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# Classic cadherins regulate tangential migration of precerebellar neurons in the caudal hindbrain

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There were a few errors in the first ePress article published on April 12, 2006. The second ePress article published on April 19, 2006, the final online version and the print version are all correct.

In the first ePress article, Fig. 4 was positioned incorrectly so that the left-hand side of the figure is missing. In addition, in the Materials and methods, the two occurrences of 'Ex utero' should have been corrected to 'Exo utero'.

We apologise to the authors and readers for these mistakes.

# Classic cadherins regulate tangential migration of precerebellar neurons in the caudal hindbrain

Hiroki Taniguchi<sup>1,2,\*</sup>, Daisuke Kawauchi<sup>3,†</sup>, Kazuhiko Nishida<sup>2,3</sup> and Fujio Murakami<sup>1,2,3,§</sup>

Classic cadherins are calcium dependent homophilic cell adhesion molecules that play a key role in developmental processes such as morphogenesis, compartmentalization and maintenance of a tissue. They also play important roles in development and function of the nervous system. Although classic cadherins have been shown to be involved in the migration of non-neuronal cells, little is known about their role in neuronal migration. Here, we show that classic cadherins are essential for the migration of precerebellar neurons. In situ hybridization analysis shows that at least four classic cadherins, cadherin 6 (Cad6), cadherin 8 (Cad8), cadherin 11 (Cad11) and N-cadherin (Ncad), are expressed in the migratory streams of lateral reticular nucleus and external cuneate nucleus (LRN/ECN) neurons. Functional analysis performed by electroporation of cadherin constructs into the hindbrain indicates requirement for cadherins in the migration of LRN/ECN neurons both in vitro and in vivo. While overexpression of full-length classic cadherins, NCAD and CAD11, has no effect on LRN/ECN neuron migration, overexpression of two dominant negative (DN) constructs, membrane-bound form and cytoplasmic form, slows it down. Introduction of a DN construct does not alter some characteristics of LRN/ECN cells as indicated by a molecular marker, TAG1, and their responsiveness to chemotropic activity of the floor plate (FP). These results suggest that classic cadherins contribute to contact-dependent mechanisms of precerebellar neuron migration probably via their adhesive property.

**KEY WORDS:** Tangential migration, Precerebellar neurons, LRN, ECN, Cadherin, Electroporation, Rat, Mouse

## INTRODUCTION

During development of the central nervous system (CNS), neurons undergo a series of dynamic processes, including cell division, migration, axon and dendrite elongation, and synapse formation, to construct precisely connected neural circuits. Among these, neuronal migration is crucial for expanding the size of the brain and for correct arrangement of cells to specific sites, which are usually far from the site of their origins, thus contributing to the formation of functional and anatomical units such as layers and nuclei (reviewed by Hatten, 1999; Marín and Rubenstein, 2003). Many studies at molecular and cellular levels have identified several mechanisms that control the direction, final position and motility of the migrating neurons and proposed two major mechanisms, contact-dependent and contact-independent regulations (reviewed by Hatten, 1999; Marín and Rubenstein, 2003). The latter is mediated by attractive or repulsive activities of diffusible molecules, which have been shown to regulate the direction of neuronal migration (Alcantara et al., 2000; Causeret et al., 2002; de Diego et al., 2002; Lu et al., 2001; Marín et al., 2001; Taniguchi et al., 2002; Yee et al., 1999; Zhu et al., 1999). The former can be mediated by cell-adhesion molecules (CAMs), which have been shown to positively control neuronal migration by enhancing neuronal motility (Marín and Rubenstein, 2003). NCAM, a neural cell-adhesion molecule that belongs to immunoglobulin superfamily

(IgSF), for example, regulates the migration of olfactory interneurons via its conjugated polysialic acids (Hu et al., 1996; Ono et al., 1994); TAG1, an axonal glycoprotein that is also a member of IgSF, also positively regulates tangential migration of cortical GABAergic interneurons and a subset of precerebellar neurons in the caudal hindbrain (Denaxa et al., 2001; Kyriakopoulou et al., 2002). However, our knowledge on the roles of CAMs in neuronal migration is still limited.

Classic cadherins,  $\text{Ca}^{2+}$ -dependent homophilic CAMs (called as cadherins hereafter unless otherwise noted), are potential candidates to be tested for their involvement in contact-dependent neuronal migration, as they are expressed throughout the developing nervous system and have been shown to play important roles in several processes of neuronal development in both vertebrates and invertebrates (Inoue et al., 1997; Matsunami and Takeichi, 1995; Redies and Takeichi, 1996; Iwai et al., 2002). In the fruit fly, for example, *Drosophila* N-cadherin mediates the axonal fasciculation of the ventral nerve cord and the target recognition between retinal axons and optic medulla (Iwai et al., 2002; Lee et al., 2001). In the vertebrate, cadherins regulate the formation and maintenance of brain compartments (Inoue et al., 2001), axonal elongation (Matsunaga et al., 1988; Riehl et al., 1996) and synapse formation (Togashi et al., 2002). Their potential roles in neuronal migration were tested in the neural crest cells, which derive from the dorsal neural tube and migrate towards their peripheral targets (Nakagawa and Takeichi, 1998), and it was suggested that cadherins control the timing of neural crest cells emergence from their origin rather than the maintenance of migration. Nevertheless, there still remains the possibility that cadherins are involved in the neuronal migration in the brain.

In this study, we have examined the role of cadherins in the neuronal migration using the lateral reticular nucleus (LRN) and external cuneate nucleus (ECN) neurons that relay information to the cerebellum as a model system. Precerebellar neurons, including the LRN/ECN neurons, originate from the embryonic lower rhombic

<sup>1</sup>Division of Behavior and Neurobiology, National Institute for Basic Biology, Myodaiji-cho, Okazaki 444-8585, Japan. <sup>2</sup>CREST, Japan Science and Technology, Japan.

<sup>3</sup>Laboratory of Neuroscience, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan.

\*Present address: Cold Spring Harbor Laboratory, Beckman 220, One Bungtown Road, Cold Spring Harbor, NY, 11724, USA

<sup>†</sup>Department of Developmental Biology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan

<sup>§</sup>Author for correspondence (e-mail: murakami@fbs.osaka-u.ac.jp)

lip located at the most dorsal site of the caudal hindbrain and migrate towards the ventral midline floor plate (FP) along the marginal or submarginal pathway (Altman and Bayer, 1987a; Altman and Bayer, 1987b; Altman and Bayer, 1987c; Altman and Bayer, 1987d; Bourrat and Sotelo, 1988; Bourrat and Sotelo, 1990). We first characterize the expression pattern of mRNAs for cadherins in the migrating LRN/ECN neurons in vivo and then show that expression of DN forms of cadherins in these neurons perturbs their migration both in vitro and in vivo. The perturbation of migration is not caused by altered TAG1 expression, which has been shown previously to be involved in the migration of these neurons (Kyriakopoulou et al., 2002), or by the change in responsiveness to the guidance molecules from the FP that attract them. These findings provide the first evidence that cadherins positively control the neuronal migration in the CNS.

