

Development 139, 1225 (2012) doi:10.1242/dev.080036
© 2012. Published by The Company of Biologists Ltd

Dpy19l1, a multi-transmembrane protein, regulates the radial migration of glutamatergic neurons in the developing cerebral cortex

Keisuke Watanabe, Hirohide Takebayashi, Asim K. Bepari, Shigeyuki Esumi, Yuchio Yanagawa and Nobuaki Tamamaki

There was an error in *Development* **138**, 4979-4990.

Hirohide Takebayashi's correct e-mail address is takebaya@med.niigata-u.ac.jp

We apologise to the authors and readers for this mistake.

Dpy19l1, a multi-transmembrane protein, regulates the radial migration of glutamatergic neurons in the developing cerebral cortex

Keisuke Watanabe^{1,‡}, Hirohide Takebayashi^{1,2,*}, Asim K. Bepari^{1,3}, Shigeyuki Esumi¹, Yuchio Yanagawa^{4,5} and Nobuaki Tamamaki¹

SUMMARY

During corticogenesis, the regulation of neuronal migration is crucial for the functional organization of the neocortex. Glutamatergic neurons are major excitatory components of the mammalian neocortex. In order to elucidate the specific molecular mechanisms underlying their development, we used single-cell microarray analysis to screen for mouse genes that are highly expressed in developing glutamatergic neurons. We identified dpy-19-like 1 (*Dpy19l1*), a homolog of *C. elegans dpy-19*, which encodes a putative multi-transmembrane protein shown to regulate directed migration of Q neuroblasts in *C. elegans*. At embryonic stages *Dpy19l1* is highly expressed in glutamatergic neurons in the mouse cerebral cortex, whereas in the subpallium, where GABAergic neurons are generated, expression was below detectable levels. Downregulation of *Dpy19l1* mediated by shRNA resulted in defective radial migration of glutamatergic neurons *in vivo*, which was restored by the expression of shRNA-insensitive *Dpy19l1*. Many *Dpy19l1*-knockdown cells were aberrantly arrested in the intermediate zone and the deep layer and, additionally, some extended single long processes towards the pial surface. Furthermore, we observed defective radial migration of bipolar cells in *Dpy19l1*-knockdown brains. Despite these migration defects, these cells correctly expressed *Cux1*, which is a marker for upper layer neurons, suggesting that *Dpy19l1* knockdown results in migration defects but does not affect cell type specification. These results indicate that *Dpy19l1* is required for the proper radial migration of glutamatergic neurons, and suggest an evolutionarily conserved role for the *Dpy19* family in neuronal migration.

KEY WORDS: *dpy-19*, Neuronal migration, Corticogenesis, Excitatory neuron, Mouse

INTRODUCTION

The mammalian cerebral cortex is the center for higher functions of the brain. The cortical area has been evolutionarily expanded, acquiring a distinct six-layer structure (Preuss and Kaas, 1999). During early cortical development, neuronal migration plays an important role in functional organization of the cortex. Several genetic malformations, such as lissencephaly and subcortical band heterotopia (SBH), are caused by deficient neuronal migration (Ross and Walsh, 2001; Kato and Dobyns, 2003; Guerrini and Marini, 2006). For example, mutations in the *LIS1* (*PAFAH1B1* – Human Gene Nomenclature Committee) gene or in the X-linked gene doublecortin (*DCX*) lead to classical lissencephaly in humans, which has been shown to be caused by neuronal migration defects (Reiner et al., 1993; des Portes et al., 1998; Gleeson et al., 1998).

During corticogenesis, glutamatergic neurons are generated from progenitors called radial glia and intermediate progenitors in the ventricular/subventricular zone of the dorsal pallium. These neurons then migrate radially into the cortical plate in an inside-out pattern (Rakic, 1972; Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001; Nadarajah and Parnavelas, 2002). By contrast, most GABAergic interneurons arise from the ganglionic eminence, then migrate tangentially and settle in the cerebral cortex (Anderson et al., 1997; Tamamaki et al., 1997; Marín and Rubenstein, 2003). The radial migration of glutamatergic neurons is strictly controlled by coordinated extracellular and intracellular molecules to establish the distinct layers of the cerebral cortex (Hatten, 2002). For example, the secreted protein reelin is well known to act as a guidance cue for the radial migration of cortical neurons (Rice and Curran, 2001), and cyclin-dependent kinase 5 (CDK5) and the cytoskeletal regulator RhoA are also involved in cortical radial migration (Gilmore et al., 1998; Hand et al., 2005). Neuronal polarization is also important for neuronal migration (Reiner and Sapir, 2009). Knockdown of the polarity kinases Par1/Mark2 and Par4/Lkb1 (Stk11) disrupts radial migration by affecting centrosome positioning and microtubule dynamics *in vivo* (Asada et al., 2007; Sapir et al., 2008). However, much less is known about the regulation of cerebral cortex-specific neuronal migration during development.

To elucidate the molecular mechanisms specific to glutamatergic neuron development, single-cell microarray analysis was used to screen for genes expressed strongly and/or specifically within glutamatergic neurons. From this screen we identified *Dpy19l1* as being prominently expressed in glutamatergic neuronal lineage cells in the developing mouse cerebral cortex. *DPY-19*, a multi-transmembrane protein, was first identified in *C. elegans*. Mutation

¹Department of Morphological Neural Science, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan. ²PRESTO, Japan Science and Technology Agency (JST), Saitama 332-0012, Japan. ³Global COE Cell Fate Regulation Research and Education Unit, Kumamoto University, Kumamoto 860-0811, Japan. ⁴Department of Genetic and Behavioral Neuroscience, Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan. ⁵CREST, JST, Tokyo 102-0075, Japan.

*Present address: Division of Neurobiology and Anatomy, Graduate School of Medical and Dental Sciences, Niigata University, Niigata 951-8510, Japan

[‡]Authors for correspondence (watak@kumamoto-u.ac.jp; takebaya@med.niigata.ac.jp)

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial Share Alike License (<http://creativecommons.org/licenses/by-nc-sa/3.0>), which permits unrestricted non-commercial use, distribution and reproduction in any medium provided that the original work is properly cited and all further distributions of the work or adaptation are subject to the same Creative Commons License terms.

in any of the *dpy* genes causes the ‘dumpy’ phenotype in *C. elegans*, which is characterized by short body length (Brenner, 1974). In *C. elegans*, Q neuroblasts (QL and QR) are generated in bilaterally symmetrical positions and then undergo asymmetrical migration posteriorly and anteriorly, respectively. In *dpy-19* mutants, the polarization of Q neuroblasts becomes randomized, resulting in defective migration, suggesting involvement of *dpy-19* in the polarization and migration of neuroblasts (Honigberg and Kenyon, 2000). Four *dpy-19*-like genes (*Dpy1911-4*) encoding putative multi-transmembrane proteins have been identified in both the human and mouse genomes (Carson et al., 2006). The *Dpy1911* gene encodes a protein of 746 amino acids; however, its expression and function during development of the mammalian cerebral cortex have yet to be determined.

In this study we investigated the role of Dpy1911 in mouse corticogenesis. Dpy1911 knockdown mediated by short hairpin RNA (shRNA) resulted in defective radial migration of glutamatergic neurons in vivo, which could be rescued by the expression of shRNA-insensitive Dpy1911. This migration defect was partly caused by the abnormal migration of bipolar cells in Dpy1911-knockdown brains. Dpy1911-knockdown cells correctly expressed the layer-specific marker *Cux1* despite their aberrant positioning. Two to 3 weeks after electroporation, most of the downregulated cells in the intermediate zone showed an abnormal rounded or multipolar morphology, whereas many of the cells arrested in the deep layer were of the characteristic pyramidal morphology. Our study identifies the significance of the transmembrane protein Dpy1911 in the proper migration of glutamatergic neurons during mammalian corticogenesis.

MATERIALS AND METHODS

Animals

Timed-pregnant ICR and C57BL/6 mice were obtained from Japan SLC. *NEX/Neurod6-Cre* (*NEX-Cre*) mice were kindly provided by Dr K. A. Nave (Schwab et al., 2000). Genotyping of *NEX-Cre* and *GAD67-GFP* (*GAD67* is also known as *Gad1*) knock-in mice was as described previously (Schwab et al., 2000; Tamamaki et al., 2003). *NEX-Cre* and *GAD67-GFP* heterozygous embryos were obtained by time-mating C57BL/6 wild-type female mice with *NEX-Cre* homozygous and *GAD67-GFP* heterozygous male mice, respectively. Noon on the day of vaginal plug was considered embryonic day (E) 0.5. All procedures were approved by the Animal Research Committee of Kumamoto University.

Plasmids

shRNA sequences against *Dpy1911* were as follows (5' to 3'): Dpy1911 sh647, GGACTCAGTCCGATTGAGA; Dpy1911 sh1769, GGTTCAGCAAACCTACAAA; Dpy1911 sh2069, GAGTCATGGTGCATAAGAA; and the scrambled sequence Dpy1911-control shRNA, ACAGCTAG-GCTCGGATATG. Each shRNA oligonucleotide was inserted into p*Silencer* 1.0-U6 (Ambion). All plasmids contained a 9 nt hairpin loop sequence (5'-TTCAAGAGA-3'). These constructs were co-transfected with a reporter plasmid by in utero electroporation as described below.

To generate the Dpy1911-EGFP fusion construct, both the *EcoRI-BspHI Dpy1911* 5' fragment (I.M.A.G.E. cDNA clone ID 6856095, Invitrogen) and the *BspHI-ApaI* 3' fragment (PCR-amplified fragment) were cloned into the *EcoRI* and *ApaI* sites of pEGFP-N1 (Takara Clontech).

To generate the Dpy1911 shRNA-insensitive construct (Dpy1911-i), a mutated 3' fragment was synthesized. Both the *EcoRI-BspHI Dpy1911* 5' fragment and the *BspHI-NotI* mutated 3' fragment were cloned into the *EcoRI* and *NotI* sites of the pCAG-RB vector (a pCAG-GS vector containing a modified multiple cloning site).

In utero electroporation

Timed-pregnant mice were deeply anesthetized using pentobarbital (50 mg/kg). Plasmid DNA (1–2 µg/µl) was microinjected into the lateral ventricles of E13.5 or E14.5 forebrains and electroporated (five 50-

msec pulses of 30 V for E13.5 or 33 V for E14.5, with an interval of 950 msec; CUY21 electroporator, Nepagene). Embryos were dissected 24 hours to 3 weeks after electroporation, then single-cell microarray and histological analyses were carried out as described below. In order to obtain sparse labeling of GFP-positive cells, we used Cre-mediated recombination in which 10–50 ng/µl pCAG-Cre along with 2 µg/µl GFP reporter plasmids and either Dpy1911 shRNA or control shRNA plasmid were electroporated at E14.5.

