0. The protocols used for whole embryo culture (WEC) and neuroepithelial cell labelling have been described in detail previously (Osumi-Yamashita et al., 1997; Inoue et al., 2000). Briefly, DiI (Molecular Probes, D-282) or DiO (Molecular Probes, D-275) was dissolved to saturation in dimethylformamide and stored at 4°C. For labelling of the telencephalic neuroepithelium, an embryo ready for culture was held with a pair of tweezers and microinjected manually with a 10-fold diluted dye stock solution (Fig. 2A), yielding 10-50 labelled cells. Each labelled spot was imaged under a binocular microscope (Leica, MZ 8) equipped with the CCD camera (Leica, 3CCD high gain colour camera), and the image was recorded by a colour video copy processor (Mitsubishi, SCT-CP7000; Fig. 2B-E). Each embryo was then cultured in a separate glass bottle. After culture, the number of somites and morphology of the embryos were carefully checked, and those embryos exhibiting abnormal development were excluded from the study. In our culture system, >90% of embryos at E10.5 developed normally for 24 hours, and >75% for 48 hours. The harvested embryos were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 12 hours at 4°C. The brain of each embryo was dissected and cut sagittally in half. Photographs were taken using a fluorescent microscope (MZ FLIII, Leica) fitted with a filter suitable for DiI or DiO.

Establishment of *cad6* mutant mice

To generate *cad6* gene targeting mice, embryonic stem cells were transfected with the targeting construct (see Fig. 3A) using the method described by Swiatek and Grindley (1993). Targeted embryonic stem cells were genotyped by Southern blotting (Fig. 3B). Both embryos and adult animals were genotyped by PCR (Fig. 3C). To detect the 185 bp band indicative of a wild-type allele, we employed primers corresponding to the first exon of *cad6* gene, 6-44 (5′-AGCCCTA-CCCAACTTTCT-3′) and 6-228r18 (5′-CTTGCCCACGTACTGATA-3′). To detect the 680 bp band indicative of the mutant allele, primers corresponding to the neomycin phosphotransferase gene, Neo3347 (5′-CCTGCTTGCCGAATATCA-3′) and 6-44. For western blotting analysis, the rabbit anti-cad6 polyclonal antibody (cad6-2; see Inoue et al., 1998) was applied at 1:1000 dilution.

Vectors

The oligonucleotides encoding triple HA antigens and their antisense oligonucleotides were synthesised. The annealed fragments were digested by *Nhe*I and cloned into a *Spe*I site of the pBluescript SK (Stratagene). This construct was digested by *Xba*I, and self-ligated after the cut ends were filled in to generate a stop codon in frame with the HA antigens (pHAstop). Other fragments encoding cad6 protein with synthesised 5′ *Hin*dIII and 3′ *Sma*I linkers were amplified by PCR, using a phage cDNA clone containing the full-length *cad6* (Inoue et al., 1997) as a template. After the *Hin*dIII/*Sma*I digestion, they were inserted into the *Hin*dIII/*Sma*I site of pHAstop. A region corresponding to the *cad6* open reading frame without its stop codon, three HA tags and the generated stop codon were then excised by *Xba*I/*Not*I digestion. After blunting, the region was cloned into a *Hpa*I site of the pCA-pA vector containing the cytomegalovirus (CMV) enhancer and β-actin promoter, yielding pCA-cad6-HA. When pCA-cad6-HA was transiently transfected into a fibroblastic cell line, L-cell, a strict cadherin-based cell-cell adhesion was induced with intense HA expressions at cell-cell contact sites (data not shown). pCA-cad6Δ-HA lacking all of the cytoplasmic domain but 9 amino acid residues

juxtaposing the transmembrane domain of pCA-cad6-HA was similarly constructed. The expression vectors for non-tagged mouse R-, N-, and E-cadherins have been described previously (Nagafuchi et al., 1987; Miyatani et al., 1989; Matsunami et al., 1993). The FLAG tagged dominant negative cadherin (Fujimori and Takeichi, 1993) and green fluorescent protein (GFP) were also cloned into the pCA-pA vector to yield pCA-ΔNcad-FLAG and pCA-GFP, respectively. These expression vectors were finally dissolved in PBS at 5 mg/ml for in vivo electroporation.

Gene transfer

The strategy for transferring genes into cultured mammalian embryos is based on electroporation in chick embryos (Muramatsu et al., 1997; Itasaki et al., 1999), which is shown schematically in Fig. 4A (also see Akamatsu et al., 1999). Briefly, precultured E10.5 embryos were transferred to the chamber-type electrodes (Fig. 4A; 8×20 mm electrodes made of platinum, and 25 mm distance between the electrodes). 0.3 μl of the plasmid vector solution was injected into the telencephalic vesicles, and square pulses (50 mseconds, 85 V, five times, 1 second intervals) were immediately sent using Electro Square Porator model T820 and the Optimizor 500 (BTX). To detect GFP expression, embryos were fixed with 4% PFA in PBS for several hours at 4°C, and their brains were cut in half sagittally to be observed from their ventricular sides for fluorescence using binocular microscopy (MZ FLIII, Leica). Some embryos were processed for anti-HA or anti-Rcad staining as described above.