## MATERIALS AND METHODS

### Explant culture

All experiments were performed following the guidelines of the National Institute for Basic Biology. The culture methods for flat whole-mount preparations followed those of Taniguchi et al. (Taniguchi et al., 2002) with minor modifications. Briefly, the myelencephalon taken from an embryonic day 14 (E14) Wistar rat was cut along the dorsal midline and opened onto membrane filters (Millipore, pore size, 10  $\mu$ m). Explants were embedded with collagen gels and cultured on the membranes floating on the culture medium at 37°C in a 5% CO<sub>2</sub> and 95% humidity incubator. Collagen gel co-culture was performed as described before (Taniguchi et al., 2002). Images were taken by an epifluorescent microscope equipped with a camera or a confocal microscope (LSM510, Zeiss).

### Immunohistochemistry

Cultured explants and hindbrains from embryos manipulated with exo utero electroporation were fixed overnight with 4% paraformaldehyde (PFA) in 0.12 M phosphate buffer (PB) (pH 7.4) at 4°C. The fixed whole hindbrains, in which enhanced green fluorescent protein (EGFP)-NCAD(t) was transferred by exo utero electroporation, were then blocked with 10% normal goat serum in PBS containing 0.3% Triton X-100, followed by the incubation with a rabbit polyclonal antibody against GFP (1:1000, Molecular Probes) and an Alexa 488-conjugated anti-rabbit IgG antibody (Molecular Probes). Cryostat sections (20  $\mu$ m) were made from the cultured explants or whole hindbrains that had been fixed and immersed overnight in 30% sucrose/4% PFA at 4°C. The sections were placed on poly-L-lysine-coated slides and blocked with 10% normal goat serum in PBS containing 0.1% Triton X-100, followed by the incubation with primary and secondary antibodies. Primary antibodies used were a rabbit polyclonal antibody against GFP (1:1000, Molecular Probes), a mouse monoclonal antibody (mAb) against pan-cadherin (CH-19; 1:500; Sigma-Aldrich), a mouse mAb against FLAG (M2; 1:750; Sigma) and a mouse mAb against TAG1 (4D7; 1:30; Developmental Studies Hybridoma Bank). Secondary antibodies used were a Cy3-conjugated goat anti-mouse IgG (1:300; Jackson), a Cy3-conjugated goat anti-mouse IgM (1:300; Jackson) and an Alexa 488-conjugated anti-rabbit IgG antibody (Molecular Probes).

### In situ hybridization

To obtain cDNA fragments for *Ecad*, *VEcad*, *Rcad*, *BRcad*, *Cad8*, *Cad11*, *Mcad* and *Cad6*, we performed PCR using cDNAs from E15 rat brains or E14 whole rat embryos as templates. Following primers were used: *Ecad*, (forward) 5'-ggagggtttcactgtgttg-3' and (reverse) 5'-catctgcctaagaatcaggtgtgc-3'; *VEcad*, (forward) 5'-cgccagaatgctaagtgtgcta-3' and (reverse) 5'-gattgagtaaaagcgggaagtgtg-3'; *Rcad*, (forward) 5'-cagcacactgtgaacacaagaagg-3' and (reverse) 5'-tgatgaactcgggacgttg-3'; *BRcad*, (forward) 5'-cctgtctctctggttctgttg-3' and (reverse) 5'-ctggcactgttccataggtgc-3'; *Cad8*, (forward) 5'-acgttttgacaccttggaactc-3' and (reverse) 5'-gggcattgtctgtgtgtgtg-3'; *Cad11*, (forward) 5'-cattctctcaggtgaaggagcg-3' and (reverse) 5'-acttggtggtgtgtgtgac-3'; *Mcad*, (forward) 5'-cctaagaccaatgaggtgtgtgc-3' and (reverse) 5'-

caaaaagggtgaagcagggc-3'; *Cad6*, (forward) 5'-tcttgctgctcttttgggtcg-3' and (reverse) 5'-catcgcatcaggtgtgtgac-3'. All PCR products were subcloned into the pGEM-T Easy vector (Promega). A *SacI/EcoRI* fragment of rat *Ncad* (a kind gift from Dr K. Asai) was subcloned into the pBluescriptSK-. Sense or antisense cRNA probes labeled with digoxigenin (DIG)-11-UTP were prepared by in vitro transcriptions with SP6 RNA polymerase, T7 RNA polymerase or T3 RNA polymerase.

Brains were dissected out from E15 Wistar rat embryos and fixed overnight with 4% PFA in 0.12 M PB, followed by immersion in 30% sucrose/4% PFA at 4°C overnight. They were embedded in OCT Compound (Tissue-Tek), frozen in a cryostat at -20°C and cut into 20  $\mu$ m sections. The sections were collected on poly-L-lysine-coated slides and dried for 3 hours. After rehydration in PBS, they were postfixed with 4% PFA in 0.12 M PB for 10 minutes (min) and washed with KPBS [20 mM KH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl (pH 7.3)]. They were then treated with 10  $\mu$ g/ml proteinase K in 100 mM Tris-Cl (pH 8.0), 50 mM EDTA at 37°C for 5 minutes and fixed again for 10 minutes, followed by a wash with KPBS for 20 minutes. After brief washes with distilled water and then 0.1 M triethanolamine (pH 8.0), they were acetylated with 0.25% acetic anhydride, 0.1 M triethanolamine for 10 minutes. They were next washed with 2 $\times$ SSC [1 $\times$ SSC; 150 mM NaCl, 15 mM Na citrate (pH 7.0)] for 4 minutes, dehydrated with 50%, 70%, 95% and 100% ethanol sequentially for 3 minutes each, and dried in the air. After preincubation with hybridization buffer [50% formamide, 10% dextran sulfate, 300 mM NaCl, 1 $\times$ Denhardt's solution, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 500  $\mu$ g/ml yeast tRNA, 0.5% SDS] at 56°C for 1 hour, they were hybridized with cRNA probes labeled with DIG at 56°C for 16 hours. After hybridization they were washed with 4 $\times$ SSC three times for 20 minutes each and treated with 20  $\mu$ g/ml RNase in 1 M NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA at 37°C for 30 minutes, followed by two washes with 2 $\times$ SSC each for 5 minutes, 1 $\times$ SSC for 5 minutes, 0.5 $\times$ SSC for 5 minutes, 0.05 $\times$ SSC at 70°C for 30 minutes and 0.05 $\times$ SSC for 3 minutes. They were then washed with solution 1 [100 mM Tris-Cl (pH 7.5), 150 mM NaCl], blocked with solution 2 [1% blocking reagent (Roche) in solution 1] for 1 hour and incubated with an anti-DIG antibody conjugated with alkaline phosphatase (1:1000; Roche) at 4°C overnight. After extensive washes with solution 1, they were briefly washed with solution 3 [100 mM Tris-Cl (pH 9.5), 100 mM NaCl, 50 mM MgCl<sub>2</sub>, 1 mM levamisole] and then incubated with detection solution [2% NBT/BCIP stock solution (Roche), 10% polyvinyl alcohol (70-100kDa) in solution 3]. The detection was stopped by incubation with Tris-EDTA [100 mM Tris-Cl (pH 8.0), 50 mM EDTA]. Finally the sections were briefly washed with distilled water. All steps were performed at room temperature unless otherwise noted. Images were taken by an epifluorescent microscope equipped with a camera.