Single-cell microarray analysis

Single-cell microarray analysis was performed as described previously (Esumi et al., 2008). E13.5 *NEX-Cre* embryos (C57BL/6 background) were electroporated with pCAG-loxP-RFP-loxP-EGFP (Wu et al., 2005). After 24 hours, the cortices of *NEX-Cre* and *GAD67-GFP* knock-in mice were dissected and dissociated into single-cell suspensions by digestion with 0.25% trypsin and 0.01% DNaseI at 37°C for 5 minutes. GFP-positive cells were identified under a fluorescence microscope and picked up using the Quixell Automated Cell Selection and Transfer System (Stoelting).

Total RNA from a single cell was reverse transcribed into cDNA using the Super SMART PCR cDNA Synthesis Kit (Takara Clontech). We then assessed the quality of the Super SMART PCR products by capillary electrophoresis (Bioanalyzer 2100, Agilent Technologies) and quantitative real-time PCR (qPCR) analysis (ABI 7500, Applied Biosystems). The following PCR profile was used: 10 minutes at 95°C; 50 cycles of 15 seconds at 95°C, 1 minute at 60°C. Primers used in this study are listed in supplementary material Table S1.

The quality-checked products were subjected to one cycle of a T7 RNA polymerase reaction to produce biotinylated cRNA. The cRNA was subsequently fragmented and hybridized to a GeneChip Mouse Genome MOE430 2.0 array (Affymetrix), according to the manufacturer's protocol. The microarray image data were processed with a GeneChip Scanner 3000 (Affymetrix). We performed per-chip normalization (normalized to the fiftieth percentile). Absolute calls (present, absent and marginal) were calculated with GCOS (Affymetrix) in the default setting. Further data analysis was carried out using GeneSpring software (Agilent).

RT-PCR

Total RNA from E14.5 or adult mouse cerebral cortex was extracted with TRIzol reagent (Invitrogen) following the manufacturer's protocol. Single-stranded cDNAs were prepared using SuperScript reverse transcriptase III (Invitrogen) and amplified by PCR. Primers used for RT-PCR are listed in supplementary material Table S1.

Histology

Mouse embryos were fixed with 4% paraformaldehyde (PFA) in PBS for 60 minutes to overnight at 4°C, cryoprotected in 20% sucrose, then embedded in OCT compound (Sakura Finetechnical). Frozen sections were cut at 20 or 50 µm on a cryostat (CM1850, Leica) and then mounted onto MAS-coated glass slides (Matsunami). Immunohistochemical staining was performed as described (Watanabe et al., 2006). Primary antibodies used were: rat anti-GFP (1:2000, Nacalai Tesque), rabbit anti-GFP (1:1000, Invitrogen/Molecular Probes), mouse anti-beta III tubulin (1:1000, Covance), mouse anti-TAG-1 (Ctnn2; 4D7, 1:5, DSHB), mouse RC2 (1:500, DSHB), mouse anti-BrdU (1:500, BD Pharmingen), rabbit anti-Cux1 (M-222, 1:50, Santa Cruz Biotechnology), rabbit anti-Ctip2 (1:200, Novus Biologicals), rabbit anti-calretinin (1:500, Swant), mouse anti-MAP2 (1:1000, Sigma), rabbit anti-Tbr1 [1:2000, a gift from Dr R. Hevner (Englund et al., 2005)] and rabbit anti-Tbr2 (1:2000, a gift from Dr R. Hevner). Rabbit anti-Dpy1911 serum was generated against two mouse Dpy1911 C-terminal peptides (SRKAPEDVKKELMKLKVC and VEDPDNAGKTPLC; 1:2000, MBL). For immunofluorescence, sections were labeled with species-specific secondary antibodies conjugated to Alexa Fluor 488 or 594 (Invitrogen) and counterstained with Hoechst 33342 (Sigma). Sections were processed by the ABC method (Vector Laboratories) following the manufacturer's protocol. Detection with horseradish peroxidase was performed by incubation in 0.05% diaminobenzidine (DAB) and 0.015% hydrogen peroxide in PBS.

In situ hybridization (ISH) was performed as described (Watanabe et al., 2006). Mouse *Dpy1911* and *Dpy1913* cDNAs were generated by RT-PCR and used as probes. Primers for ISH are listed in supplementary material

Table S1. DIG-labeled RNA hybrids were reacted with alkaline phosphatase-conjugated anti-DIG antibody (Roche). The reaction product was visualized by incubating the sections with nitroblue tetrazolium chloride (NBT; Roche) and 5-bromo-4-chloro-3-indolylphosphate (BCIP; Roche).

Images were taken with a digital camera (DP72, Olympus). Confocal images were captured with a confocal laser-scanning microscope (FV300, Olympus).

BrdU labeling

Pregnant mice were given two intraperitoneal injections of BrdU (50 μ g/g body weight; Sigma) with a 30-minute interval. One hour after the first injection, embryos were isolated and fixed for immunostaining.

Time-lapse imaging

Slice culture was performed as described previously (Tabata and Nakajima, 2003) with minor modifications. Electroporation with shRNA and pCX-EGFP plasmids was performed at E14.5. After 3 days, embryonic brains were dissected and 250 μ m coronal neocortical slices were prepared using a vibratome (Dosaka EM). The slices were placed on a Millicell-CM filter (Millipore), embedded in collagen gel and cultured in Neurobasal medium supplemented with B27, 2 mM Glutamax and penicillin-streptomycin (Invitrogen) at 37°C under 5% CO₂. Seven optical z-sections at 5 μ m intervals were captured at intervals of 30 minutes for 6-12 hours using the FV300.

Quantification

To quantify GFP-positive cells in the cortex at postnatal day (P) 2, we counted cells in eight areas formed by dividing the images of the cortical plate and intermediate zone of the lateral cerebral cortex into eight equal squares, and then analyzed the data from at least three embryos using Student's *t*-test.

Neuron morphology was defined as having an apical dendrite and/or triangular morphology as observed in 50 μ m sections.

RESULTS

Identification of genes specific to glutamatergic neuronal lineages

To study the molecular mechanisms underlying the development of cortical glutamatergic neurons, we performed single-cell microarray analysis to identify genes that are specifically expressed in glutamatergic neurons (Esumi et al., 2008). We labeled newly born glutamatergic neuron precursors by GFP expression utilizing *NEX-Cre* mice, and labeled GABAergic neuronal lineages using *GAD67-GFP* knock-in mice (Fig. 1A-D) (Schwab et al., 2000; Tamamaki et al., 2003). To label glutamatergic neurons, E13.5 *NEX-Cre* embryos were electroporated with a pCAG-loxP-RFP-loxP-EGFP construct; single GFP-positive cells were then selected after 24 hours (Fig. 1A,B).

Immunohistochemical analysis was used to examine the properties of GFP-positive cells in littermates. Many NEX-GFP-positive cells were also positive for Tbr2 (Eomes – Mouse Genome Informatics), a marker of intermediate/basal progenitors (Englund et al., 2005), and some also colabeled with the neuronal marker beta III tubulin, suggesting that most GFP-positive cells were glutamatergic neurons/progenitors (Fig. 1C-D'') (Wu et al., 2005).

Additionally, we collected single GABAergic neurons from the medial ganglionic eminence of E12.5 *GAD67-GFP* mice (Fig. 1A) and performed single-cell microarray analysis on cells from both lineages: seven NEX-positive cells and six GAD67-positive cells were analyzed. Cluster analysis (including data from P0 GAD67-positive cells) (Esumi et al., 2008) revealed distinct gene expression profiles in the two lineages and identified 123 genes that were present only in NEX-positive cells (present call in at least three out of seven NEX-positive cells, absent call in all E12.5 and