RESULTS

Expression profiles of cadherins define the ctx and lge as compartment units in the telencephalon

We have previously demonstrated that expression of cad6 and Rcad mRNAs in ventricular cells demarcates two neighbouring areas, the lge and ctx, in the E12.5 mouse telencephalon (Inoue et al., 1997). In this study, we examined the protein expression of these two cadherins at earlier stages by whole-mount immunostaining. At E10.5, the morphological boundary between the ctx and lge in the telencephalic vesicle was not yet obvious. At this stage, cad6 expression already demarcated a brain region in the telencephalon (Fig. 1A), while Rcad delineated the neighbouring area (Fig. 1B). The ctx/lge boundary becomes obvious morphologically by E11.5. At this stage, the expression of cad6 and Rcad bordered each other, making a sharp interface at the morphologically identifiable ctx/lge boundary (Fig. 1C,D). Thus, complementary expression of the two cadherins in neuroepithelial cells of the ctx and lge occurs by E10.5 in mice.

According to an earlier study, ventricular cells of the ctx and lge do not intermingle in slice preparations of E15 mouse embryonic brain (Fishell et al., 1993). In addition, dissociated cells of the ctx and lge isolated from E14 rat (roughly corresponding to E12 mouse) embryos can segregate in a Ca^{2+}-dependent manner (Götz et al., 1996). Based on these data, we next examined whether the interface between Rcad and cad6 expression can be used to define the boundary for cell lineage restriction at E10.5. Ventricular cells of the E10.5 telencephalon were labelled with vital dyes, DiI and/or DiO, and the distribution of sibling cells was observed after 48 hours of WEC (Fig. 2A). This culture period was of sufficient duration to allow the development of a morphologically identifiable ctx/lge boundary, and a single labelled spot, initially containing 10~50 labelled cells (Fig. 2B-E), generated a large group of labelled siblings after the culture (Fig. 2B′-E′). We categorised the siblings of each labelled spot into five classes: (i and ii) those distributed only within the ctx or lge (plotted as upward and downward pointing triangles in Fig. 2F), (iii and iv) those distributed to border the ctx/lge boundary (plotted as upward and downward pointing bold triangles in Fig. 2F), and (v) those distributed to straddle the ctx/lge boundary (plotted as squares in Fig. 2F). In a total of 51 labellings, we found that almost all siblings from the spots labelled within Rcad- or cad6-positive regions at E10.5 did not violate the ctx/lge boundary after 48 hours of WEC (Fig. 2B′; triangles in Fig. 2F). In some cases, the labelled siblings obeyed the ctx/lge boundary (bars with yellow arrows in Fig. 2C′,D′; Fig. 2E′; bold triangles in Fig. 2F). In others, the siblings were distributed to straddle the ctx/lge boundary only when cells were labelled just along the Rcad/cad6 expression limit at E10.5 (squares in Fig. 2F). We noticed that several populations of cells, which were originally labelled in the cad6-positive region, crossed the boundary in the mantle zone (data not shown). These cells are regarded as differentiated neurones and such neuronal migration from the lge to the ctx has been reported previously (Tamamaki et al., 1997; Anderson et al., 1997; Chapouton et al., 1999). Thus, we concluded that the expression interface of the two cadherins (Rcad and cad6) in the mouse E10.5 telencephalon corresponds to the prospective ctx/lge compartment boundary within the ventricular zone.

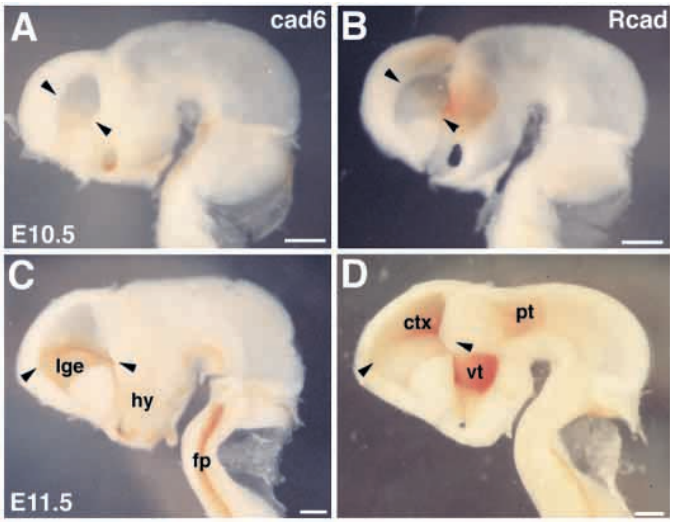

Fig. 1. Restricted expression patterns of cad6 and Rcad in the mouse embryonic brain. (A-D) Expression of cad6 and Rcad at E10.5 (A, B) and E11.5 (C,D) was examined in the whole-mount brain samples cut in half sagittally. As for E11.5 samples, the medial walls of the telencephalic vesicles were removed. The right halves of stained samples are observed from their ventricular sides (the anterior is to the left). Note that in ventricular cells of E10.5~11.5 mouse telencephalon, cad6 demarcates the lge (A,C), while Rcad delineates the ctx (B, D). Arrowheads in A-D indicate the expression limit for cad6 and Rcad in the telencephalon. The positively immunostained regions are marked by abbreviations: ctx, future cerebral cortex; fp, floor plate; hy, hypothalamus; lge, lateral ganglionic eminence; pt, pretectum; vt, ventral thalamus. Scale bars, 500 μm.

Generation of *cad6* mutant mice

To determine the significance of the complementary expression patterns of Rcad and cad6 in the telencephalic compartments, we performed both loss-of-function and gain-of-function analyses. In the loss-of-function study, *cad6* mutant mice were generated. We constructed a targeting vector in which the portion of the *cad6* gene encoding the first exon was replaced with a *PGKNeobpA* gene cassette in a reverse orientation relative to *cad6* transcription (Fig. 3A). The targeting construct was used to transfect ES cells, and targeted ES clones were selected by Southern blot (Fig. 3B). We prepared lines of chimeric mice using the targeted ES clones, and found that one of the targeted clones produced germline chimeras with high efficiency. Animals heterozygous for the targeted *cad6* allele were established by mating germline-transmitting chimeras with C57Bl/6 mice. F_1 heterozygotes were intercrossed, and offspring were genotyped by Southern blot (data not shown) and PCR analysis (Fig. 3C). We found that the mutant mice were viable and fertile, even though the cad6 protein could not be detected in their whole-brain homogenate from postnatal day 7 (Fig. 3D).