### Expression vectors

The whole coding regions for the FLAG-tagged chicken NCAD (NCAD-FLAG), FLAG-tagged mouse CAD11 (CAD11-FLAG), FLAG tagged dominant negative chicken NCAD (cN390 $\Delta$ -FLAG) (all FLAG-tagged cadherin cDNAs are kind gifts from Dr M. Takeichi) were cloned into the pCAGGS vector (a kind gift from J. Miyazaki), yielding pCAGGS-NCAD-FLAG, pCAGGS-CAD11-FLAG and pCAGGS-cN390 $\Delta$ -FLAG. The cytoplasmic form of DN-NCAD [pCAGGS-EGFP-NCAD(t)] was constructed by fusing the intracellular domain of rat NCAD with EGFP (Clontech). The coding sequences for EGFP, DsRed (Clontech) and Venus YFP (a kind gift from Dr A. Miyawaki) were cloned into the pCAGGS vector to yield pCAGGS-EGFP, pCAGGS-DsRed and pCAGGS-Venus YFP, respectively.

### Gene transfer by electroporation in the flat whole-mount culture

The gene transfer based on electroporation in rat embryos was carried out as described previously with minor changes (Taniguchi et al., 2002). In brief, pCAGGS-EGFP and one of the cadherin constructs used in the present study were mixed at 2.5 mg/ml each in PBS with 0.02% Trypan Blue. In the experiments to confirm co-expression, pCAGGS-DsRed and pCAGGS-EGFP-NCAD(t) were mixed. The cDNA solution was injected into the fourth ventricle of rat embryos using a capillary glass pipette. Electric pulses (voltage: 40 V; duration: 50 mseconds; 12 pulses) were applied in PBS by using forceps-shaped electrodes. Although we found that one side (right

side) of the lower rhombic lips was predominantly labeled with EGFP, the other side (left side) occasionally showed weak GFP labeling. To avoid mixing of the labeled neurons derived from both sides, the left rhombic lip was removed before culture.

### Exo utero electroporation

Exo utero electroporation was performed as described previously (Kawauchi et al., 2006). In brief, either pCAGGS-Venus YFP or pCAGGS-EGFP-NCAD(t) was injected into the fourth ventricle of E12.5 mouse embryos that had been exposed by cutting the uterine wall. The DNA solution was mixed with 0.01% Fast Green or Indigo Carmine for visualization of the injection. Electric pulses (20 V, 50 mseconds, three pulses) were applied by using an electroporator. Embryos were taken out of pregnant mice at E15.5 or E18.5. Embryos that have the unilaterally labeled lower rhombic lip were selected for analysis and their whole hindbrains were imaged using an epifluorescent microscope (VB-G25, KEYENCE) after dissection and fixation.

### Quantification

The fluorescent and bright images of the same area were taken with a CCD camera (C4880-40-26A, Hamamatsu Photonics) for the analysis. Bright-field illumination was used to identify the position of the ventral midline (VM). For quantification we first determined a  $150\ \mu\text{m} \times 300\ \mu\text{m}$  rectangular area that is the farthest from the VM but includes more than 10 migrating neurons, and then measured the distance between the VM and the forefront of the cells which reside within the rectangle.

## RESULTS

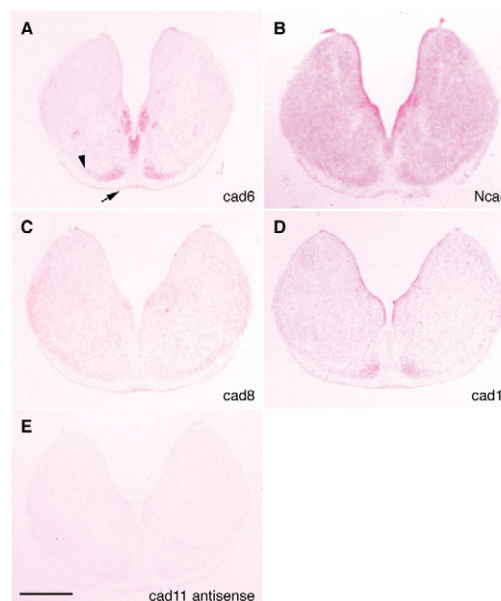
### Classic cadherins are expressed in the migratory streams of LRN/ECN neurons at the caudal medulla

To identify the expression of mRNAs for cadherins in the migrating precerebellar neurons, we performed in situ hybridization in the rat hindbrains at E15 when early born LRN/ECN neurons reach the ventral midline. We tested several classic cadherins including ECAD (CDH1 – Mouse Genome Informatics), VECAD (Pcdh12 – Mouse Genome Informatics), RCAD (CDH4 – Mouse Genome Informatics), NCAD (CDH2 – Mouse Genome Informatics), BRCAD, CAD8 (Acad8 – Mouse Genome Informatics), CAD11 (CDH11 – Mouse Genome Informatics), MCAD (CDH15 – Mouse Genome Informatics) and CAD6 (CDH6 – Mouse Genome Informatics). However, among them, transcripts for only NCAD, CAD8, CAD11 and CAD6 were detected in the migratory streams of LRN/ECN neurons (Fig. 1). No obvious signals were obtained with sense probes (Fig. 1E). Thus, several types of classic cadherins are expressed in the migrating LRN/ECN neurons.

### Overexpression of cadherin constructs in the flat whole-mount culture by electroporation

Previously, we developed a flat whole-mount culture preparation of the rat medulla, in which migrating LRN/ECN neurons were almost exclusively labeled with EGFP by introducing an *Egfp* cDNA into the lower rhombic lip by electroporation (Fig. 2A) (Taniguchi et al., 2002). In this culture, LRN/ECN neurons migrated just beneath the pial surface towards the contralateral side over a distance of 2 mm in 3 days in vitro (div). We thought that this in vitro system would enable us to study the role of cadherins in the neuronal migration if cadherin constructs could be co-expressed with EGFP in the neurons. First, we introduced EGFP into the flat whole-mount preparation and after 2 div it was immunostained with an antibody for pan cadherin to examine expression of endogenous cadherins in LRN/ECN neurons. LRN/ECN neurons migrating just beneath the pial surface expressed cadherin proteins (Fig. 2B-D).