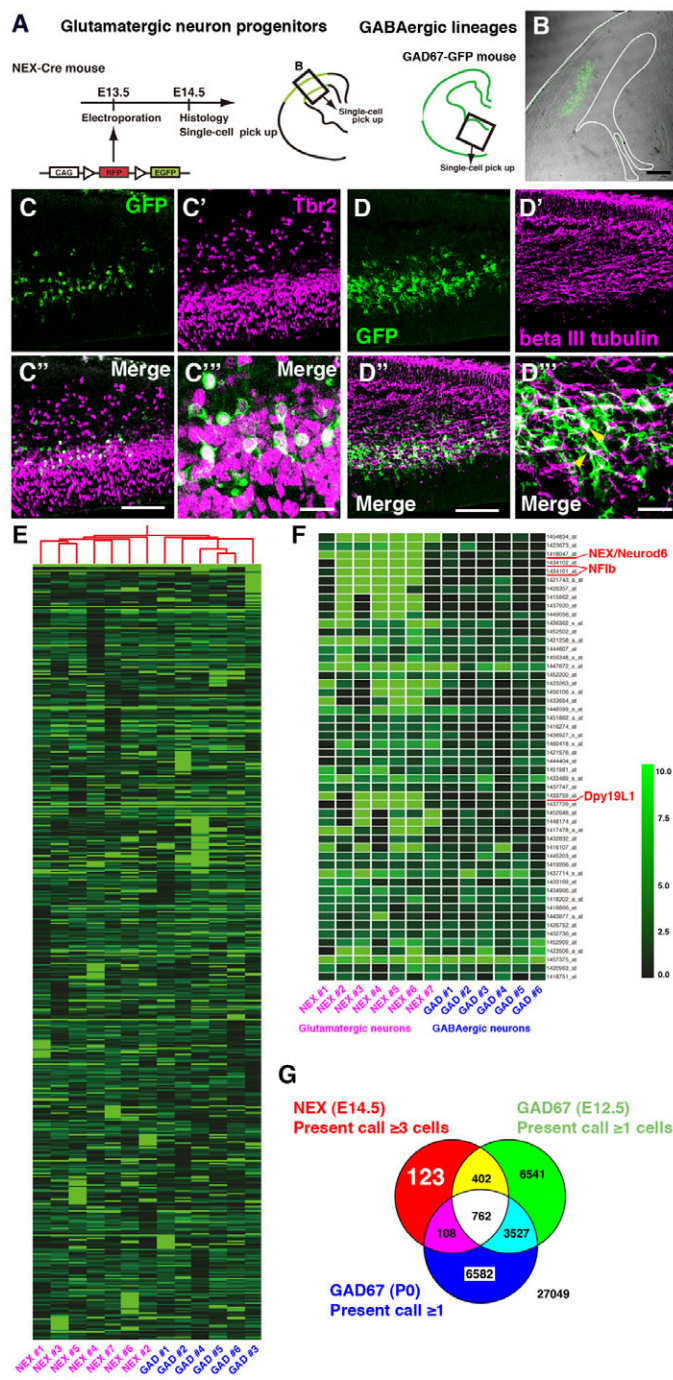


Fig. 1. Subtraction screening of cortical glutamatergic neuron-specific genes by single-cell microarray. (A) The labeling procedure for glutamatergic or GABAergic neuron lineage cells. (B) Localization of GFP-positive cells in the *NEX-Cre* cerebral cortex 24 hours after electroporation with a reporter plasmid. The border of the section is outlined. (C-D'') Immunohistochemistry of cortical sections of E14.5 *NEX-Cre* mouse embryos labeled with a GFP reporter showing overlay of Tbr2 (C-C'') or beta III tubulin (D-D'') and GFP. C'' and D'' show magnified images. Yellow arrowheads indicate cells positive for GFP and beta III tubulin. (E) Cluster analysis. Distinct gene expression profiles for glutamatergic and GABAergic neuron lineage cells were detected. (F) Expression levels of genes strongly expressed in glutamatergic neuronal lineage cells. (G) We selected 123 genes that were specifically expressed in glutamatergic neurons by filtering with present/absent calls. Scale bars: 200 μ m in B; 100 μ m in C-C'', D-D''; 20 μ m in C'', D''.

P0 GAD67-positive cells; Fig. 1E-G). We subsequently examined the expression pattern of ~40 genes of interest at E14.5 by ISH, from which we identified several genes that were expressed in cortical glutamatergic neurons. Although some of these genes are expressed in the subpallium as well as the dorsal cortex, we were able to identify several genes that are expressed predominantly in glutamatergic neurons (supplementary material Fig. S1; data not shown). Among these genes, we focused on the *Dpy19* family, as *Dpy1911* mRNA is highly expressed in the developing cerebral cortex (Fig. 2).

***Dpy1911* is highly expressed in glutamatergic neurons of the developing cerebral cortex**

The *Dpy19* gene is evolutionarily conserved in *C. elegans*, rodents and human; four genes (*Dpy1911-14*) are found in mouse (Mouse Genome Informatics). We first examined the expression of *Dpy19* family members in the mouse telencephalon by ISH and RT-PCR. At E14.5, strong expression of *Dpy1911* and *Dpy1913* were observed in the developing cerebral cortex (Fig. 2B,C), whereas *Dpy1912* and *Dpy1914* mRNA could not be detected with our ISH probes. However, mRNAs of all the *Dpy19* family members were detected in both the E14.5 and adult cerebral cortex by RT-PCR (Fig. 2A) (Sugiura et al., 2001). *Dpy1912* mRNA was detected at relatively low levels at both stages. At E12.5, *Dpy1911* expression was detected in the cortical plate (CP) of the dorsal telencephalon (Fig. 2D). At E14.5, *Dpy1911* was expressed in a lateromedial gradient within the cerebral cortex, whereas *Dpy1913* showed an opposite expression gradient (Fig. 2B,C). At this stage, high levels of expression were observed in both the CP, where neurons terminate their migration, and the intermediate zone (IZ), through which neurons migrate to reach the CP, and weak expression was observed in the proliferative ventricular zone (VZ) and the subventricular zone (SVZ) (Fig. 2B,E). Additionally, the surface of the VZ showed intense expression of *Dpy1911* (Fig. 2E, arrows). By contrast, very low expression of *Dpy1911* was detected in the ganglionic eminence, the source of cortical GABAergic interneurons (Fig. 2B). We observed a very restricted expression of *Dpy1911* in other regions, such as the diencephalon and the dorsal midline of the brain stem (supplementary material Figs S2, S4). During perinatal stages, *Dpy1911* expression was apparent in the deep cortical layer and the surface of the VZ, whereas weaker expression was detected in the upper layer, particularly in the lateral regions of the cortex (Fig. 2F, arrows in 2G). At this stage, intense expression of *Dpy1911* mRNA could also be detected in regions other than the cerebral cortex (Fig. 2F). *Dpy1911* expression was also observed in the adult cerebral cortex, with stronger signals in the deep layer compared with the upper layer (Fig. 2H,I).

To determine the cellular distribution of Dpy1911 protein, we generated an antibody against the C-terminal region of Dpy1911 and then used it for immunohistochemistry. The pattern of immunostaining was similar to that of ISH (supplementary material Fig. S2). Dpy1911 protein was primarily localized in the IZ, the CP, the marginal zone (MZ) and the surface of the VZ in the E14.5 cerebral cortex, with weak expression detected in the VZ/SVZ (Fig. 2J,K; supplementary material Fig. S2A,B). Localization in the ganglionic eminence was barely detectable (Fig. 2J,L). Dpy1911 protein also appeared to be localized in the axons of the CNS and peripheral tissues (supplementary material Figs S2, S3, arrows; data not shown).

To confirm that *Dpy1911* is expressed in glutamatergic neurons, we examined the colocalization of *Dpy1911* mRNA with cortical subtype-specific markers. At E14.5, many *Dpy1911*-expressing

cells in the CP co-expressed *Tbr1*, a marker of layer VI neurons, or *Ctip2* (Bcl11b – Mouse Genome Informatics), a marker of layer V neurons (Fig. 3A,B). At E17.5, *Dpy1911*-positive cells expressed *Cux1*, a marker of upper layer neurons (layers II-IV), although *Dpy1911* expression in the upper CP was weaker at E17.5 than at E14.5 (Fig. 3C). These results suggest that *Dpy1911* is strongly expressed in glutamatergic neuronal lineage cells during corticogenesis.

Dpy1911 regulates the radial migration of cortical neurons

To study the function of Dpy1911 during the development of glutamatergic neurons, we performed shRNA-mediated knockdown of Dpy1911. Three shRNA constructs designed against Dpy1911 and a scrambled shRNA plasmid as a control were tested. Co-transfection of COS-7 cells with any of the Dpy1911 shRNAs along with the Dpy1911-EGFP fusion plasmid specifically suppressed *Dpy1911-EGFP* mRNA expression after 3 days, whereas co-transfection with the control shRNA did not (Fig. 4A).

We next examined whether Dpy1911 regulates the development of cortical glutamatergic neurons. The lateral cortices of E14.5 wild-type mice were electroporated with either a Dpy1911 shRNA construct (sh1769 had a strong RNAi effect; sh2069 had a moderate effect; Fig. 4A) or control shRNA along with the pCX-EGFP plasmid. Two days to 3 weeks post-electroporation, the distribution of GFP-positive cells in the cerebral cortex was analyzed by GFP immunostaining (Fig. 4B). Neurons in the upper layers were labeled as a result of the experimental conditions. At P2, 7 days after electroporation, a time point at which most GFP-positive cells have terminated their migration, shRNA-mediated knockdown by two of the Dpy1911 shRNAs (sh1769 and sh2069) resulted in cells with neuronal migration defects, whereas the control shRNA had no effect (Fig. 4C-I). A large number of Dpy1911-knockdown GFP-positive cells remained in the IZ, and some were ectopically located in the deep cortical layer (arrowheads in Fig. 4G,H), whereas almost all GFP-labeled cells transfected with control shRNA had reached the upper layer (arrowheads in Fig. 4F). The migration defect was less severe in brains transfected with sh2069 than with sh1769 (Fig. 4D-I; see Discussion). Moreover, this migration defect could also be induced between P8 and P14 by downregulating Dpy1911, suggesting that aberrant neural migration upon Dpy1911 knockdown cannot be entirely attributed to developmental delay (Fig. 7A-D; supplementary material Fig. S7).

To determine whether the migration defect caused by Dpy1911 shRNAs is a direct result of Dpy1911 deficiency, we performed a rescue experiment in which a construct was introduced that expressed shRNA-insensitive Dpy1911 (Dpy1911-i). We electroporated the control, pCAG or pCAG-Dpy1911-i plasmid along with Dpy1911 sh1769 and pCX-EGFP at E14.5 and observed the localization of GFP-positive cells at P2. Re-expression of Dpy1911 in Dpy1911-knockdown cells partially rescued the migration defect, whereas the injection of control pCAG and Dpy1911 shRNA plasmids perturbed radial migration (Fig. 4J-L). These results suggest that Dpy1911 is required for the proper radial migration of glutamatergic neurons during corticogenesis.

Disruption of radial migration of bipolar cells by Dpy1911 downregulation

Prior to arriving at their terminal destination, glutamatergic neuron progenitors transit to a multipolar stage in the SVZ, transform into a bipolar shape in the IZ and then migrate into the CP while