In this cad6 protein null mutant, gross morphology of the brain remained normal throughout development, and region-specific markers such as Pax6 and Dlx2 in the E11.5 telencephalon exhibited expression profiles indistinguishable from those of the wild type (data not shown). By vital dye labelling, the ctx/lge compartment boundary was also confirmed to exist in the *cad6* mutant (bold triangles in Fig. 3E). A minor, yet interesting phenotype is that the cell movement appeared to be enhanced in the mutant lge (Fig. 3E). That the ctx/lge boundary remains in *cad6* mutant embryos is likely due to unaltered expression of Rcad within the ctx (data not shown); as N-cadherin (Ncad, Cdh2) is expressed in ventricular cells of the entire brain (Matsunami and Takeichi, 1995), differential adhesiveness between the ctx (Rcad + Ncad) and the lge (Ncad) may still exist in the *cad6* mutant mouse. These results indicated that the loss of a single cadherin, Cad6, is insufficient to deform the ctx/lge compartment boundary.

Focal overexpression of cadherins at the ctx/lge boundary in wild-type and *cad6* mutant embryos

A gain-of-function study was performed by overexpressing various cadherin subclasses into cultured mice embryos (Fig. 4A). In order to establish optimal conditions for electroporation, we transferred an expression vector for GFP (pCA-GFP) into the mouse E10.5 telencephalon. As expected,

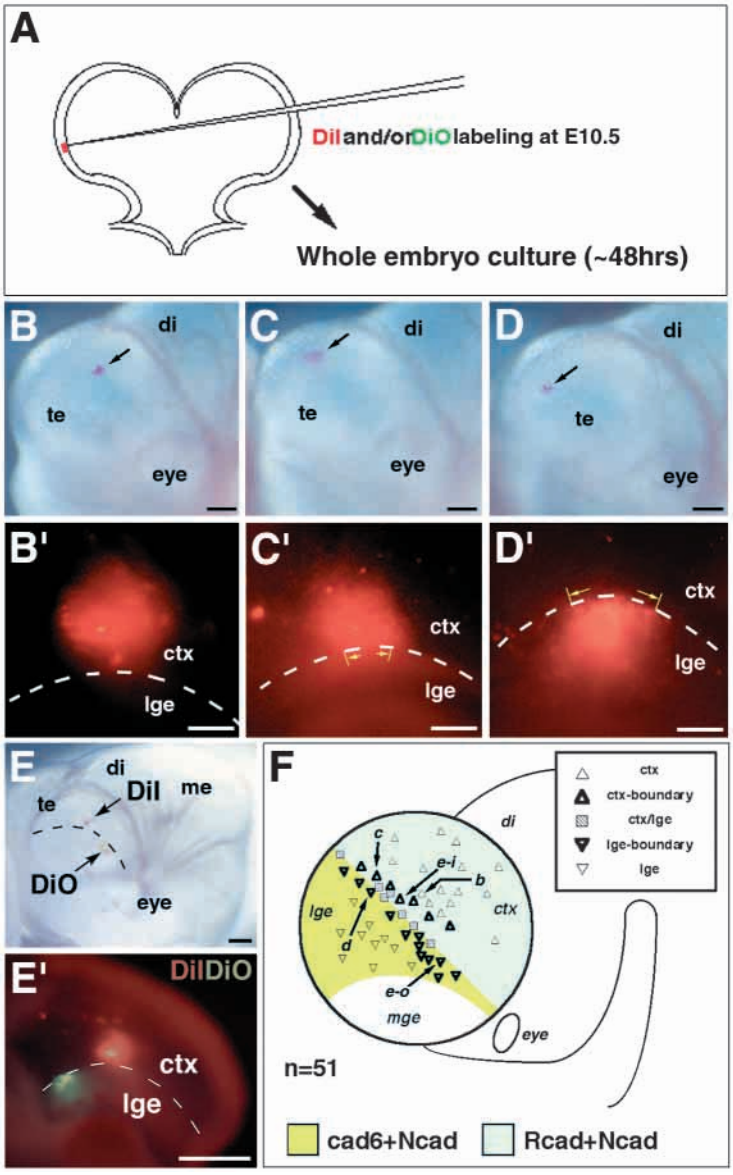

Fig. 2. Expression limits of cad6 and Rcad in the mouse embryonic telencephalon can be used to define the boundary between compartments at E10.5. (A) Schematic drawing of the method used to label neuroepithelial cells in the E10.5 telencephalon. Each labelled spot contains 10~50 cells. (B-D) DiI-labelled spots at the beginning of the whole embryo culture (WEC) are indicated by arrows. (B′-D′) Distribution pattern of the siblings of the labelled cells in B-D after 48 hours in WEC. (B′) A round shaped clone in the ctx and (C′,D′) clones bordering the ctx/lge boundary. Yellow bars with arrows indicate the smooth edge of labelled cells bordering the ctx/lge boundary. (E) Two spots within the left telencephalic vesicle indicated by arrows were labelled with DiI and DiO simultaneously at the beginning of WEC (E10.5). The dashed line shows the virtual expression limit of cadherins at E10.5. In this case, the DiI-labelled spot is within the Rcad-positive area and the DiO-marked spot is within the cad6-positive area. (E′) The same embryo as in E after 48 hours of WEC. The sibling cells of the labelled spots border the ctx/lge boundary. The sample is viewed from its ventricular side (the anterior is to the right) and the white dashed line indicates the ctx/lge boundary at this stage. (F) Summary of cell tracing analysis. According to the distribution of siblings in the telencephalon, initial labelled spots are categorised into five classes: those with siblings distributed (i) only within the ctx (white triangles) or (ii) within the lge (inverted triangles; B′), (iii and iv) bordering the ctx/lge boundary (bold triangles; C′-E′), and (v) straddling the ctx/lge boundary (squares). The position of the DiI-labelled spots in B-D are indicated by arrows labelled b-d in F. The DiI- and DiO-labelled spots in E are indicated by arrows labelled e-i and e-o, respectively. ctx, future cerebral cortex; di, diencephalon; lge, lateral ganglionic eminence; me, mesencephalon; mge, medial ganglionic eminence; te, telencephalon. Scale bras, 100 μm for (B′-D′), 500 μm for (B-E, E′).