We next examined if EGFP and the cadherin constructs prepared here could be co-expressed in LRN/ECN neurons. Full-length NCAD, CAD11 and the membrane-bound form of DN cad



**Fig. 1. mRNA expressions of classic cadherins in the marginal and submarginal streams of the migrating precerebellar neurons in the myelencephalon.** Transverse sections of the E15 rat caudal hindbrain hybridized with antisense digoxigenin-labeled riboprobes for *Cad6* (A), *Ncad* (B), *Cad8* (C) and *Cad11* (D), and sense digoxigenin-labeled probes for *Cad11* (E). Signals of cadherin mRNAs are seen in both marginal (arrow) and submarginal (arrowhead) streams with antisense probes (A-D), whereas sense probes produce no significant signals (E). Scale bar: 400  $\mu\text{m}$ .

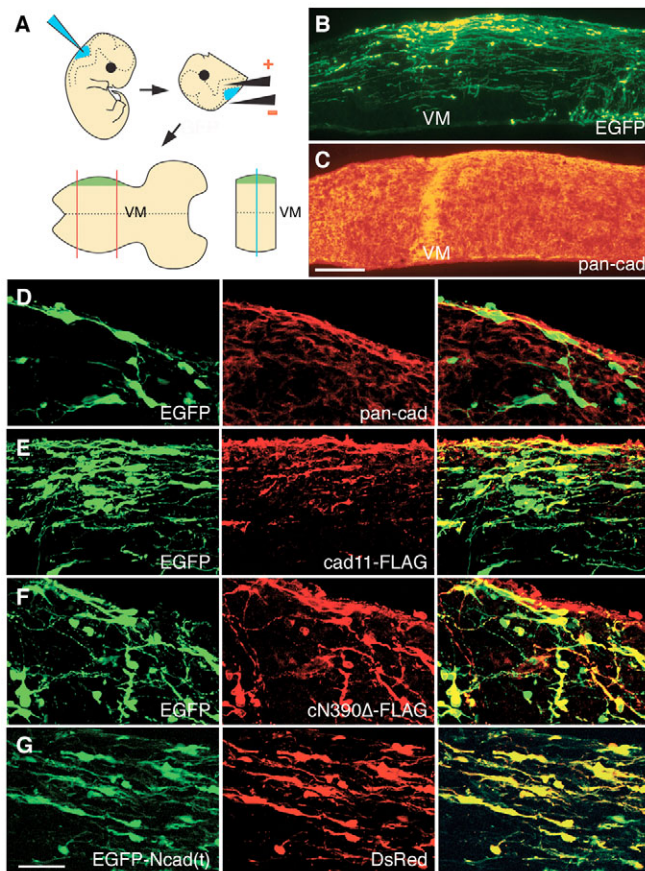
(cN390 $\Delta$ ) were tagged with FLAG to detect their expression. EGFP was used as a tag to detect the cytoplasmic form of DN cad [EGFP-NCAD(t)]. Each tagged cadherin construct was introduced into the flat whole-mount preparations together with EGFP, except for EGFP-NCAD(t), which was co-expressed with DsRed. After 2 div, many LRN/ECN neurons expressed FLAG-tagged constructs and EGFP (Fig. 2E,F). EGFP-NCAD(t) was also well co-expressed with DsRed in the migrating neurons (Fig. 2G). As early as the first day in vitro, LRN/ECN neurons that have just left the rhombic lip expressed these cadherins and fluorescent markers (data not shown). Thus, cadherin constructs can be co-expressed with fluorescent proteins in migrating LRN/ECN neurons, which endogenously express cadherins.

### Effect of blocking cadherin function on migration of LRN/ECN neurons

We then analyzed the effect of overexpression of cadherin constructs on migration of LRN/ECN neurons. When EGFP was introduced alone into the rhombic lip, EGFP-labeled cells normally reached the contralateral end of the explants after 3 div (Fig. 3A, Fig. 4). Overexpression of either full-length NCAD or CAD11 caused no notable effect on the distance of migration (Fig. 3B,C, Fig. 4). The appearance of the chain migration, which is characteristic of the migrating LRN/ECN neurons, also looked normal (data not shown). However, cN390 $\Delta$ -FLAG, which should suppress the function of all classic cadherins by sequestering endogenous  $\beta$ -catenins, appeared to slow migration of LRN/ECN neurons down (Fig. 3D, Fig. 4, compare with Fig. 3A-C). A similar but stronger phenotype was observed when EGFP-NCAD(t), a cytoplasmic form of a DN construct that should have the same function as cN390 $\Delta$ -FLAG, was used (Fig. 3E, Fig. 4).

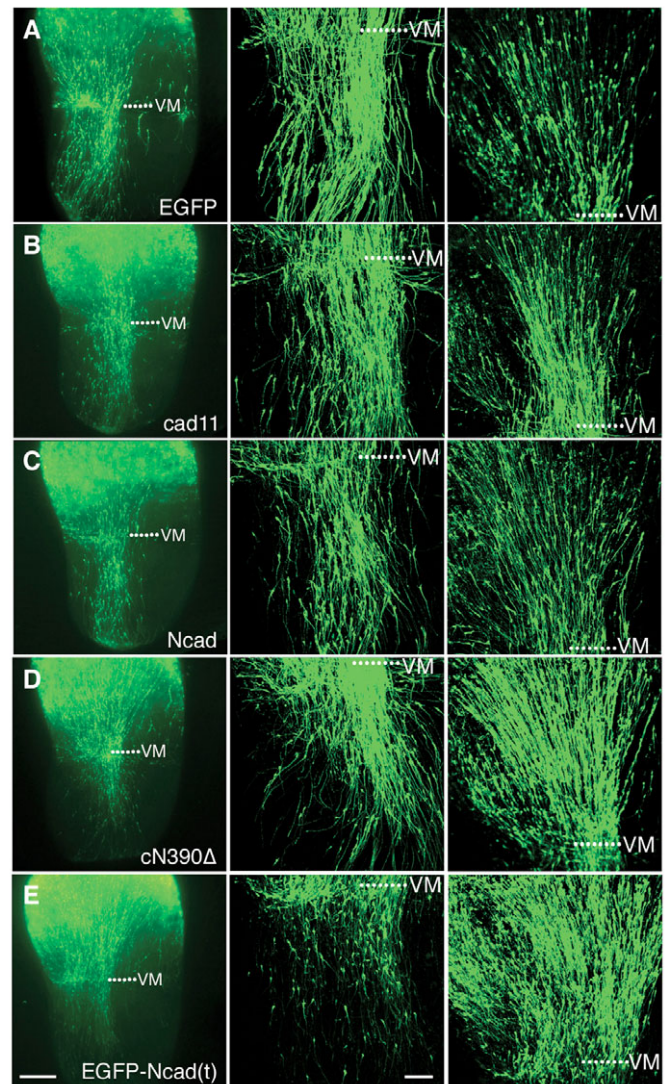


One might argue that the phenotype resulted from disruption of local interactions between LRN/ECN neurons and the FP, which play an important role in their midline crossing (Taniguchi et al., 2002), rather than slowed migration. If this is the case, it should be expected that the phenotype occurs only when they cross the ventral midline. However, the delayed migration of neurons expressing EGFP-NCAD(t) occurred before they reached the ventral midline, refuting this possibility (compare Fig. 5C,D with Fig. 5A,B). Taken together, these results indicate that classic cadherins are involved in the initiation and maintenance of migration of LRN/ECN neurons in the medulla.

