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Integration of neuronal clones in the radial cortical columns by EphA/ephrin-A signaling

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The cerebral cortex is a laminated sheet of neurons composed of the arrays of intersecting radial columns^{1,2,3}. During development, excitatory projection neurons originating from the proliferative units at the ventricular surface of the embryonic cerebral vesicles migrate along elongated radial glial fibers⁴ to form a cellular infrastructure of radial (vertical) ontogenetic columns in the overlying cortical plate (CP)⁵. However, a subpopulation of these clonally related neurons also undergoes a short lateral shift and transfers from their parental to the neighboring radial glial fibers⁶, and intermixes with neurons originating from neighboring proliferative units^{5,7}. This columnar organization serves as the primary information processing unit in the cortex^{1,8,9}. The molecular mechanisms, role and significance of this lateral dispersion for cortical development are not understood. Here we provide the evidence that EphA/ephrin-A signalling-dependent shift in allocation of clonally related neurons is essential for the proper assembly of cortical columns. In contrast to the relatively uniform labelling of the developing CP by various molecular markers and retrograde tracers in wild-type mice, we found alternating labelling of columnar compartments in *ephrin-A* knockout mice that are caused by impaired lateral dispersion of migrating neurons rather than altered cell production or death. Furthermore, *in utero* electroporation revealed that lateral dispersion depends on the expression levels of EphAs and ephrin-As during neuronal migration. This heretofore unrecognized mechanism for lateral neuronal dispersion appears to be essential for the proper intermixing of neuronal types in the cortical columns, which, when disrupted, might contribute to neuropsychiatric disorders associated with abnormal columnar organization^{8,10}.

Functional columns in the neocortex emerge developmentally from a proliferative zone protomap, and consist of multiple classes of interconnected neurons^{1,11,12} defined anatomically and physiologically. In the present study, we have explored possible roles of A-type Eph receptors and their ligands, ephrin-As, in the integration of neurons in radial columns because they have been implicated in controlling cell positioning in a variety of developmental contexts by either sorting cell types^{13,14,15}, restricting their intermingling¹⁶, or regulating their migration¹⁷. First, to determine their expression during the period when the majority of postmitotic neurons migrate radially to the CP, sections were incubated with human Fc-tagged ephrin-A and EphA proteins that bind to all EphAs and ephrin-As, respectively. We observed

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Author Contributions

M.T. initiated the project, conducted experiments, analyzed the data, and wrote the manuscript. K.H.T. conducted experiments, analyzed the data, and helped to write the manuscript. P.L. and P.R. contributed to the interpretation of results and writing of the manuscript.

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an elaborate but stereotypical binding pattern of both receptors and ligands across the thickness of the neocortex with a peak expression in the intermediate zone (IZ), where they exhibited opposing gradients (Supplementary Fig. 1). In fact, multiple *EphA* receptors and *ephrin-A* ligands were detected in the subventricular zone (SVZ) and/or IZ through which neurons originated in the ventricular zone (VZ) migrate to the CP (Supplementary Fig. 1). To examine the roles of *EphA*s/*ephrin-A*s in neuronal migration in the neocortex, we analyzed the triple knockout (TKO) mouse¹⁸ for *ephrin-A2*, *A3*, and *A5* that account for almost all *ephrin-A*s in the developing neocortex¹⁹. To minimize the effects of mistargeted afferent and efferent projections^{19,20,21}, we performed most analyses at postnatal day 0 (P0) or embryonic stages before the establishment of these projections. It has been demonstrated recently that *EphA*/*ephrin-A* signaling has a proapoptotic effect in the embryonic cortex without affecting the proliferation or cell cycle progression²². Consistently, proliferation and cell cycle progression were not affected in the TKO cortex (data not shown). In addition, similar to the *EphA7* knockout mice²², less than 10% of the 52 embryos showed an exencephalic phenotype at E14.5, which is likely caused by decreased apoptosis of neural progenitors²². These animals were excluded from our analyses. The remaining TKO embryos did not exhibit any difference in the number of apoptotic cells in the cortex, gross brain shape, brain mass, cortical surface size and cell numbers in a cortical hemisphere compared to wild-type embryos (Supplementary Fig.2).

The overall patterning of cortical areas at P0, revealed by *in situ* hybridization, appeared unaffected in TKO (Supplementary Fig.2, 3). However, more detailed analysis revealed spotted, irregular distribution of cells labeled by these markers, and alternating thickening and thinning of the CP in TKO mice (Supplementary Fig. 3). Analysis of the wild-type ($n \geq 7$) and TKO ($n \geq 9$) brains showed highly uneven distribution of *Ctip2*⁺ neurons in layer 5 along the tangential axis in the TKO, although their radial positioning was unaffected (Fig. 1a). Most domains with an excess of *Ctip2*⁺ neurons were found adjacent to domains with reduced *Ctip2*⁺ neurons (Fig. 1b, c). Similarly, labeling for *Sox5* and *Cutl1*, markers of neurons in layers 5–6 and 2–4, respectively, showed normal radial but irregular tangential distribution of labeled neurons in the TKO (Fig. 1d, e). Importantly, double labeling with *Ctip2* and *Cutl1*, and *Ctip2* and *Lmo4* (which is expressed in neurons of layers 2–6), revealed that the irregular tangential distribution of marker-positive and -negative neuronal subtypes has a certain independency among cortical layers (Fig. 1e, f). Labeling of cell nuclei exhibited uneven CP thickness and cell packing among neighboring columnar domains (Fig. 1f, Supplementary Fig. 4). However, their increase and decrease, and those of marker-positive neurons, were not parallel, so that the thicker cortical domains with higher cell density often contained less *Lmo4*⁺ neurons in the upper cortical layers (Fig. 1f, compare *Lmo4* and nuclei labeling). To gain insight into the functional organization of the TKO neocortex, corticotectal projection neurons (CTPN) and callosal projection neurons (CPN) were retrogradely labeled at P2 and observed at P4 ($n = 6$ per genotype). Whereas layer distribution of labeled neurons was normal, their numbers were highly variable along the tangential axis in the TKO neocortex (Fig. 1g). Tangential axis-specific disruption of cortical organization was further confirmed at P14 by combining *Cutl1* immunohistochemistry with 5-bromodeoxyuridine (BrdU) injection made at E14.5 (Supplementary Fig. 4). In addition, no apparent defect was observed in the organization of the marginal zone and pial surface, Reelin expression in Cajal-Retzius cells, and the organization of radial glial fibers in the TKO neocortex at P0 (Supplementary Fig. 5).