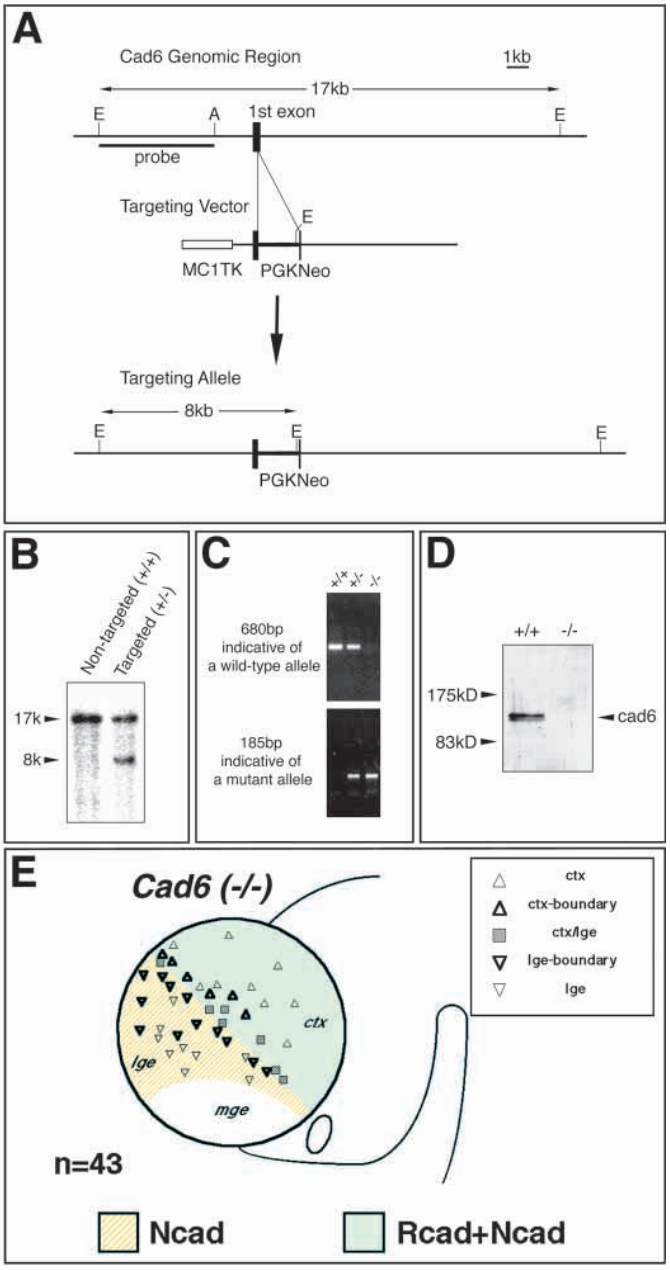

Fig. 3. Generation of *cad6* mutant mice. (A) Design of the gene targeting construct. (Top) Normal genomic structure of the *cad6* gene. The vertical column represents the first exon, although the location of other exons is not determined in this genomic clone. The position of a flanking probe for target selection by Southern blot analysis is indicated. (Middle) Targeting replacement vector. A *PGKNeobpA* gene cassette is inserted into the *Bam*HI site which is 203 nucleotides from the beginning of the *cad6* gene, in the reverse orientation relative to *cad6* transcription. An *MC1TK* cassette was added for negative selection to enrich the targeted cells. (*Bottom*) Structure of the mutated *cad6* gene. A, *Afl*II; E, *Eco*RV. (B) Southern blot analysis for the selection of targeted embryonic stem cell clones. DNA digested with *Eco*RV and probed with a 4.5 kb *Eco*RV-*Afl*II fragment 5′ to first exon, not included in the targeting vector, yielded diagnostic 17 kb restriction fragments from wild-type (+/+) and 8 kb fragments from mutant alleles (–/–). (C) PCR analysis for the selection of mutant mice. (D) Western blot analysis of wild-type and mutant brain homogenates. No band was detected with whole brain homogenates from the postnatal day 7 mutant mouse in the expected position. (E) The ctx/lge boundary is not affected, while cellular motility within the lge ventricular zone seems to be enhanced in the *cad6* mutant. Vital dye labelling was performed and the results are summarised as in Fig. 2F.

expression of GFP was only seen in ventricular cells of the region facing the anodal side 24 hours later (Fig. 4B-E). Notably, gene expression was observed in almost all ventricular cells in the targeted area (Fig. 4F).