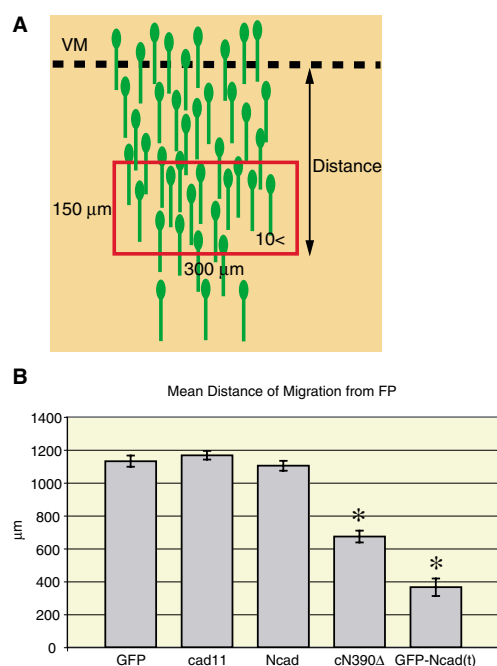


**Fig. 2. Electroporation-based gene transfer in the flat whole-mount culture of the embryonic rat medulla.** (A) Schematic diagrams showing the procedure to make the flat whole-mount preparation of the hindbrain into which expression vectors are transferred. The DNA solution containing expression vectors and a dye is injected into the fourth ventricle. The head is cut, immersed in PBS and electroporated using forceps-shaped electrodes. The hindbrain is dissected out, cut at the dorsal midline and opened in the medium. The medulla is excised from it by cutting at the red lines and placed on the membrane in an open book configuration. Fixed preparations are sectioned along the blue line to make cryostat sections for immunostaining. (B-D) Transverse sections of 2 div explants immunostained with an anti-pan-cadherin Ab. An *egfp* cDNA is introduced into the explants. Lower (B,C) and higher (D) magnification. EGFP-positive migrating neurons express cadherin proteins. VM, ventral midline. (E-G) Transverse sections of 2 div explants expressing EGFP+CAD11-FLAG (E), EGFP+cN390Δ-FLAG (F) and EGFP-NCAD(t)+DsRed (G). FLAG signal is detected with an anti-FLAG antibody. Most of the cells labeled with fluorescent reporter proteins express exogenous cadherin constructs. Scale bars: 200  $\mu$ m in B and C; 50  $\mu$ m in D-G.

To validate this hypothesis, we next examined the effect of a DN construct on migration of LRN/ECN neurons *in vivo*, taking advantage of *ex utero* electroporation in mice. *In situ* hybridization



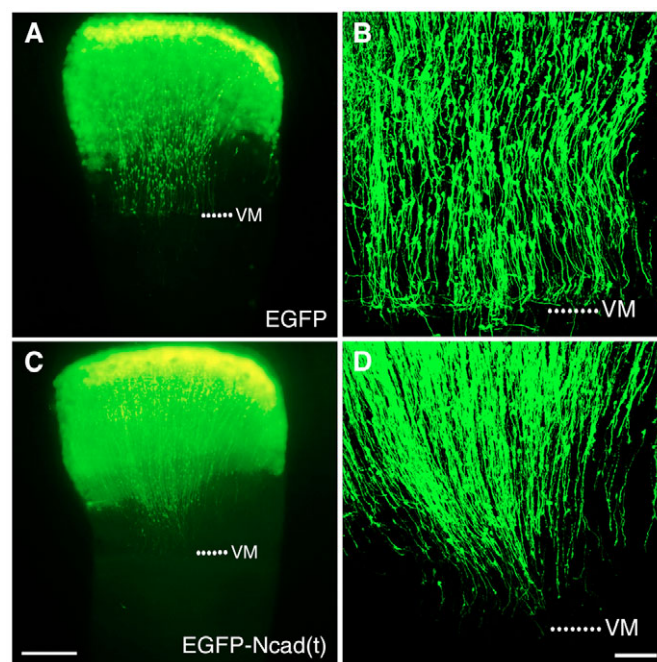
**Fig. 3. Overexpression of DN cadherin constructs but not full-length cadherin constructs slows down the migration of LRN/ECN neurons.** Flat whole-mount preparations of rat hindbrains electroporated with expression vectors encoding EGFP (A), EGFP+CAD11 (B), EGFP+NCAD (C), EGFP+cN390Δ (D) and EGFP+EGFP-NCAD(t) (E) at 3 div. (Left column) Lower magnification views of flat whole-mount preparations in which migrating neurons are labeled with EGFP. (Middle and right columns) Higher magnification views of contralateral and ipsilateral sides, respectively, in the flat whole-mount preparations in the left column. Many migrating neurons with EGFP, EGFP+CAD11 and EGFP+NCAD that have crossed the ventral midline are seen on the contralateral side (middle column in A-C). By contrast, fewer cells with EGFP+cN390Δ and EGFP+EGFP-NCAD(t) are observed in the contralateral part, apart from the ventral midline (middle column in D and E). Denser cell clumps are seen on the ipsilateral side of the preparations with DN cadherins (right column in D and E) compared with the control preparations with only EGFP or the preparations with exogenous full-length cadherins (right column in A-C). Broken lines labeled VM indicate the position of the ventral midline. Scale bars: 400  $\mu$ m in left column; 50  $\mu$ m in middle and right columns.



**Fig. 4. Quantification of the effect of cadherin constructs on the migration of LRN/ECN neurons in the flat whole-mount preparations.** (A) Schematic diagrams showing the method for quantification (see detail in text). (B) Bar graph indicating the mean distance between the VM and the forefront neuron in the rectangle with more than 10 neurons, which is the farthest from the ventral midline.  $n=10$ , one-way ANOVA,  $P<0.0001$ ; Dunnett's Multiple Comparison Test,  $*P<0.01$ .

showed that the mRNA for NCAD is also expressed in LRN/ECN neurons in mouse hindbrains (see Fig. S1A in the supplementary material). Mouse embryos were electroporated with either Venus YFP or EGFP-NCAD(t) at E12.5, and analyzed at E15.5. Electroporation at this stage enabled us to specifically label migrating LRN/ECN neurons but not inferior olivary nucleus neurons in the myelencephalon (data not shown). In all the control samples (12/12), migrating neurons on the contralateral side outnumbered those on the ipsilateral side (Fig. 6A,C,E,G). By contrast, neurons expressing EGFP-NCAD(t) showed delayed migration; LRN/ECN neurons were predominantly distributed on the ipsilateral side (7/12) or were present evenly on ipsilateral and contralateral sides (5/12) (Fig. 6B,D,F,H). To explore further which type of cadherin is essential for the migration of LRN/ECN neurons, we carried out the RNAi-based gene knock down for NCAD in vivo using exo utero electroporation. LRN/ECN neurons expressing a short hairpin RNA for NCAD also exhibited delayed migration, suggesting that NCAD is a major cadherin responsible for their migration (see Fig. S1B-D in the supplementary material). Taken together, these results suggest that classic cadherins play an important role in the migration of LRN/ECN neurons in vivo.