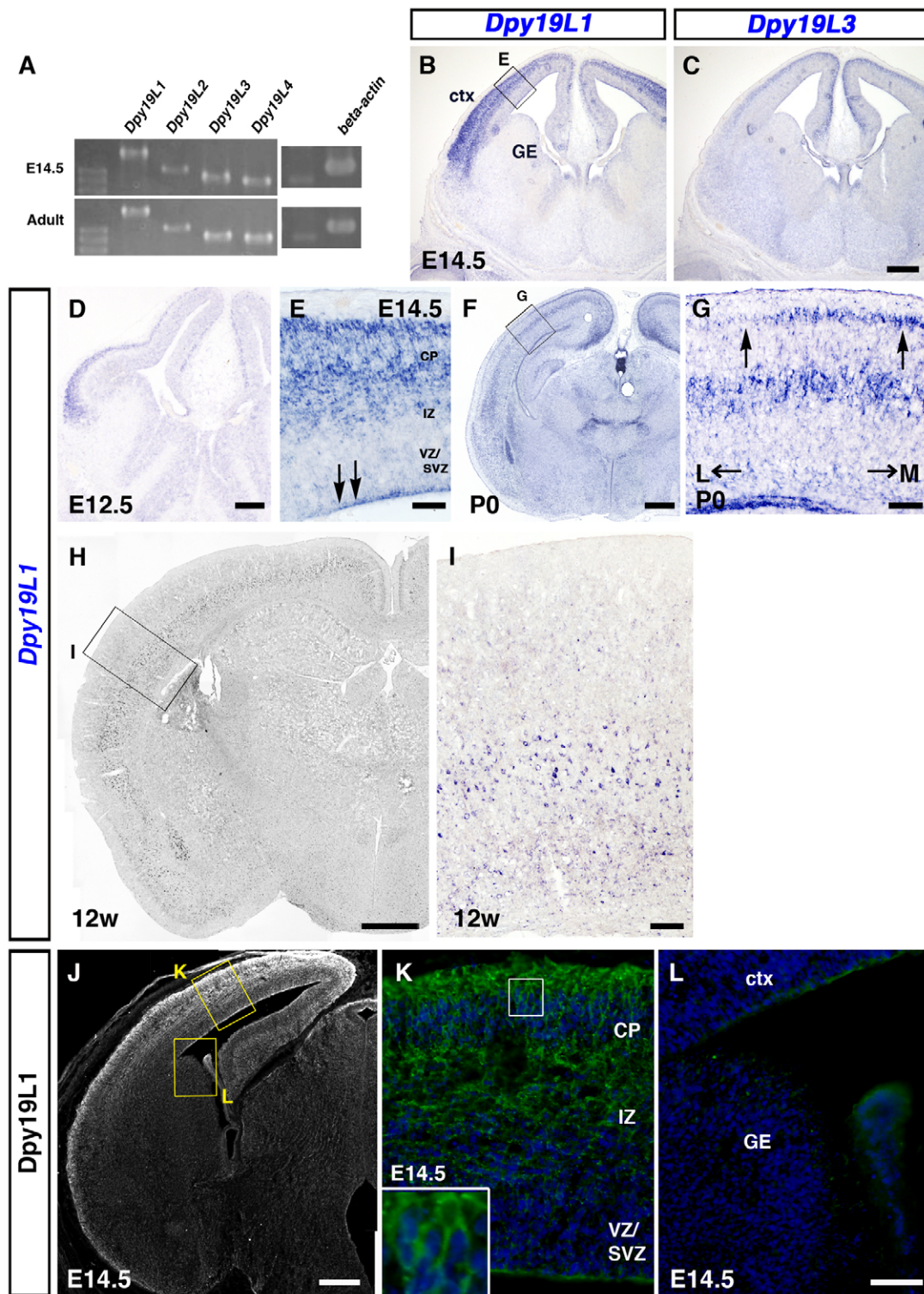


Fig. 2. Expression analyses of the Dpy19 family in the developing cortex. (A) RT-PCR of *Dpy19* family genes in E14.5 and adult mouse brains. beta-actin provides a control. (B-L) Expression of *Dpy19l1* and *Dpy19l3* mRNAs visualized by in situ hybridization (ISH) in the telencephalon. (B-E) At E12.5, expression of *Dpy19l1* was observed in the dorsal telencephalon. At E14.5, *Dpy19l1* mRNA was intensely expressed in the CP, the IZ and weakly in the VZ/SVZ of the cerebral cortex. *Dpy19l3* mRNA showed an opposite gradient of expression. The boxed area in B is magnified in E. (F,G) Expression of *Dpy19l1* was also observed in the P0 cortex, especially in the deep layer. The boxed area in F is magnified in G. (H,I) *Dpy19l1* expression persists in the adult cerebral cortex at 12 weeks. The boxed area in H is magnified in I. (J-L) Distribution of Dpy19l1 protein at E14.5. In J, Dpy19l1 immunoreactivity is white. Dpy19l1 was mainly distributed in the IZ as well as the CP of the cortex. Inset in K shows a magnified view of the CP. Boxed areas in J are magnified in K and L. This section was counterstained with Hoechst 33342. CP, cortical plate; ctx, cerebral cortex; GE, ganglionic eminence; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; L, lateral; M, medial. Scale bars: 300 μ m in B,C; 200 μ m in D,J; 50 μ m in E,K,L; 500 μ m in F; 100 μ m in G,I; 1 mm in H.

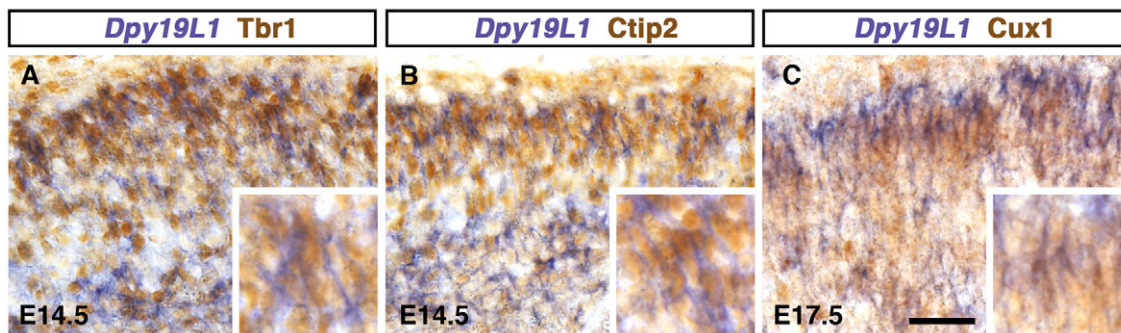


Fig. 3. *Dpy1911* is expressed in cortical glutamatergic neurons. (A–C) Immunostaining for Tbr1 (A), Ctip2 (B) and Cux1 (C) after ISH for *Dpy1911* in the developing mouse cerebral cortex at E14.5 (A,B) and E17.5 (C). Insets are magnified views. *Dpy1911*-positive cells co-express different glutamatergic neuron markers: Tbr1, layer VI; Ctip2, layer V; Cux1, layers II–IV. Scale bar: 30 μ m.

extending their axons (Tabata and Nakajima, 2003; Kriegstein and Noctor, 2004). To investigate which of these processes is affected by the downregulation of *Dpy1911*, we performed morphological analyses of *Dpy1911*-knockdown cells. *Dpy1911* shRNA-transfected cells were labeled using Cre-mediated GFP recombination in order to facilitate the morphological characterization of GFP-labeled cells (see Materials and methods). Four days post-electroporation (at E18.5), we observed that many *Dpy1911*-knockdown cells did not pass through the subplate (SP) and instead were arrested within the upper IZ (Fig. 5A,B). Moreover, many of these cells assumed a bipolar morphology, although some of them extended a long process towards the pial surface (arrowheads in Fig. 5C). Some of the cells that were aberrantly stalled in the IZ were adjacent to the radial fibers of radial glial cells (arrowheads in Fig. 5D–D’).

To examine this migration further we performed time-lapse imaging. E14.5 embryos were electroporated with shRNA, and cortical slices were cultured 3 days later. We observed that some bipolar cells in the IZ and the CP continued to extend a leading process towards the pial surface, but their cell body was almost immobile (Fig. 5F,G; supplementary material Movies 2–4). By contrast, many bipolar cells in the control brain translocated their cell soma during radial migration (Fig. 5E; supplementary material Movie 1). These results suggested that the defective migration in *Dpy1911*-knockdown brains was caused, in part, by the abnormal migration of bipolar cells.

Normal cell type specification in *Dpy1911*-knockdown cells

The impaired migration in *Dpy1911*-knockdown brains could be the indirect result of abnormal specification of glutamatergic neuron progenitors. However, 2 days after electroporation, no significant change in the rate of BrdU incorporation was observed in the VZ of the *Dpy1911*-knockdown cortex compared with the control brain (Fig. 6A–C). We next labeled GFP-positive cells with markers for cortical neurons/progenitors. In the E17.5 SVZ (3 days post-electroporation), the majority of *Dpy1911*-knockdown/GFP-positive cells expressed Tbr2, a marker of intermediate/basal progenitors, similar to control shRNA-transfected cells (supplementary material Fig. S5). Furthermore, almost all GFP-positive cells expressed Cux1 in both control and *Dpy1911*-knockdown brains at E17.5 (Fig. 6D; supplementary material Fig. S6) (Nieto et al., 2004). At P2, *Dpy1911*-knockdown cells in the upper cortical layer expressed Cux1, as did control embryos (Fig.

6E,F). GFP-positive cells that were aberrantly stalled in the deep CP and the IZ also expressed Cux1 (Fig. 6F–H’). These results indicated that cells settling in aberrant positions maintained the ability to differentiate into layer-specific cortical neurons, even though knockdown of *Dpy1911* caused a defect in neuronal migration.

Morphology of *Dpy1911*-downregulated cells

To investigate if cells with defective migration can differentiate into mature neurons, we performed morphological analysis of shRNA-transfected cells. Two weeks after electroporation (at P8), most control neurons in the superficial layer had a triangular soma and a single apical dendrite (Fig. 7A,C). The majority of *Dpy1911*-knockdown cells arrested in the deeper CP had a similar morphology, although they also had a longer process extending from the deeper layer towards the pial surface (arrows in Fig. 7D,E). Furthermore, most cells ectopically positioned within the IZ were either round in shape or exhibited a multipolar morphology (arrowheads in Fig. 7F). Some cells in the IZ had a long apical dendrite-like process extending towards the pial surface (arrows in Fig. 7G) and an axon-like structure extending towards the dorsal midline (arrows in Fig. 7H).

To accurately evaluate dendrite formation, we analyzed P14 embryos that had been labeled by Cre-mediated GFP recombination. In both control and *Dpy1911*-knockdown embryos, many GFP-positive cells in the cortical upper layer assumed the characteristic pyramidal morphology, characterized by the extension of one apical dendrite and several basal dendrites, which were positive for the dendritic marker MAP2 (Mtap2 – Mouse Genome Informatics) (Fig. 7I,J; supplementary material Figs S7, S8). Many *Dpy1911*-knockdown cells in the deep layer had a similar pyramidal morphology (116/150, 77.3%; Fig. 7K; supplementary material Fig. S7B–B’). However, most GFP-positive cells that were arrested in the white matter showed an abnormal rounded or multipolar morphology (70/80, 87.5%; Fig. 7L–L’; supplementary material Fig. S7B–D’).