We then electroporated pCA-cad6-HA at E10.5 to generate cells with ectopic cad6 expression straddling the ctx/lge boundary by E11.0, and examined the distribution of cad6-overexpressing cells after 24 hours. In this case, HA-positive cells were scarcely observed within the ctx, but were detected within the lge facing the ctx/lge boundary (Fig. 5A,B). Curiously, the HA-positive cells along the ctx/lge boundary formed cellular clumps or tubes on the ventricular side (arrow in Fig. 5B). When electroporated cells were monitored by co-injection of pCA-cad6-HA with pCA-GFP, GFP-positive cells again predominated within the lge (Figs 5C-E, 6A). This finding was significantly different from that of the control experiment; when pCA-GFP was targeted to straddle the ctx/lge boundary, the GFP-positive cells were present both in the ctx and lge (Figs 4F, 6A). One possible explanation for this finding could be that cells expressing ectopic cad6 in the ctx region could not survive. This possibility was unlikely because when pCA-cad6-HA, together with pCA-GFP, was targeted to be expressed within the dorsal ctx (not straddling the boundary), GFP- and HA-positive cells remained in the ctx (Fig. 6B) without evidence of apoptosis or necrosis (data now shown). An interesting point to be emphasised here is that the preferential sorting was only seen when groups of cells with ectopic cad6 expression had contacted the lge cells that endogenously express the same cadherin. We therefore proposed another scenario; the cells with ectopic cad6 in the ctx were actively sorted into the lge when cad6 was targeted to the ctx/lge boundary. This was actually confirmed by time-course analysis; 9 hours after electroporation of cad6/GFP at the boundary, GFP-positive cells still remained both in the ctx and lge (Fig. 6A). In addition, the expression limit for R-cad shifted beyond the morphological ctx/lge boundary 24 hours after electroporation (Fig. 5F-H). Taken together, our results showed that the cells expressing ectopic cad6 are sorted into the lge where endogenous cad6 is expressed.

When Rcad was targeted at the ctx/lge boundary, the direction of the cell movement tended to be reversed; Rcad/GFP-positive cells were sorted from the lge to the ctx (Fig. 5I-K). However, the sorting effect was less obvious than with cad6 overexpression (compare Fig. 6C with Fig. 6A). This may be due to heterophilic interactions of Rcad with another cadherin, Ncad (Matsunami et al., 1993), which is expressed ubiquitously in ventricular cells of the entire telencephalon (Matusnami and Takeichi, 1995). When Ncad was introduced to the ctx/lge boundary, these cells remained at the boundary (Fig. 6C). This was also the case for ectopic E-cadherin (Ecad, Cdh1), which is not expressed in either the ctx or lge (Matsunami and Takeichi, 1995; Fig. 6C). These results suggested that the cells expressing exogenous cadherin subclasses can be sorted on the basis of the endogenous expression pattern of cadherins.

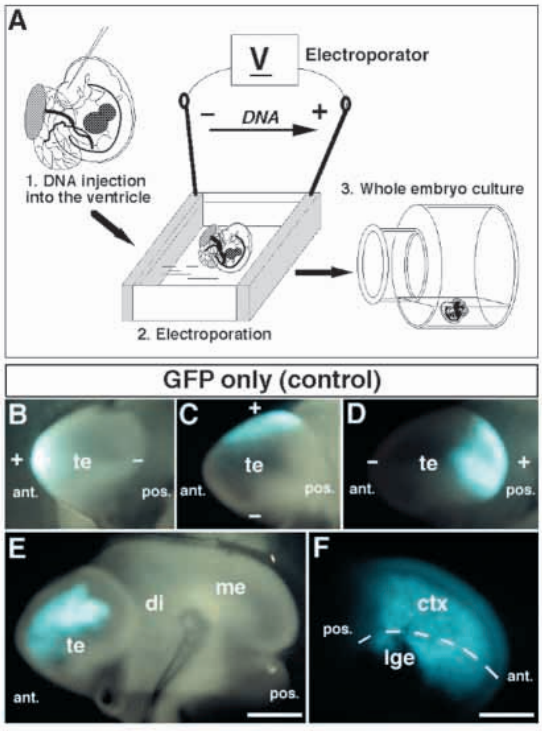

Fig. 4. Establishment of the gene transfer method into cultured mammalian embryos by electroporation. (A) Schematic flow chart of the method. (Step 1) Dissection of embryos at a defined developmental stage, and microinjection of the solution of expression vector(s) into the ventricle at a preselected part of the neural tube. (Step 2) Setting the embryo with the targeted wall of the ventricle facing the anode in the chamber, and applying square pulses immediately. The injected DNA is expected to move from the cathode (–) to anode (+). (Step 3) Gene expression commences after several hours of WEC. (B-F) pCA-GFP was transferred into various restricted regions of mouse E10.5 telencephalon and GFP signals were observed in whole-mounts after 24 hours of WEC. In B-D the directions of the electric field is indicated by + and –. (E) pCA-GFP was targeted to straddle the ctx/lge boundary. (F) A view from the ventricular side of the same embryo as in E. The dashed curved line indicates to the ctx/lge boundary. Note that the GFP signal is observed precisely at the targeted area and almost all cells within the positive region express the exogenous gene. ant., anterior side of the brain; ctx, future cerebral cortex; di, diencephalon; lge, lateral ganglionic eminence; me, mesencephalon; pos., posterior side of the brain; te, telencephalon. Scale bars, 1mm for (E), 500 μm for (F).