Owing to the delayed migration, considerable numbers of neurons expressing EGFP-NCAD(t) remained on the ipsilateral side at E18.5 when control neurons formed nuclei on the contralateral side. To follow their final destination, we prepared transverse sections from mouse hindbrains electroporated with YFP or EGFP-NCAD(t). When EGFP-NCAD(t) was electroporated into the IRL, LRN/ECN



**Fig. 5. The delay in the migration of LRN/ECN neurons expressing DN cadherin constructs is observed before crossing of the ventral midline by control neurons expressing only EGFP.** Flat whole-mount preparations electroporated with expression vectors encoding EGFP (A,B), EGFP+EGFP-NCAD(t) (C,D) at 1.5 div. (B,D) Enlarged views of migrating neurons on the ipsilateral side near the FP in A,C, respectively. Whereas the control neurons with only EGFP reach the FP at this time (A,B), the neurons expressing EGFP+EGFP-NCAD(t) do not (C,D). Broken lines labeled VM indicate the position of the ventral midline. Scale bars: 400 μm in A,C; 50 μm in B,D.

nuclei were formed on the ipsilateral side (Fig. 7A,C,E), whereas electroporation of YFP gave rise to the nuclei on the contralateral side (Fig. 7B,D,F). These results suggest that proper regulation of migration rate appears to be prerequisite for nucleogenesis in appropriate location.

### Overexpression of DN cadherin constructs does not alter the TAG1 expression and responsiveness to attractive cues from the FP in LRN/ECN neurons

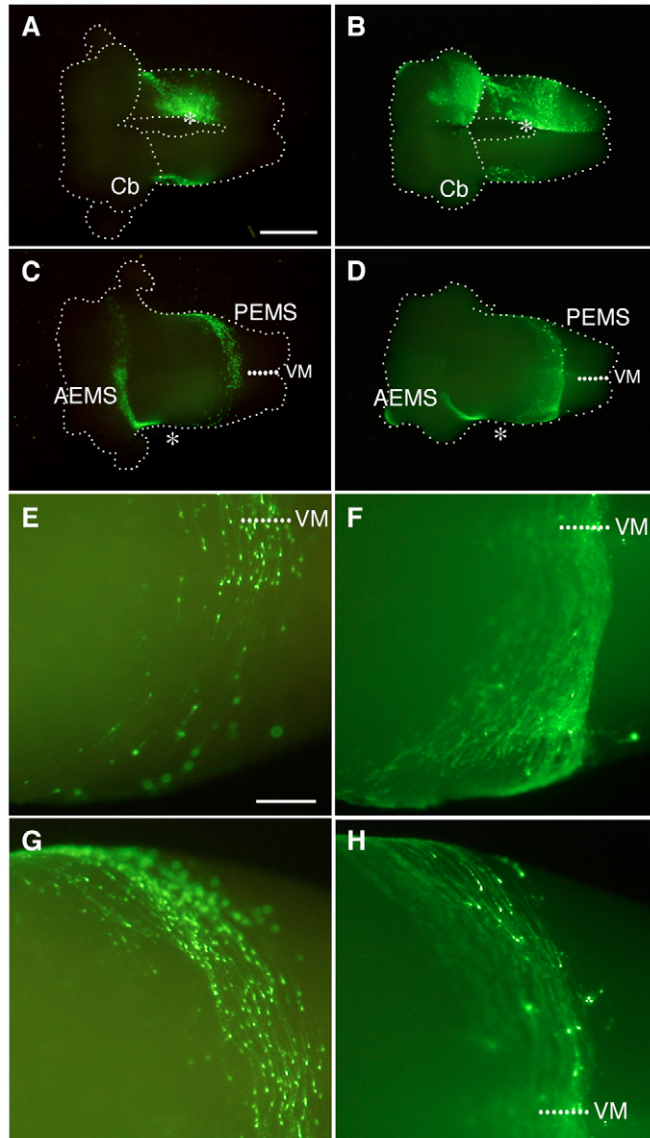
A recent work using similar flat whole-mount preparations of the caudal hindbrain has suggested that TAG1 is involved in the migration of LRN/ECN neurons (Kyriakopoulou et al., 2002). In these experiments, inhibition of the TAG1 function disrupted proper migration of LRN/ECN neurons in vitro. To test if TAG1 expression could be influenced by cadherin function, EGFP-NCAD(t) introduced preparations were fixed at 2 div and immunostained for TAG1. We found no difference in the expression of TAG1 between EGFP control neurons (Fig. 8A-C) and EGFP-NCAD(t) LRN/ECN neurons (Fig. 8D-F). Thus, suppression of cadherin function in LRN/ECN neurons does not cause changes in the TAG1 expression.

The FP promotes migration of precerebellar neurons by secreting guidance factors, including netrin 1 (Alcantara et al., 2000; de Diego et al., 2002; Taniguchi et al., 2002; Yee et al., 1999). LRN/ECN neurons can change their responsiveness to FP cues during tangential migration of these neurons (Taniguchi et al., 2002). Therefore, one might speculate that disruption of cadherin function might change their responsiveness to FP-derived guidance



molecules. To examine this possibility, an explant from the E14 rat dorsal medulla electroporated with either EGFP or a mixture of EGFP and EGFP-NCAD(t) was cultured with an FP explant for 2 days in collagen gels. In this co-culture most of EGFP labeled neurons emigrating from the dorsal medulla explant have been shown to be TAG1- and DCC-positive precerebellar neurons