DISCUSSION

In this study, we used single-cell microarray analysis to identify genes that are expressed specifically and/or strongly in cortical glutamatergic neurons. Among the candidate genes, we found that *Dpy1911* was prominently expressed in glutamatergic neurons of the developing cerebral cortex. *Dpy1911* knockdown studies showed that it is required for proper radial migration. However, *Dpy1911*

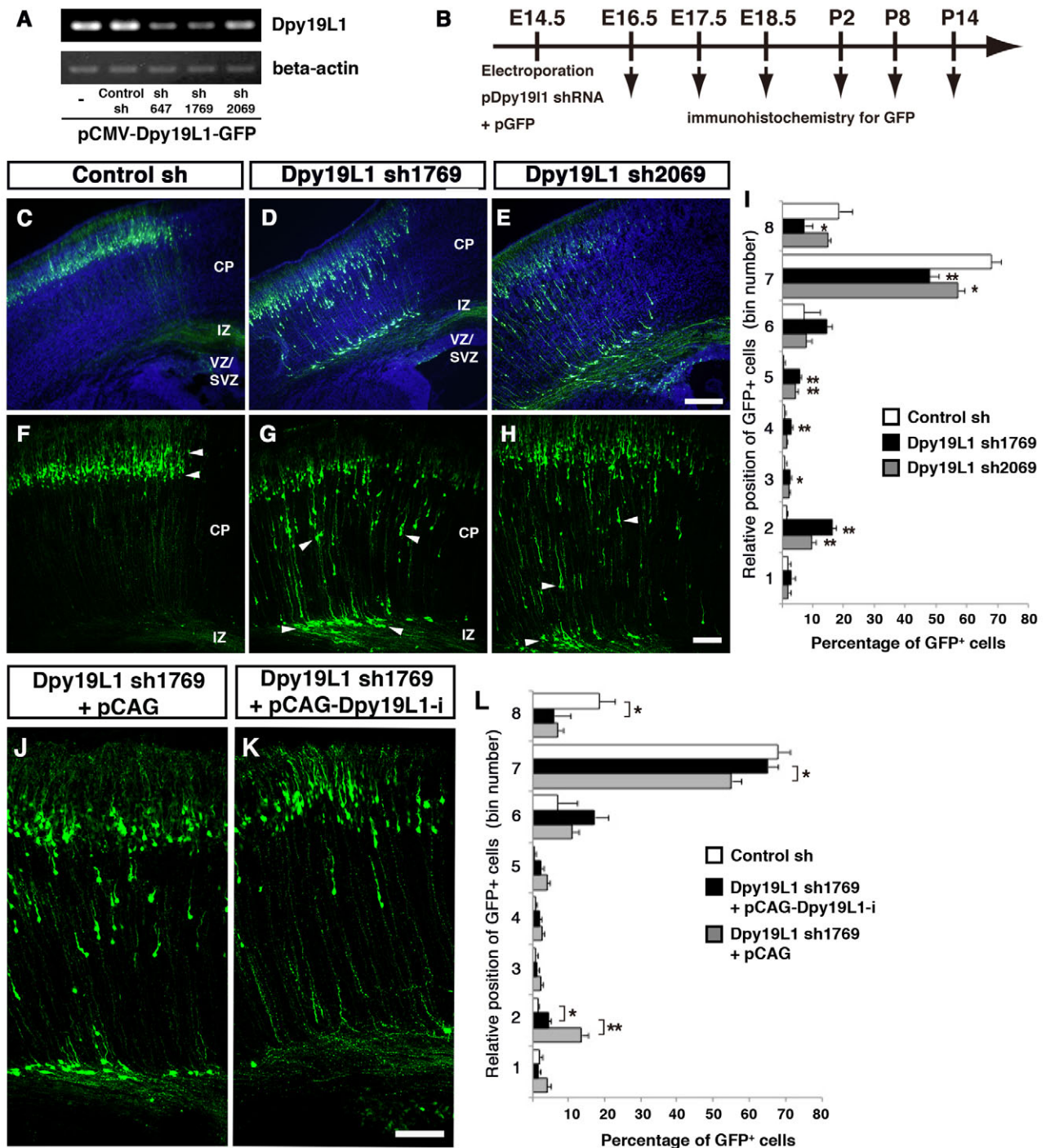


Fig. 4. Dpy19l1 regulates radial migration of cortical neurons. (A) Knockdown experiment in COS-7 cells. Either control shRNA or one of three Dpy19l1 shRNA constructs along with a CMV-Dpy19l1-GFP fusion plasmid were co-transfected into COS-7 cells, and expression was examined by RT-PCR. Each shRNA construct downregulated *Dpy19l1* expression. (B) Time line of these experiments. (C-I) Either control (C,F) or one of the Dpy19l1 shRNAs (sh1769 in D,G; sh2069 in E,H) was electroporated along with pCX-EGFP at E14.5. The distribution of cells with shRNA-mediated knockdown was analyzed at P2. Sections were counterstained with Hoechst 33342. (I) Quantification of GFP-positive cells in the P2 cerebral cortex. The percentage of GFP-positive cells is plotted as the mean \pm s.e.m. (three embryos were analyzed per shRNA). *, $P < 0.05$; **, $P < 0.01$; versus control by *t*-test. Dpy19l1 downregulation disrupted the radial migration of cortical neurons. Many Dpy19l1-knockdown cells were aberrantly positioned in the IZ and the deep CP (arrowheads in G,H), whereas almost all cells reached the cortical upper layer in control brains (arrowheads in F). (J-L) Rescue experiments. Co-electroporation of pCAG control plasmid (J) or shRNA-insensitive pCAG-Dpy19l1 (*Dpy19l1-i*) expression plasmid (K) along with Dpy19l1 shRNA1769 and pCX-EGFP constructs was performed. (L) Quantification of GFP-positive cells at P2. The percentage of GFP-positive cells is plotted as the mean \pm s.e.m. (three embryos for control shRNA, three for sh1769 + pCAG-Dpy19l1-i and six for sh1769 + pCAG). *, $P < 0.05$; **, $P < 0.01$; by *t*-test. The neuronal migration defect caused by Dpy19l1 knockdown was partially restored by re-expression of Dpy19l1-i. Scale bars: 200 μ m in C-E; 100 μ m in F-H,J,K.

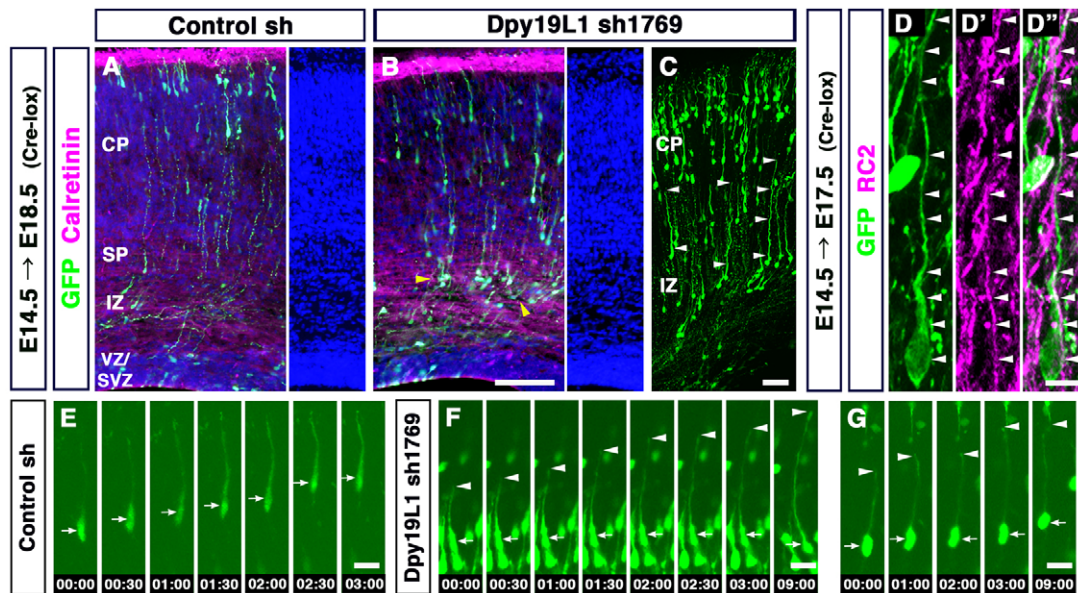


Fig. 5. Impaired neuronal migration of Dpy19L1-knockdown cells. (A–D'') Control (A) or Dpy19L1 shRNA (sh1769, B–D'') was electroporated along with Cre and GFP reporter plasmids at E14. The morphology of GFP-positive cells was analyzed at E18.5 (A–C) or E17.5 (D–D''). (A, B) The subplate (SP) was identified by immunostaining for calretinin (calbindin 2) and by nuclear staining (Hoechst 33342, blue). At E18.5, many Dpy19L1-knockdown cells were abnormally stalled in the IZ (yellow arrowheads in B). Some cells in the IZ had single long processes extending towards the pial surface (arrowheads in C). (D–D'') Double immunostaining for GFP and RC2 (Ifaprc2), a marker of radial glial cells. (E–G) Time-lapse imaging of migrating cells expressing either control (E) or Dpy19L1 (F, G) shRNA. Time-lapse imaging was performed 3 days after electroporation. In Dpy19L1-knockdown brains, the leading process of bipolar cells in the IZ (F) and the CP (G) actively grew towards the pial surface, but the cell body remained in place. Arrowheads and arrows indicate the tip of the leading process and the cell body, respectively. Time is shown in hours:minutes. Scale bars: 100 μ m in A, B; 50 μ m in C; 10 μ m in D–D''; 20 μ m in E–G.

downregulation did not affect cell type specification of cortical neurons. These results demonstrate that Dpy19L1 regulates radial migration of cortical glutamatergic neurons during development.

Identification of *Dpy19L1* as a glutamatergic neuron-specific gene

Many studies have used genome-wide screens in an attempt to identify genes that regulate cell differentiation, migration and other developmental processes and that are specifically expressed in various cell types (Gray et al., 2004; Arlotto et al., 2005). Recently, Kawaguchi et al. reported the spatiotemporal heterogeneity of cortical progenitors using single-cell microarray analysis (Kawaguchi et al., 2008). In the present study, gene expression profiles were compared between glutamatergic and GABAergic neurons using the single-cell microarray method in order to identify genes that are expressed in developing glutamatergic neurons (Fig. 1) (Esumi et al., 2008). This type of single-cell microarray analysis is useful for detecting distinct expression patterns of each neuron/progenitor, although this technique is disadvantaged by its low sensitivity. With single-cell microarray analysis, however, we identified several genes, including *Dpy19L1*, that are prominently localized in glutamatergic neurons of the E14.5 telencephalon (Fig. 2; supplementary material Fig. S1; data not shown). We predict that our results will pave the way for the identification of other genes that regulate distinct developmental processes within each neuronal subtype.