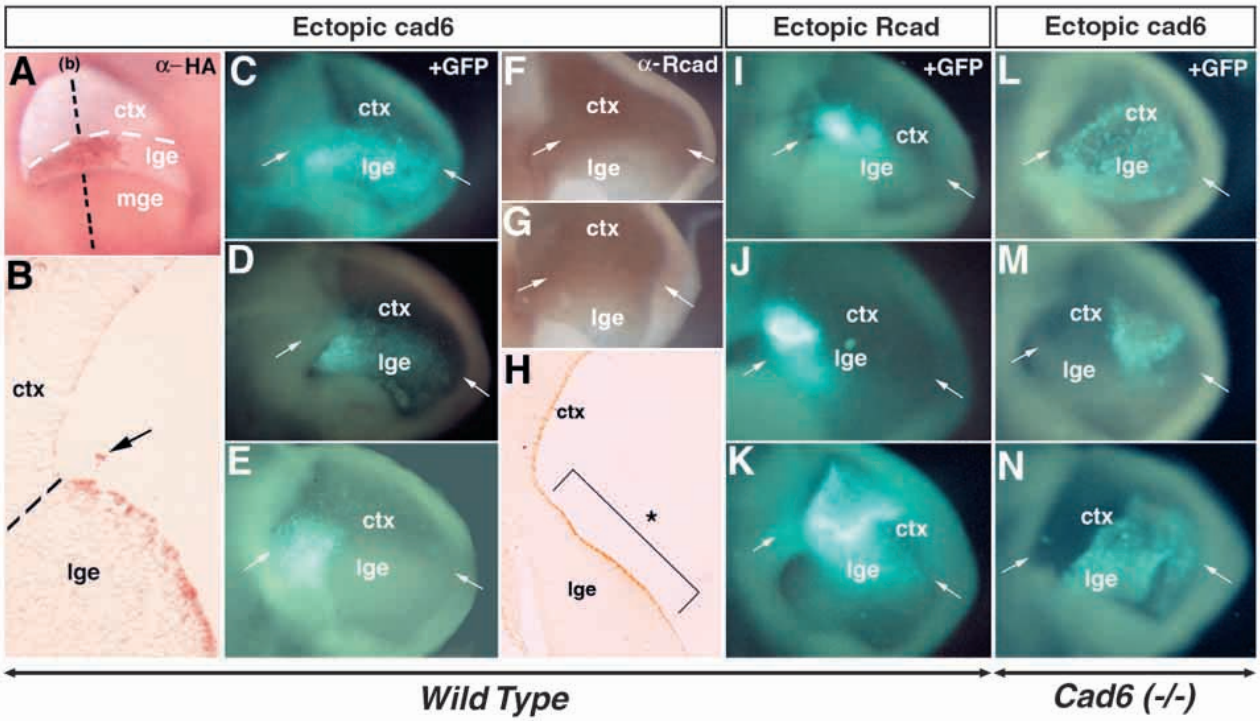

Fig. 5. Ectopic expression of cadherins at the ctx/lge boundary in wild type and *cad6* mutant mice. (A) pCA-cad6-HA was electroporated in the telencephalon including the ctx/lge boundary at E10.5. Cells expressing exogenous cad6 are detected by whole mount immunostaining for HA tags after 24 hours of WEC (line b indicates line of section in B). (B) Clumps of HA-positive cells are formed (arrow) only along the boundary. (C-E) The pCA-GFP was co-transfected with pCA-cad6-HA at the E10.5 ctx/lge boundary, and GFP-positive cells were examined 24 hours later. Note that cells expressing exogenous cad6 and GFP are predominantly located within the lge. (F-H) Cad6 was transferred as in (C-E) and Rcad expression was examined by whole-mount immunostaining after 24 hours of WEC. Note Rcad-positive cortical cells moving into the lge (a square bracket with an asterisk in H; compare F and G with Fig. 1D). (I-K) Rcad was co-transferred with GFP as in (C-E). GFP-positive cells tended to be distributed within the ctx rather than the lge. (L-N) Cad6 was targeted as in C-E in *cad6* mutant mice. The sorting effect with ectopic cad6 is no longer observed. White arrows indicate the position of the ctx/lge boundary; mge, medial ganglionic eminence. Sample scales in A-N are all comparable with those in Fig. 4F as these samples are at the same developmental stage.

To further investigate whether differential expression of cadherins is required for sorting cells ectopically expressing a particular cadherin, we performed a similar experiment in the *cad6* mutant. When cad6/GFP was introduced in cells straddling the ctx/lge boundary, those with ectopic cad6 were no longer sorted in the mutant (Figs 5L-N, 6E). This finding is in contrast with results in the wild-type background, in which cells with ectopic cad6 retreated into the lge (refer Fig. 5A-H). In addition, when non-functional cad6 (cad6Δ; lacking almost all the cytoplasmic region necessary to induce stable adhesion between cells), or a dominant negative cadherin (ΔNcad; lacking almost all the extracellular domain; Fujimori and Takeichi, 1993), was targeted to the ctx/lge boundary, the sorting effect could not be observed (Fig. 6D). Taken together, it is concluded that the differential expression of Rcad and cad6 plays a role in maintaining the ctx/lge boundary through their characteristic selective adhesiveness.