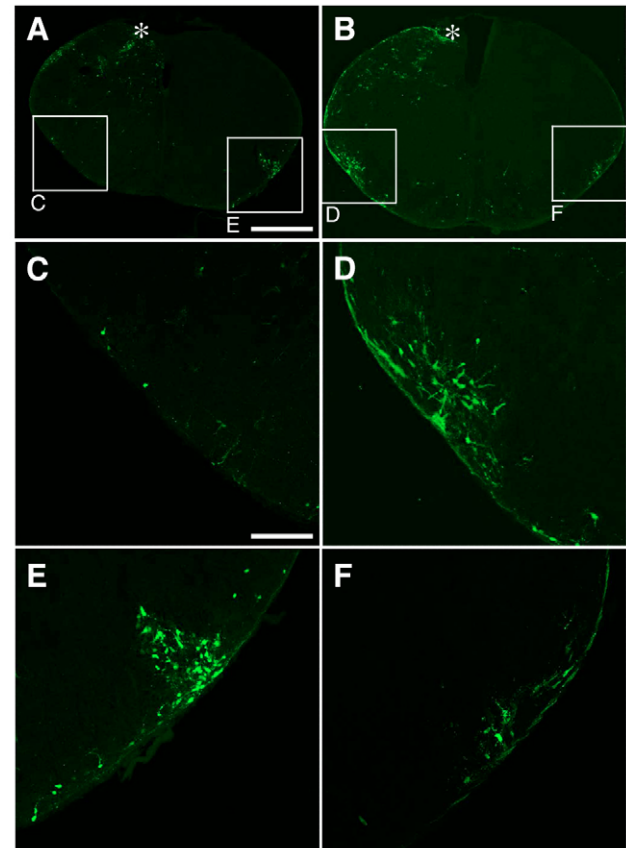
(Taniguchi et al., 2002). EGFP-NCAD(t) expressing neurons emigrated from the dorsal medulla explant toward the FP, similarly to those expressing EGFP alone (Fig. 9), indicating that their responsiveness to the FP is unaltered. Taken together, these results suggest that cadherins regulate LRN/ECN neuron migration by directly modulating cell adhesion, rather than via changes of their TAG1 expression or responsiveness to FP attractive cues.



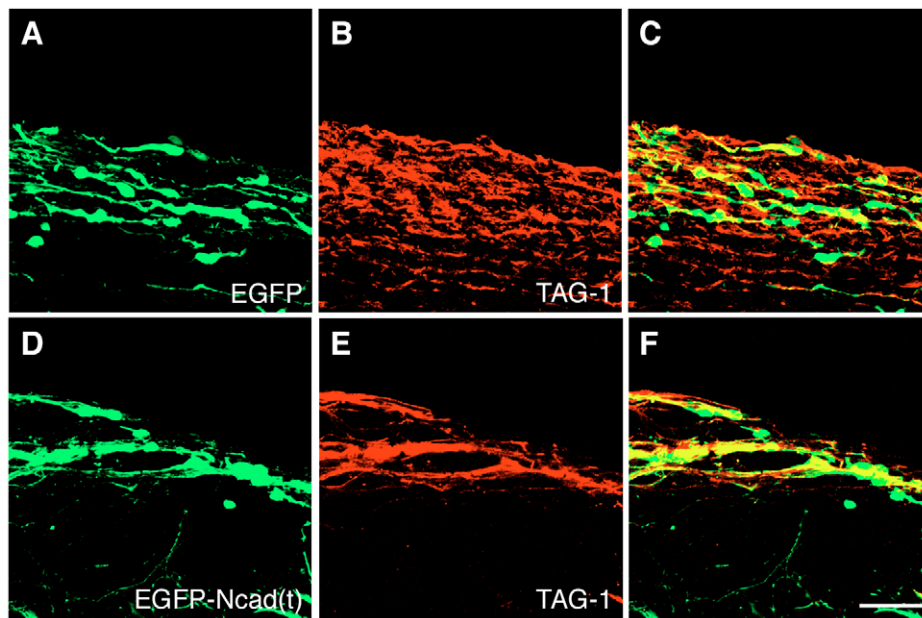
**Fig. 6. Overexpression of a DN cadherin construct disrupts the migration of LRN/ECN neurons in vivo.** E15.5 mouse hindbrains electroporated with Venus YFP (A,C,E,G) and EGFP-NCAD(t) (B,D,F,H) at E12.5. Lower magnification views from dorsal (A,B) and ventral (C,D) side. The right lower rhombic lip is exclusively labeled with fluorescent markers. PEMS and AEMS corresponding to the migratory streams of LRN/ECN neurons and of pontine gray and reticulotegmental nuclei neurons, respectively, are seen in the ventral views. Rostral is towards the left. Asterisks represent the labeled side. Cb, cerebellum; AEMS, anterior extramural migratory stream; PEMS, posterior extramural migratory stream. Higher magnification views of ipsilateral (E,F) and contralateral (G,H) sides. More control neurons are seen on the contralateral side in controls, whereas more neurons expressing EGFP-NCAD(t) are found on the ipsilateral side. Broken lines labeled VM indicate the position of the ventral midline. Scale bars: 1 mm in A-D; 200  $\mu$ m in E-H.

## DISCUSSION

Mechanisms controlling neuronal migration can be categorized into at least two types: a contact-independent mechanism, a diffusible signal generated by surrounding tissues and a contact-dependent cue coming from the cells associated with migrating neurons (reviewed by Hatten, 1999; Marín and Rubenstein, 2003). Each group of migrating neurons in different areas of CNS seems to be guided by both mechanisms (reviewed by Hatten, 1999; Marín and Rubenstein, 2003). In the hindbrain, LRN/ECN neurons are attracted by the FP in the vicinity of the ventral midline (Alcantara et al., 2000; de Diego et al., 2002; Taniguchi et al., 2002). Several lines of evidence also suggest that contact-dependent mechanisms might also play an important role in the control of their migration. First, migrating

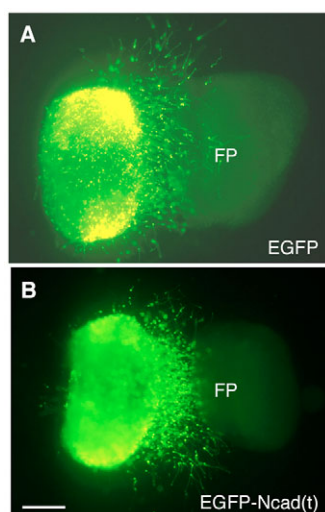


**Fig. 7. Migration of LRN/ECN neurons slowed by cadherin function blocking causes ectopic ipsilateral formation of nuclei.** Transverse sections of E18.5 mouse hindbrains electroporated with Venus YFP (A,C,E) and EGFP-NCAD(t) (B,D,F) at E12.5. Lower magnification views (A,B). LRN but not ECN is included in these sections owing to the angle of sectioning. Squares correspond to the higher magnification views in C-F. Asterisks represent the labeled side. Higher magnification views of ipsilateral (C,D) and contralateral (E,F) sides. EGFP-NCAD(t) neurons form a nucleus ipsilaterally unlike control YFP neurons. Scale bars: 600  $\mu$ m in A,B; 150  $\mu$ m in C-F.



**Fig. 8. Blocking of the cadherin function by DN cadherin has no effect on TAG1 expression in LRN/ECN neurons.** Transverse sections of 2 div explants expressing EGFP (A-C) and EGFP+EGFP-NCAD(t) (D-F) immunostained with an anti-TAG1 antibody. Confocal images of EGFP signals (A,D), TAG1 signals (B,E) and merged signals (C,F). EGFP-positive cells express TAG1 in both conditions. Scale bar: 50  $\mu$ m.