Evolutionary conservation of *Dpy19* genes

In *C. elegans*, DPY-19 is a putative multi-transmembrane protein that is required for the proper polarization and migration of Q neuroblasts (Honigberg and Kenyon, 2000). At least three *Dpy19*

genes are present in vertebrates (Carson et al., 2006). In the mammalian genome, four *Dpy19* genes (*Dpy19L1-4*), encoding 8–11 predicted transmembrane proteins, have been identified, although the Dpy19 proteins lack any predicted functional domains. For the first time, we reveal that mammalian Dpy19L1 is crucial for directed neuronal migration during corticogenesis. Our results suggest a possible conserved role for the Dpy19 family during neuronal migration in both *C. elegans* and mammals. Interestingly, *DPY19L3* has been reported to be associated with bipolar disorder by genome-wide association studies (Smith et al., 2009). More recently, *DPY19L2* deletion has been found to cause human globozoospermia, which is a severe male infertility disorder resulting from round-headed spermatozoa that lack an acrosome (Harbuz et al., 2011; Kosciński et al., 2011). These observations are indicative of the importance of Dpy19 family members in development and disease.

Gradient expression of *Dpy19L1* and *Dpy19L3* in the developing cerebral cortex

We revealed that *Dpy19L1* is mainly expressed in glutamatergic neurons, and not in GABAergic neurons, during embryonic development (Fig. 2). At E14.5, *Dpy19L1* is expressed in a lateromedial gradient within the cerebral cortex, whereas *Dpy19L3* is expressed in an opposite gradient (Fig. 2). Dpy19L1 expression also occurred in a rostral-to-caudal gradient (supplementary material Fig. S4). During early corticogenesis, when cortical neuron progenitors are initially specified, some transcription factors, such as Pax6, Emx2 and COUP-TFI (Nr2f1 – Mouse Genome Informatics), exhibit gradient expression in the VZ and regulate regional identity within the neocortex (Bishop et al., 2000;

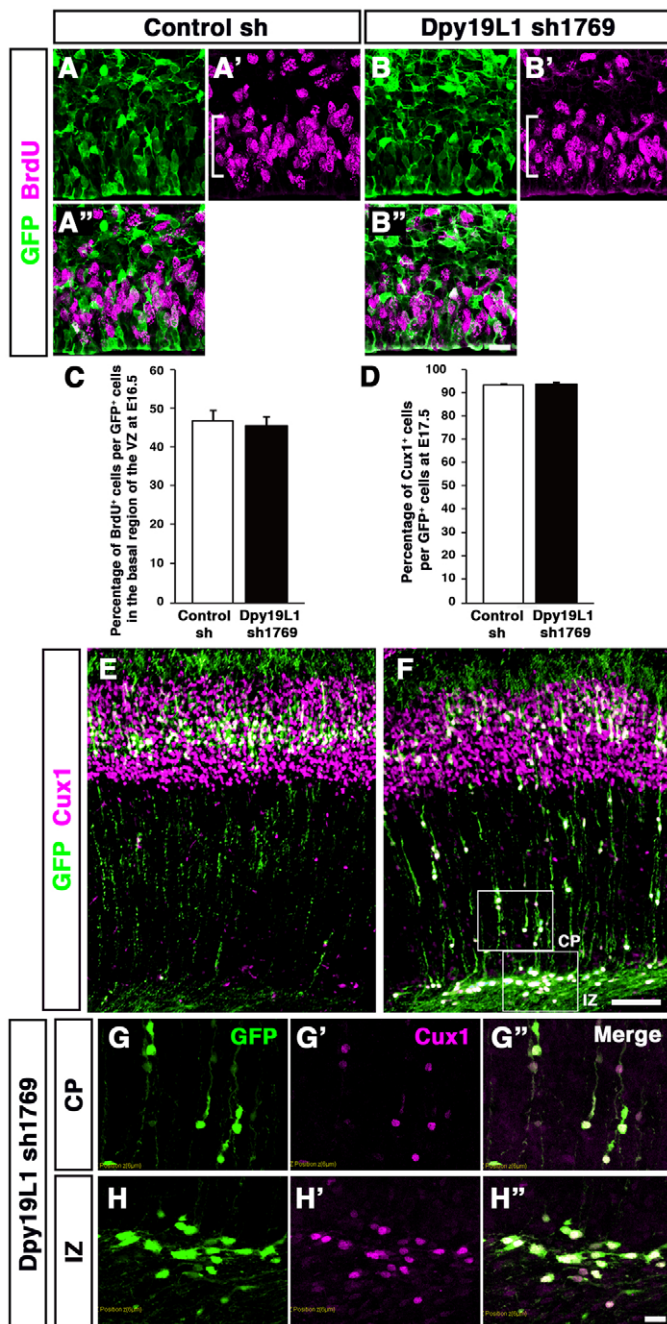


Fig. 6. Dpy1911 knockdown does not affect neuronal differentiation. E14.5 mouse embryos were electroporated with control (A-A'',E) or Dpy1911 shRNA (sh1769, B-B'',F-H'') and analyzed at E16.5 (A-B'') or P2 (E-H''). (A-B'') BrdU incorporation in VZ cells at E16.5. (C) Quantification of BrdU-labeled cells. The percentage of GFP and BrdU double-positive cells in the basal region of the VZ (brackets in A',B') is plotted as the mean \pm s.e.m. (three embryos each for control and sh1769). (D) Quantification of Cux1-positive cells. The percentage of GFP and Cux1 double-positive cells in the E17.5 CP-IZ is plotted as the mean \pm s.e.m. (three embryos each for control and sh1769). (E-H'') Double immunostaining of GFP and Cux1 at P2. In control embryos, most GFP-positive cells were immunolabeled by Cux1. In Dpy1911-downregulated brains, Cux1 was expressed in GFP-positive cells that had stalled abnormally in the IZ and the CP, as well as in neurons that were correctly positioned in the upper cortex. Boxed areas in F are magnified in G-H''. Scale bars: 20 μ m in A-B'',G-H''; 100 μ m in E,F.

Hamasaki et al., 2004). Pax6 expression shows a rostral/lateral^{high} to caudal/medial^{low} gradient, and caudal areas are expanded in Pax6 mutants (Bishop et al., 2000). This lateromedial gradient is similar to that of Dpy1911, although Dpy1911 is strongly expressed in postmitotic neurons (Fig. 2; supplementary material Fig. S4). It is possible that Dpy1911 is a downstream target of transcription factors such as Pax6 and that they might regulate neuronal migration once cell fate is determined in the specific cortical areas.

Regulation of glutamatergic neuronal migration by Dpy1911

A large number of Dpy1911-knockdown cortical neurons were stalled deep within the CP and the IZ (Fig. 4). However, Dpy1911-knockdown cells properly expressed the cell type-specific marker Cux1 (Fig. 6). These results suggest that the primary function of Dpy1911 is the regulation of neuronal migration, but not neuronal specification. Furthermore, our results suggest that the efficiency of the migration defect is directly correlated with the severity of the migration defect (Fig. 4). Moreover, when we applied Dpy1911 shRNA at reduced concentrations, the number of cells arrested in the IZ was decreased (data not shown). Thus, complete knockout of *Dpy1911* would likely result in a more severe migration defect in cortical neurons.

Defects in radial migration have also been observed in several mouse mutants and shRNA-transfected brains in which the cytoskeletal machinery has been modified, although there are differences in the severity of the migration defects (LoTurco and Bai, 2006). For example, Cdk5 loss-of-function in mice and Dcx knockdown in the rat neocortex result in severe migration defects in which a large number of cortical neurons are confined to the IZ (Gilmore et al., 1998; Bai et al., 2003). *Lis1* heterozygous mice also show abnormalities in cortical neuronal migration (Hirotsune et al., 1998). A small percentage of *Lis1* shRNA-transfected cells assumed a bipolar morphology and reached the IZ. Although the cell body was immobile, the leading process continued to grow (Tsai et al., 2005). Interestingly, we observed a phenotype similar to that of the *Lis1* knockdown in the brains of Dpy1911 knockdowns (Fig. 5). In *C. elegans*, *dpy-19* is involved in the polarization of neuroblasts (Honigberg and Kenyon, 2000). In the mammalian brain, knockdown of the polarity kinases Mark2 and Lkb1 causes the arrest of neuronal migration in the IZ (Asada et al., 2007; Sapir et al., 2008). Although, at present, we do not know what signaling pathways interact with Dpy19 proteins to cause the defective migration, it is possible that Dpy1911 participates in some of the aforementioned pathways to regulate neuronal migration during corticogenesis. It will be intriguing to investigate the possible interactions between Dpy1911 and known migration regulators during corticogenesis. We are also in the process of designing a proteomic screen to identify binding partners of the Dpy19 family.

In addition to the proteins mentioned above, extracellular diffusible factors and cell adhesion molecules are also important for proper neuronal migration. Reelin and Sema3A have been shown to guide the radial migration of cortical neurons (Rice and Curran, 2001; Chen et al., 2008). Moreover, a recent report has shown that inhibition of the endocytic pathways also causes aberrant migration via disruption of N-cadherin trafficking (Kawauchi et al., 2010). Although *dpy-19* and *unc-40* (*dcc*), the receptor for *unc-6* (*netrin*), are suggested to function in a shared signaling pathway in Q neuroblast migration in *C. elegans* (Honigberg and Kenyon, 2000), we did not observe any apparent defects in cortical layer formation in *Dcc* mutant mice (data not