DISCUSSION

Classical experiments have formulated the hypothesis that differential affinities between cells serve to retain a variety of tissue structures in a multicellular organism during development (Townes and Holtfreter, 1955). While brain compartmentalisation in vertebrates provides an ideal model system to examine how differential cellular affinities operate in morphogenesis, the precise molecular mechanisms involved in the process remained elusive. Cadherins are cell adhesion molecules that confer cell selective adhesiveness (Nose et al., 1988). We have previously demonstrated exclusive expression patterns of Rcad in the ctx region and cad6 in the lge region in the E12.5 mouse brain (Inoue et al., 1997). These telencephalic subdivisions are also defined as cell-lineage-restricted compartments in a study using slice preparations from E15 mouse embryos (Fishell et al., 1993). In order to elucidate the function of cadherins in brain compartmentalisation, we first addressed the question of whether the expression limit of Rcad and cad6 coincides with a boundary for restriction of ctx and lge cell mixing. Immunostaining studies and cell labelling experiments showed

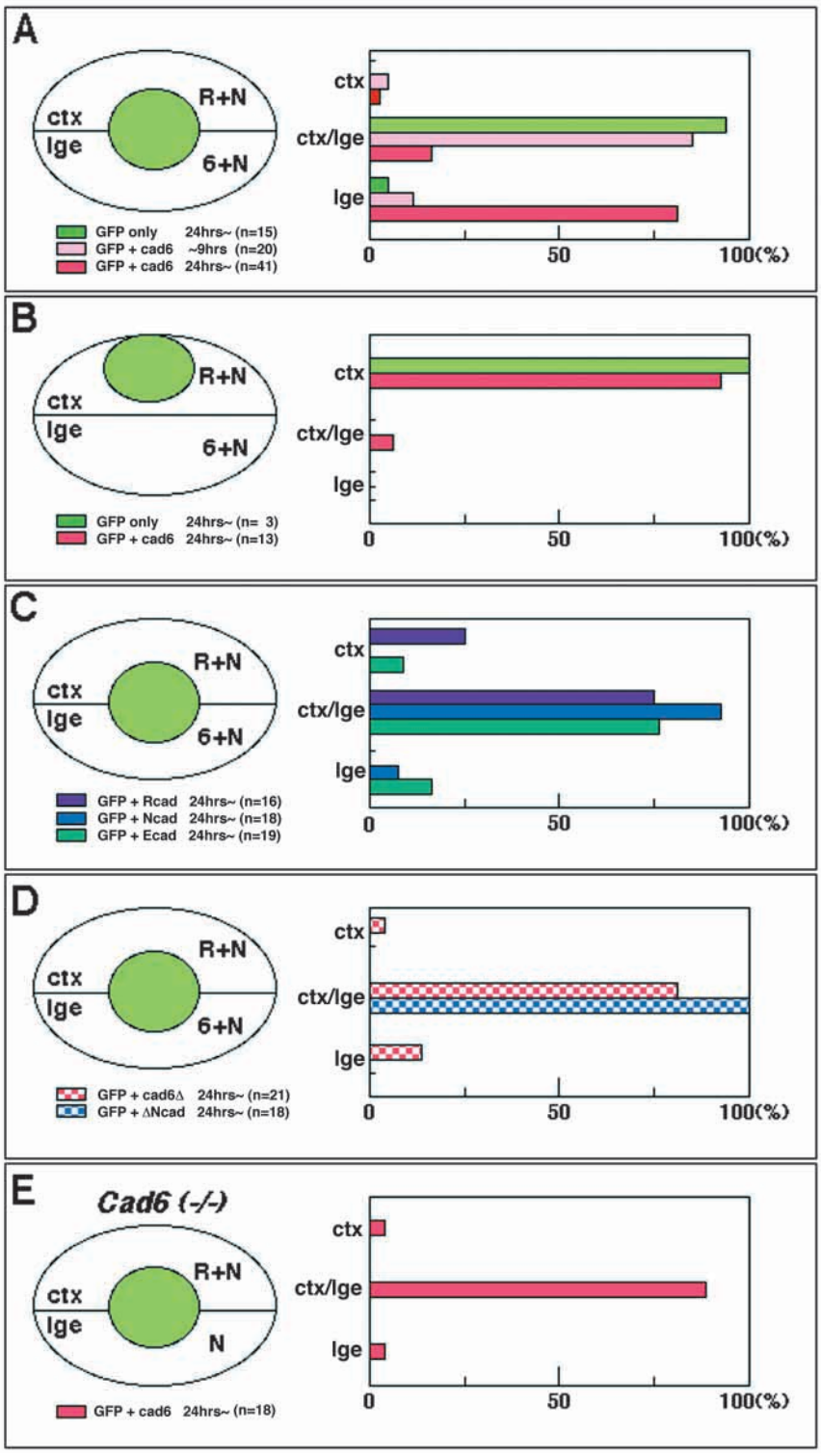

Fig. 6. Summary of cadherin overexpression experiments in the mouse E10.5 telencephalon. On the left side of each panel, the ctx and lge are represented as an oval. Expression of cadherins is indicated (i.e., ctx, Rcad+Ncad (R+N), and lge, cad6+Ncad (6+N) in wild type (A-D); Ncad (N) only in *cad6* mutant mice (E)). The green circles indicate the targeted areas for ectopic genes. Transfected genes, culture periods and numbers of cases for each experiment are specified. Bars on the right side of each panel summarise the experimental results. Distribution of cells with GFP is categorised into three classes; >90% of GFP-positive cells were distributed within the ctx (Fig. 5I for a typical example), >90% of GFP-positive cells were distributed within the lge (Fig. 5A,C,D for typical examples), or neither (ctx/lge; Fig. 5E, J,K,L-N for typical examples). Percentages of each distribution pattern for each experiment are shown by bars of different colours.

that the Rcad/cad6 expression limit exactly falls into a pre-established boundary for restriction of ctx and lge cell movement at E10.5.