LRN/ECN neurons form a chain-like structure as if they use themselves as a migration substrate (Alcantara et al., 2000; Taniguchi et al., 2002). Second, it has been suggested that the axons running just beneath the migratory stream of LRN/ECN neurons confer the substrate on the migrating neurons (Kyriakopoulou et al., 2002; Ono and Kawamura, 1989). Third, blocking the TAG1 function perturbs the migration of LRN/ECN neurons (Kyriakopoulou et al., 2002). However, molecular mechanisms for the contact-dependent regulation have remained elusive. In the present study, we showed that mRNA for CAD6, CAD8, CAD11 and NCAD were expressed in the migratory streams of LRN/ECN neurons. The expression of cadherin mRNAs together with their homophilic adhesive property prompted us to hypothesize that they regulate the migration of LRN/ECN neurons.



**Fig. 9. The attraction of LRN/ECN neurons by the FP is not disturbed by overexpression of DN cadherin.** The dorsal medulla electroporated with EGFP (A) or EGFP+EGFP-NCAD(t) (B) is co-cultured with an FP explant in collagen gels. In both conditions, many neurons migrate towards the FP explant. Scale bar: 400  $\mu$ m.

We first tested this hypothesis by electroporating cadherin constructs together with EGFP into LRN/ECN neurons in the previously established flat whole-mount culture of the medulla (Taniguchi et al., 2002). This method enabled us to visualize the entire morphology of individual neurons, and thus to clarify the effect of cadherin constructs on their migration. Consistent with the proposed hypothesis, overexpression of two different types of DN cadherin constructs in the lower rhombic lip of the caudal hindbrain perturbed the migration of LRN/ECN neurons. This phenotype appeared to be due to a defect not only in initiation but also in maintenance of migration, because the difference in the migration distance between control cells and those expressing DN-constructs became more evident after 3 div compared with 1 div. Importantly, a similar phenotype was also observed *in vivo* when a cytoplasmic form of a DN cadherin or an shRNA for NCAD was expressed in the LRN/ECN neurons using *ex utero* electroporation. These results provide the first compelling evidence suggesting that classic cadherins control initiation and maintenance of neuronal migration.

What is the mechanism by which overexpression of DN constructs influenced the migration of LRN/ECN neurons? One possibility is that the cytoplasmic domain of the cadherin constructs might disrupt the Wnt signaling pathway by sequestering  $\beta$ -catenin, which is one of its canonical components. This is unlikely because DN constructs but not full-length constructs caused a phenotype in our experiments, despite the fact that they have the same cytoplasmic sequence and similar activity to suppress the transactivation by  $\beta$ -catenins (Sadot et al., 1998).

We have found that a cytoplasmic form of a DN construct, EGFP-NCAD(t), caused more pronounced phenotype than a membrane-bound form of a DN construct, cN390 $\Delta$ . One possible explanation could be that EGFP-NCAD(t) affected the  $\beta$ -catenins signaling pathway in addition to homophilic interactions mediated by cadherins, because a cytoplasmic form suppresses the transactivation by  $\beta$ -catenins more strongly than a membrane-bound form (Sadot et al., 1998). Another could be that EGFP-NCAD(t) is able to sequester  $\beta$ -catenins more efficiently than cN390 $\Delta$  localized in the plasma membrane as it is expected to be localized diffusely in the cytoplasm, thereby interfering with cadherin interactions more potently.



One might argue that overexpression of DN constructs might have changed the TAG1 expression, which has been implicated in the migration of LRN/ECN neurons (Kyriakopoulou et al., 2002). This, however, is unlikely, because overexpression of DN constructs did not alter TAG1 expression in LRN/ECN neurons.

The migration of LRN/ECN neurons from the dorsal medulla explants elicited by an FP explant in collagen gel co-cultures was not blocked by overexpression of DN constructs. Furthermore, in our flat whole-mount culture, LRN/ECN neurons expressing DN constructs still reached the ventral midline, with some delay, after 3 div and had ability to turn toward the ectopic FP placed laterally to their migratory stream (data not shown). These results negate the possibility that DN constructs altered the responsiveness of LRN/ECN neurons to FP cues. Therefore, the most plausible mechanism for the effect of DN constructs on the migration of LRN/ECN neurons would be disruption of homophilic interactions through classic cadherins between migrating neurons and/or they and accompanying axonal fibers.

It has been shown that switching a class of cadherins from NCAD and CAD6B to CAD7 plays a crucial role in the emigration of neural crest cells from the dorsal neural tube: when full-length NCAD or CAD7 were overexpressed in the neural tube, they failed to emigrate from neuroepithelial cells (Nakagawa and Takeichi, 1998). These results contrast with our findings that overexpression of full-length cadherins was insufficient to affect the migration of LRN/ECN neurons implicating the failure to form additional strong adhesion complexes. It is also of note that appearance of chain-like structures composed of migrating LRN/ECN neurons was apparently unaltered when DN cadherins or full-length cadherins were expressed. Our results suggest that classic cadherins promote the motility of migrating LRN/ECN cells rather than stabilizing them despite their adhesive property. What, then, is the rational explanation for this difference? It has been proposed that their conformational changes and interactions with intracellular proteins such as  $\beta$ -catenins can modulate strength of cadherin-mediated adhesion (Tanaka et al., 2000; Barth et al., 1997). These mechanisms could work in migrating LRN/ECN neurons so that weak rather than strong adhesive interactions are triggered, promoting their gliding along a cadherin-adhesive path.

The technique of exo utero electroporation also allowed us to analyze the effect of the perturbation of cadherin functions in the formation of nuclei in vivo. We showed that inhibition of cadherin functions leads to the ipsilateral formation of LRN/ECN, which normally occurs contralaterally. It is likely that the ipsilateral formation of the nuclei is a consequence of slowed migration, because: (1) LRN/ECN neurons acquire the ability to respond to cues for their entry into prospective nuclear regions in a developmental time-dependent manner (see Kawauchi et al., 2006); and (2) early emigrating EGFP-NCAD(t) neurons do not form nuclei on the ipsilateral side (D.K. and F.M., unpublished). The regulation of migration speed therefore may be important for nucleogenesis at a proper site.

In conclusion, our results demonstrate that classic cadherins play an essential role in contact-dependent regulation for the tangential migration of LRN/ECN neurons. We propose that the regulated adhesion and signaling mediated through the homophilic interaction of cadherins provide the migrating neurons with forces to move forwards. Interestingly, it has been reported that the migration of Purkinje cells is disorganized in  $\alpha$ -Ncatenin knockout mice, suggesting the importance of cadherin function (Togashi et al., 2002). Thus, classic cadherins might have a general role as

substrates not only in the tangential migration as presented in this study but also the radial migration, as seen in the cerebrum and cerebellum.

#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/10/1923/DC1>

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