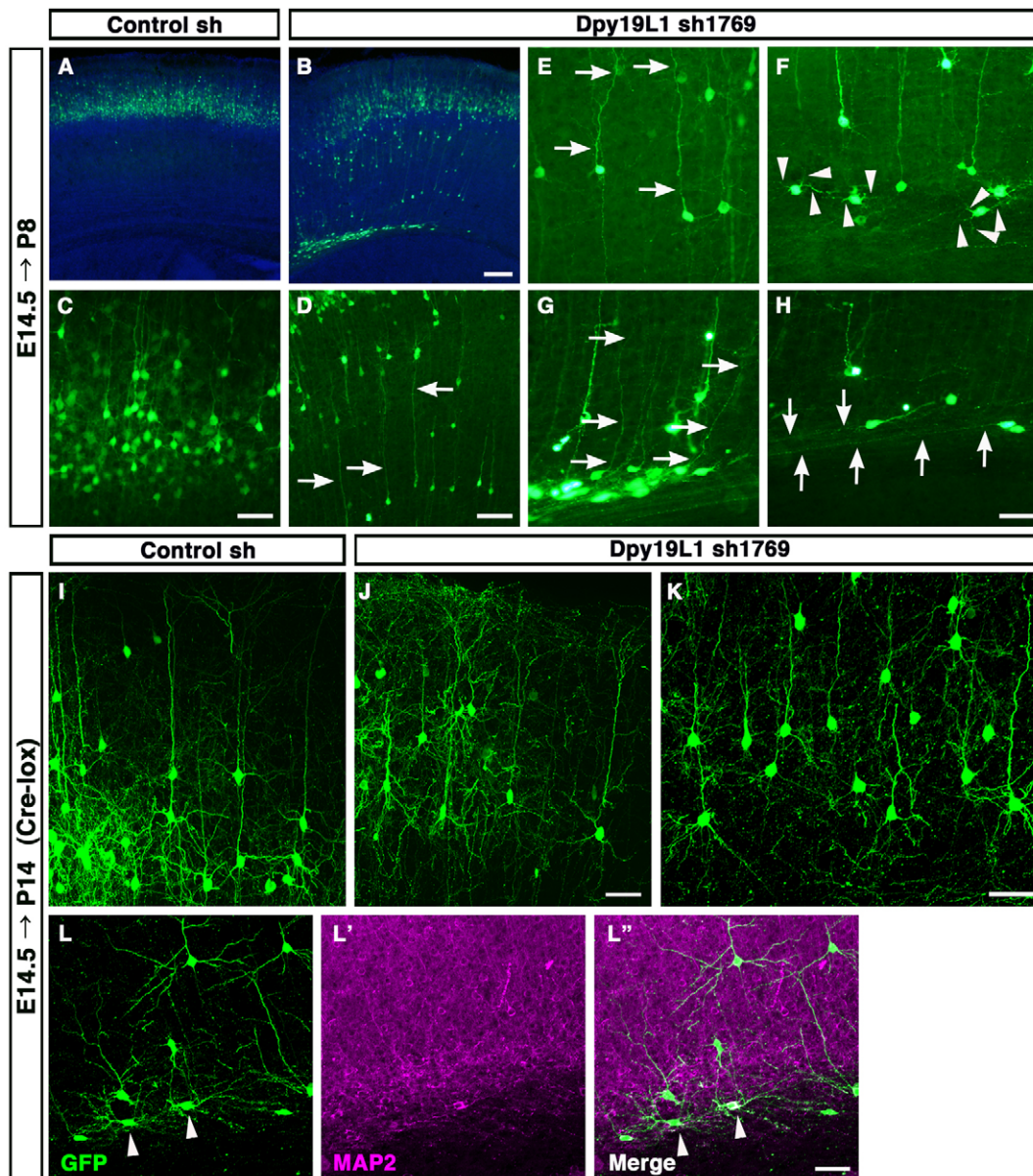


Fig. 7. Morphological changes within migration-disrupted neurons in the Dpy1911-knockdown brain. (A-H) Control (A,C) or Dpy1911 shRNA (sh1769; B,D-H) was electroporated along with pCX-EGFP at E14.5. Dpy1911-downregulated cells were analyzed at P8. (A,C) Almost all GFP-positive cells were found in the upper layer. (B,D-H) Dpy1911-downregulated cells were stalled in the deeper CP and the IZ, although some cells in the IZ extended a long process towards the pial surface and an axon-like process towards the dorsal midline (arrows). Many of these cells showed a rounded or multipolar morphology in the IZ (arrowheads in F). Sections were counterstained with Hoechst 33342. (I-L'') E14.5 embryos were electroporated with control (I) or Dpy1911 shRNA (sh1769, J-L'') together with Cre and GFP reporter plasmids and analyzed at P14. (L-L'') Double immunostaining of GFP and MAP2. Most Dpy1911-downregulated cells in the deep layer assumed the characteristic pyramidal morphology, whereas many cells in the white matter showed abnormal morphologies (arrowheads). Scale bars: 200 μ m in A,B; 100 μ m in D; 50 μ m in C,E-L''.

shown), suggesting that Dcc signaling is dispensable in the radial migration of mammalian cortical neurons. However, it is possible that Dpy1911 mediates extracellular signals and/or is a component of the cell adhesion machinery, as Dpy19 family members are putative multi-transmembrane proteins.

Dendrite and axon formation in Dpy1911-knockdown neurons

We observed that Dpy1911-knockdown cells were localized in abnormal positions within the deep CP (Fig. 5). These cells assumed the characteristic pyramidal morphology but failed to

correctly integrate into the upper layer (Figs 5, 6). Furthermore, the majority of Dpy1911-knockdown cells in the IZ showed an abnormal rounded or multipolar morphology (Fig. 7; supplementary material Fig. S7). These results suggest that Dpy1911 is involved in dendrite formation in addition to regulating neuronal migration. However, we cannot exclude the possibility that the morphological defects in the IZ are a reflection of the effects of the extracellular environment.

Since Dpy1911 protein was localized in cortical axons (Fig. 2; supplementary material Fig. S3), it is possible that Dpy1911 regulates axonal growth and/or pathfinding. We compared the

axonal growth of cortical neurons in control and Dpy1911 shRNA-electroporated neurons at P2. Many axons of Dpy1911-knockdown neurons crossed the dorsal midline, similar to what is observed in control brains (supplementary material Fig. S9). Migrating neurons in the IZ started to extend their axons towards the dorsal midline in Dpy1911 knockdowns (data not shown). However, we cannot exclude the possibility that the Dpy1911 protein remaining after shRNA knockdown might still have a role in directing proper axonal projections. Additional complete loss-of-function studies using *Dpy1911* knockout mice will be required to clarify the role of *Dpy1911* in axonal projections.

Conclusions

The mammalian neocortex has been evolutionarily expanded to acquire higher functions. However, the developmental mechanisms specific to this region of the brain are poorly understood (Martinez-Cerdeño et al., 2006). Here, we identify *Dpy1911* as a gene expressed strongly in cortical glutamatergic neurons and as a regulator of radial neuronal migration. In mammals, the four members of the Dpy19 family are known to be associated with various diseases (Smith et al., 2009; Harbuz et al., 2011; Kosciński et al., 2011). Future study of Dpy19 family members might lead to further elucidation of the developmental mechanisms and functional organization of the mammalian neocortex.

Acknowledgements

We thank Dr Klaus-Armin Nave for generously providing *NEX-Cre* mice and Dr Marc Tessier-Lavigne for kindly providing *Dcc*-deficient mice; Dr Robert Hevner for the gift of anti-Tbr1 and anti-Tbr2 antibodies; Dr Jun-ichi Miyazaki for the pCAG-GS vector; the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa for monoclonal antibodies 4D7 (anti-TAG-1) and RC2; Drs Takeshi Kawauchi, Koji Oishi, Hitoshi Gotoh, Kenji Shimamura and Yohei Shinmyo for helpful comments; and Mr Noriyoshi Usui, Ms Mari Miyamoto, Dr Takashi Seki, Mr Shingo Usuki and members of Gene Technology Center at Kumamoto University for their contributions.

Funding

This work was supported by a Grant-in-Aid for Scientific Research [19700297 to K.W., 23590237 to H.T. and 20300121 to N.T.]; Scientific Research on Priority Area – Advanced Brain Science Project [18021030 to N.T.] from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan [to N.T.]; the Global Center of Excellence (COE) Program in Kumamoto University (Cell Fate Regulation Research and Education Unit); the Takeda Science Foundation [to K.W. and H.T.]; and the Naito Foundation [to K.W.]. Deposited in PMC for immediate release.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.068155/-/DC1>