Involvement of cadherins in sorting cells in the developing telencephalon has been examined previously. Dissociated cells from the ctx and lge of E14 rat embryos (roughly corresponding to E12 mouse embryos) segregated in a Ca^{2+}-dependent manner (Götz et al., 1996). Rcad expression is downregulated in the *Pax6* mutant cortex (Stoykova et al., 1997; our unpublished results in *Pax6* mutant rat), and ctx cells dissociated from the *Pax6* mutant exhibit different adhesiveness from those of the wild type (Stoykova et al., 1997). Rcad is thus suggested as one of the candidate molecules for selective affinity between the ctx and lge. The function of a single cadherin was previously assayed in *Xenopus* embryos, where F-cadherin acts to localise a particular population of cells precisely into the sulcus limitans during the formation of the neural tube (Espeseth et al., 1998). In the present study, we investigated the role of more than one cadherin subclass, which are differentially expressed in the ctx and lge, in formation/maintenance of the ctx/lge compartment boundary in the mouse embryo. When exogenous cad6 was electroporated to the area including the ctx/lge boundary, these cells were preferentially sorted into the lge, and the reverse effect was observed in case of Rcad electroporation. Importantly, this sorting effect was not observed with overexpression of Ncad (ubiquitously expressed in the entire telencephalon) or with ectopic expression of Ecad (not expressed in the ctx and lge). Furthermore, the sorting effect with ectopic cad6 was abolished in *cad6* mutant mice. These results provide the first in vivo evidence that the differential expression of cadherins in the embryonic telencephalon is responsible for maintaining the ctx/lge boundary.

The preferential sorting of ectopic cad6 into the lge supports the notion that homophilic adhesion normally restricts cad6-expressing cells to the lge. However, in this experimental condition, the electroporated cells on the ctx side of the boundary continued to express endogenous Rcad as well as ectopic cad6 (Fig. 5F-G). Therefore, the ctx/lge boundary is not rigorously dependent on cell lineage but on cell surface conditions of the populations. Why then do these Rcad-expressing cells segregate away from their original Rcad domain? Previous experiments have shown that when cells expressing a higher level of a particular cadherin contact those expressing a lower level of the same cadherin, the latter spread progressively over the surface of the former to make these two populations into a stable state (Steinberg and Takeichi, 1994). This would minimise the contact among cells with different sets of cadherin subclasses, and is also likely to be used in preferential sorting in vivo. That is, there are four populations with different cadherin expression profiles after cad6 electroporation: (i) native ctx cells (low Rcad + Ncad), (ii) electroporated cells at the ctx side (low Rcad + Ncad + high cad6), (iii) electroporated cells at the lge side (Ncad + high cad6), and (iv) native lge cells (Ncad + low cad6). The second population may be the most combinative with the third population because both populations exhibit a high level of cad6, and therefore the second population moved toward the lge. Eventually, the ctx cells with ectopic cad6, which were sorted to encounter with the lge cells, would further segregate into the ventricle as a clump or tube-like structure at the native ctx/lge boundary (arrow in Fig. 5B). In contrast, a much weaker sorting effect of ectopic Rcad was observed. In this case, electroporated lge cells (Ncad + low cad6 + high Rcad) could not make stable contact with the electroporated ctx cells (Ncad + high Rcad) or with the native ctx cells (Ncad + low Rcad) probably because homophilic affinity of cad6 is stronger than that of Rcad. Heterophilic interaction between Rcad and Ncad (Matsunami et al., 1993) also explains the weaker sorting effect of ectopic Rcad.

Inconsistent with the above-described experimental model (Steinberg and Takeichi, 1994), native lge cells with lower cad6 expression did not spread over the electroporated lge cells with higher cad6 expression. In other words, cad6 overexpression at the ctx/lge boundary did not perturb the bulge patterns in the telencephalon (Fig. 5A-N). This may suggest an alternative mechanism, other than the quantitative and qualitative differences in cadherins, which function synergistically or dominantly to form or maintain the ctx/lge boundary. A recent study indicated that eph receptor tyrosine kinases, and their ligands (ephrins) play a crucial role in forming and/or maintaining rhombomere boundaries in zebrafish embryos (Xu et al., 1999). Rcad and cad6 also transiently delineate odd- and even-numbered rhombomeres alternately in mice embryos (Inoue et al., 1997; Rhinn et al., 1999). It would be possible that the eph/ephrin system also functions in cell sorting at the ctx/lge boundary in parallel with the cadherin system or modulating each other.

The differential expression patterns of cadherins in the embryonic telencephalon are thought to be under the strict control of regulatory gene cascades. In the *Drosophila* wing disc, opposing transcriptional outputs of hedgehog signalling and engrailed closely regulate cell sorting at the anterior/posterior (A/P) boundary (Dahmann and Basler, 2000). Although no cadherin is reported to be expressed complementarily at the A/P boundary, a quantitative difference of *D*E-cadherin expression has been suggested to work downstream of the transcriptional factors that are opposingly expressed at the boundary (Dahmann and Basler, 2000). In the mouse brain, several transcription factors are known to demarcate the telencephalic compartments. Among them, Pax6 may be one of the upstream candidates for Rcad expression within the ctx because Rcad expression was missing in the *Pax6* mutant ctx (Stoykova et al., 1997; our unpublished observation in a *Pax6* mutant rat). Interestingly, neuronal migration from the lge to ctx, which was relatively restricted in the wild type, was significantly enhanced in the *Pax6* mutant mouse (Chapouton et al., 1999). Hence, in order to elucidate the genetic program responsible for cell sorting with cadherins, detailed analyses of downstream cascades of Pax6 would be the focus of future studies.

We thank Drs Yoshio Wakamatsu and Tetsuya Tabata for critical reading of the manuscript in the original version, Ms Junko Asami and Mr Takashi Nagase for technical assistance, and all other members of Shindan laboratory in the National Institute of Neuroscience for thoughtful discussions. This work was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture (Nos 1022029, 11470002, 1158090), CREST from Japan Science and Technology Corporation, and Human Frontier Science Program Organization (RG0145/1998-B) awarded to N. O. T. I. is a recipient of the domestic research fellowship from Japan Science and Technology Corporation.

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