References

- Anderson, S. A., Eisenstat, D. D., Shi, L. and Rubenstein, J. L. (1997). Interneuron migration from basal forebrain to cerebral cortex: dependence on *Dlx* genes. *Science* **278**, 474-476.
- Arlotta, P., Molyneaux, B. J., Chen, J., Inoue, J., Kominami, R. and Macklis, J. D. (2005). Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. *Neuron* **45**, 207-221.
- Asada, N., Sanada, K. and Fukada, Y. (2007). LKB1 regulates neuronal migration and neuronal differentiation in the developing neocortex through centrosomal positioning. *J. Neurosci.* **27**, 11769-11775.
- Bai, J., Ramos, R. L., Ackman, J. B., Thomas, A. M., Lee, R. V. and LoTurco, J. J. (2003). RNAi reveals doublecortin is required for radial migration in rat cerebral cortex. *Nat. Neurosci.* **6**, 1277-1283.
- Bishop, K. M., Goudreau, G. and O'Leary, D. D. M. (2000). Regulation of area identity in the mammalian cerebral cortex by *Emx2* and *Pax6*. *Science* **288**, 344-349.
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.
- Carson, A. R., Cheung, J. and Scherer, S. W. (2006). Duplication and relocation of the functional *DPY19L2* gene within low copy repeats. *BMC Genomics* **7**, 45.
- Chen, G., Sima, J., Jin, M., Zheng, W., Wang, K. Y., Xue, X. J., Ding, Y. Q. and Yuan, X. B. (2008). Semaphorin-3A guides radial migration of cortical neurons during development. *Nat. Neurosci.* **11**, 36-44.
- des Portes, V., Pinard, J. M., Billuart, P., Vinet, M. C., Koulakoff, A., Carrié, A., Gelot, A., Dupuis, E., Motte, J., Berwald-Netter, Y. et al. (1998). A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome. *Cell* **92**, 51-61.
- Englund, C., Fink, A., Lau, C., Pham, D., Daza, R. A., Bulfone, A., Kowalczyk, T. and Hevner, R. F. (2005). *Pax6*, *Tbr2*, and *Tbr1* are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing cerebral cortex. *J. Neurosci.* **25**, 247-251.
- Esumi, S., Wu, S. X., Yanagawa, Y., Obata, K., Sugimoto, Y. and Tamamaki, N. (2008). Method for single-cell microarray analysis and application to gene-expression profiling of GABAergic neuron progenitors. *Neurosci. Res.* **60**, 439-451.
- Gilmore, E. C., Ohshima, T., Goffinet, A. M., Kulkarni, A. B. and Herrup, K. (1998). Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex. *J. Neurosci.* **18**, 6370-6377.
- Gleeson, J. G., Allen, K. M., Fox, J. W., Lamperti, E. D., Berkovic, S., Scheffer, I., Cooper, E. C., Dobyns, W. B., Minnerath, S. R., Ross, M. E. and Walsh, C. A. (1998). Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. *Cell* **92**, 63-72.
- Gray, P. A., Fu, H., Luo, P., Zhao, Q., Yu, J., Ferrari, A., Tenzen, T., Yuk, D. I., Tsung, E. F., Cai, Z. et al. (2004). Mouse brain organization revealed through direct genome-scale TF expression analysis. *Science* **306**, 2255-2257.
- Guerrini, R. and Marin, C. (2006). Genetic malformations of cortical development. *Exp. Brain Res.* **173**, 322-333.
- Hamasaki, T., Leingartner, A., Ringstedt, T. and O'Leary, D. D. M. (2004). *EMX2* regulates sizes and positioning of the primary sensory and motor areas in cerebral cortex by direct specification of cortical progenitors. *Neuron* **43**, 359-372.
- Hand, R., Bortone, D., Mattar, P., Nguyen, L., Heng, J. I., Guerrier, S., Bouff, E., Peters, E., Barnes, A. P., Parras, C. et al. (2005). Phosphorylation of Neurogenin2 specifies the migration properties and the dendritic morphology of pyramidal neurons in the neocortex. *Neuron* **48**, 45-62.
- Harbuz, R., Zouari, R., Pierre, V., Ben Khelifa, M., Kharouf, M., Coutton, C., Merdassi, G., Abada, F., Escoffier, J., Nikas, Y. et al. (2011). A recurrent deletion of *DPY19L2* causes infertility in man by blocking sperm head elongation and acrosome formation. *Am. J. Hum. Genet.* **88**, 351-361.
- Hatten, M. E. (2002). New directions in neuronal migration. *Science* **297**, 1660-1663.
- Hirotsune, S., Fleck, M. W., Gambello, M. J., Bix, G. J., Chen, A., Clark, G. D., Ledbetter, D. H., McBain, C. J. and Wynshaw-Boris, A. (1998). Graded reduction of *Pafah1b1* (*Lis1*) activity results in neuronal migration defects and early embryonic lethality. *Nat. Genet.* **19**, 333-339.
- Honigberg, L. and Kenyon, C. (2000). Establishment of left/right asymmetry in neuroblast migration by *UNC-40/DCC*, *UNC-73/Trio* and *DPY19* proteins in *C. elegans*. *Development* **127**, 4655-4668.
- Kato, M. and Dobyns, W. B. (2003). Lissencephaly and the molecular basis of neuronal migration. *Hum. Mol. Genet.* **12**, R89-R96.
- Kawaguchi, A., Ikawa, T., Kasukawa, T., Ueda, H. R., Kurimoto, K., Saitou, M. and Matsuzaki, F. (2008). Single-cell gene profiling defines differential progenitor subclasses in mammalian neurogenesis. *Development* **135**, 3113-3124.
- Kawauchi, T., Sekine, K., Shikanai, M., Chihama, K., Tomita, K., Kubo, K., Nakajima, K., Nabeshima, Y. and Hoshino, M. (2010). Rab GTPases-dependent endocytic pathways regulate neuronal migration and maturation through N-cadherin trafficking. *Neuron* **67**, 588-602.
- Kosciński, I., Elinati, E., Fossard, C., Redin, C., Muller, J., Velez de la Calle, J., Schmitt, F., Ben Khelifa, M., Ray, P. F., Kilani, Z. et al. (2011). *DPY19L2* deletion as a major cause of globozoospermia. *Am. J. Hum. Genet.* **88**, 344-350.
- Kriegstein, A. R. and Noctor, S. C. (2004). Patterns of neuronal migration in the embryonic cortex. *Trends Neurosci.* **27**, 392-399.
- LoTurco, J. J. and Bai, J. (2006). The multipolar stage and disruptions in neuronal migration. *Trends Neurosci.* **29**, 407-413.
- Marin, O. and Rubenstein, J. L. (2003). Cell migration in the forebrain. *Annu. Rev. Neurosci.* **26**, 441-483.
- Martinez-Cerdeño, V., Noctor, S. C. and Kriegstein, A. R. (2006). The role of intermediate progenitor cells in the evolutionary expansion of the cerebral cortex. *Cereb. Cortex* **16 Suppl 1**, i152-i161.
- Miyata, T., Kawaguchi, A., Okano, H. and Ogawa, M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* **31**, 727-741.
- Nadarajah, B. and Parnavelas, J. G. (2002). Modes of neuronal migration in the developing cerebral cortex. *Nat. Rev. Neurosci.* **3**, 423-432.
- Nieto, M., Monuki, E. S., Tang, H., Imitola, J., Haubst, N., Khoury, S. J., Cunningham, J., Gotz, M. and Walsh, C. A. (2004). Expression of *Cux-1* and

- Cux-2 in the subventricular zone and upper layers II-IV of the cerebral cortex. *J. Comp. Neurol.* **479**, 168-180.
- Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S. and Kriegstein, A. R.** (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409**, 714-720.
- Preuss, T. M. and Kaas, J. H.** (1999). Human brain evolution. In *Fundamental Neuroscience* (ed. M. J. Zigmond, F. E. Bloom, S. C. Landis, J. L. Roberts and L. R. Squire), pp. 1283-1311. San Diego: Academic Press.
- Rakic, P.** (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. *J. Comp. Neurol.* **145**, 61-83.
- Reiner, O. and Sapiro, T.** (2009). Polarity regulation in migrating neurons in the cortex. *Mol. Neurobiol.* **40**, 1-14.
- Reiner, O., Carrozzo, R., Shen, Y., Wehnert, M., Faustinella, F., Dobyns, W. B., Caskey, C. T. and Ledbetter, D. H.** (1993). Isolation of a Miller-Dieker lissencephaly gene containing G protein beta-subunit-like repeats. *Nature* **364**, 717-721.
- Rice, D. S. and Curran, T.** (2001). Role of the reelin signaling pathway in central nervous system development. *Annu. Rev. Neurosci.* **24**, 1005-1039.
- Ross, M. E. and Walsh, C. A.** (2001). Human brain malformations and their lessons for neuronal migration. *Annu. Rev. Neurosci.* **24**, 1041-1070.
- Sapiro, T., Sapoznik, S., Levy, T., Finkelshtein, D., Shmueli, A., Timm, T., Mandelkow, E. M. and Reiner, O.** (2008). Accurate balance of the polarity kinase MARK2/Par-1 is required for proper cortical neuronal migration. *J. Neurosci.* **28**, 5710-5720.
- Schwab, M. H., Bartholomae, A., Heimrich, B., Feldmeyer, D., Druffel-Augustin, S., Goebbels, S., Naya, F. J., Zhao, S., Frotscher, M., Tsai, M. J. and Nave, K. A.** (2000). Neuronal basic helix-loop-helix proteins (NEX and BETA2/Neuro D) regulate terminal granule cell differentiation in the hippocampus. *J. Neurosci.* **20**, 3714-3724.
- Smith, E. N., Bloss, C. S., Badner, J. A., Barrett, T., Belmonte, P. L., Berrettini, W., Byerley, W., Coryell, W., Craig, D., Edenberg, H. J. et al.** (2009). Genome-wide association study of bipolar disorder in European American and African American individuals. *Mol. Psychiatry* **14**, 755-763.
- Sugiura, N., Patel, R. G. and Corriveau, R. A.** (2001). N-methyl-D-aspartate receptors regulate a group of transiently expressed genes in the developing brain. *J. Biol. Chem.* **27**, 14257-14263.
- Tabata, H. and Nakajima, K.** (2003). Multipolar migration: the third mode of radial neuronal migration in the developing cerebral cortex. *J. Neurosci.* **23**, 9996-10001.
- Tamamaki, N., Fujimori, K. E. and Takauji, R.** (1997). Origin and route of tangentially migrating neurons in the developing neocortical intermediate zone. *J. Neurosci.* **17**, 8313-8323.
- Tamamaki, N., Nakamura, K., Okamoto, K. and Kaneko, T.** (2001). Radial glia is a progenitor of neocortical neurons in the developing cerebral cortex. *Neurosci. Res.* **41**, 51-60.
- Tamamaki, N., Yanagawa, Y., Tomioka, R., Miyazaki, J., Obata, K. and Kaneko, T.** (2003). Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. *J. Comp. Neurol.* **467**, 60-79.
- Tsai, J. W., Chen, Y., Kriegstein, A. R. and Vallee, R. B.** (2005). LIS1 RNA interference blocks neural stem cell division, morphogenesis, and motility at multiple stages. *J. Cell Biol.* **170**, 935-945.
- Watanabe, K., Tamamaki, N., Furuta, T., Ackerman, S. L., Ikenaka, K. and Ono, K.** (2006). Dorsally derived netrin 1 provides an inhibitory cue and elaborates the 'waiting period' for primary sensory axons in the developing spinal cord. *Development* **133**, 1379-1387.
- Wu, S. X., Goebbels, S., Nakamura, K., Nakamura, K., Kometani, K., Minato, N., Kaneko, T., Nave, K. A. and Tamamaki, N.** (2005). Pyramidal neurons of upper cortical layers generated by NEX-positive progenitor cells in the subventricular zone. *Proc. Natl. Acad. Sci. USA* **102**, 17172-17177.

Table S1. Primers

Gene	GenBank accession	Primers (5' to 3')	Expected size (bp)
RT-PCR			
beta-actin	NM_007393	GCCAACCGTGAAAAGATGAC GCACTGTGTTGGCATAGAGG	556
<i>Dpy1911</i>	NM_172920	TGCATCATTATTGCCGTGT CAGTCACTGTCTGAGAACTT	1234
<i>Dpy1912</i>	NM_001166207	ACAAAACCTGGTTGCAGCAT CCTCTTTGCTCCACACAGAA	1012
<i>Dpy1913</i>	NM_178704	ATGGCTTTCTCACCAGTGCT GTGGCTCCGTGTATCAGGTT	934
<i>Dpy1914</i>	NM_001081201	CCCGAACTTTGGATGACACT CGCTACAGGGAAGCTTTACG	906
qPCR			
beta-actin	NM_007393	GACAGGATGCAGAAGGA TGATCCACATCTGCTGGAA	142
<i>Neurod6 (Nex)</i>	NM_009717	GGGGAGGTTATCAGTGCAT GCTTTAAACTGTAGGATGG	147
In situ hybridization			
<i>Dpy1911</i>	NM_172920	GCTGCTGCTAGCTTCCTTTG CAAGCAGGATCGCCCAAT	402
<i>Dpy1911</i>	NM_172920	TGCATCATTATTGCCGTGT CAGTCACTGTCTGAGAACTT	1234
<i>Dpy1913</i>	NM_178704	ATGGCTTTCTCACCAGTGCT GTGGCTCCGTGTATCAGGTT	934