To test the possibility that neurons in TKO mice might be allocated into unusual cortical columns, we examined the positional relationship among the clonally related CP neurons by infecting precursors with a Green Fluorescent Protein (GFP)-retrovirus at E12.5 and analyzing the location of GFP⁺ neurons at P0. The results show that the width of ontogenetic columns of GFP⁺ clones in TKO was smaller (Fig. 2a, $n = 7$) than that in the wild-type neocortex (Fig. 2a, $n = 10$). Furthermore, in the majority of the TKO CP ($n = 5/7$), the labeled neurons were

aligned in straight columns with one to two cell diameters, which was never encountered in the wild-type CP (Fig. 2b). This straight alignment of neurons was not due to a crowding effect of increased cell packing density (Supplementary Fig. 6). Because lateral movement of radially migrating neurons occurs within the SVZ and IZ during “the multipolar stage”²³, we measured the lateral distance between migrating neurons and the parental radial fibers at the top of the IZ at E14.5 in the neocortex infected with GFP retrovirus at E12.5. The distance was significantly less in TKO than that in wild-type (Fig. 2c), indicating that EphA/ephrin-A signaling is required for lateral dispersion/intermingling of neuronal clones during migration. To address the mechanism of lateral dispersion, we performed cell ablation experiments, in which the diphtheria toxin A-chain fragment (DTA) and an Enhanced Yellow Fluorescent Protein (EYFP) reporter plasmids were electroporated into the cortex at E13.5, and the effects on the migrating neurons and upper layer neurons were examined at E15.5 and at P0. As DTA causes inhibition of protein synthesis followed by apoptotic cell death²⁴, we observed death of infected progenitor cells in the VZ and, importantly, also of the migrating neurons in the IZ, before they arrive in the CP (Fig. 2d, see also Methods in detail). With this system, we found that, despite significant numbers of dying cells in the DTA-electroporated domains, surviving NeuroD⁺ young neurons²⁵ in the lower IZ were distributed continuously along the tangential axis of the cortex in the wild-type neocortex at E15.5, irrespective of the size of electroporated areas (Fig. 2d, e, $n = 12$). In contrast, most electroporated TKO cortices showed interrupted distribution of NeuroD⁺ neurons (Fig. 2d, $n = 8/9$), even in the brains that have very limited electroporated areas (Fig. 2e). Consistent with these observations, Cutl1-labeled upper layers was apparently thinner in the TKO cortex at P0, specifically within the domains of DTA electroporation (Fig. 2f). Finally, we observed no difference in proliferation and neuronal production in the progenitors in the electroporated domains between wild-type and TKO (data not shown). These results demonstrate that the developing neocortex possesses a striking capacity to fill in the vacated space of ablated neurons with neighbouring migrating neurons, but that this capacity is lost in the TKO neocortex.

To further examine the role of EphA/ephrin-A signaling in lateral dispersion of neocortical neurons, we co-electroporated either a control or an EphA7 expression plasmid with an EYFP reporter plasmid at E12.5, and examined the distribution of EYFP labeled neurons at E18.5. In contrast to the random distribution of EYFP⁺ neurons in control brains (Fig. 3a, $n \geq 20$), EphA7 overexpressed neurons formed striking multiple EYFP⁺ columns (Fig. 3a, $n \geq 20$) in all cortical regions examined (Supplementary Fig. 7). Introducing another EphA receptor, EphA4, generated the same phenotype (data not shown). The exaggerated columnar pattern of EYFP⁺ neurons appears one day after EphA7 electroporation, and continues during embryonic and postnatal stages (Fig. 3a, Supplementary Fig. 7, $n = 7$ [E13.5], $n \geq 20$ [P4]). Non-labeled neurons situated between EYFP⁺ columns had a similar cell packing density (Fig. 3a, Supplementary Fig. 7 and 8) and the overall cytoarchitecture in the CP was unaffected as previously reported²¹. Organization of apical dendrites and formation of radial glial fibers also were not affected grossly by EphA7 overexpression (Supplementary Fig. 8). TUNEL and BrdU labeling at E18.5 following EphA7 electroporation showed no difference between EYFP⁺ and EYFP⁻ domains (Supplementary Fig. 8). Furthermore, the cell cycle index and cell death rate displayed no change in the EphA7 electroporated neocortex (Supplementary Fig. 8). Finally, sequential electroporation with the EphA7 expression plasmid, with the EYFP (first) and DsRed2 (second) marker plasmids, demonstrated that the isolated columns of EphA7 electroporated neurons consist of multiple clones (Supplementary Fig. 7). EphA7 electroporation into the TKO cortex did not form the columnar segregation (Supplementary Fig. 8). Together, these results indicate that EYFP⁺ columns are not formed by increased production or death of a subset of EYFP⁺ clones, but rather, by tangential segregation of EYFP⁺ and EYFP⁻ neurons.

Since EphAs and ephrin-As can serve as both receptors and ligands to mediate forward and reverse signaling²⁶ and both are required for cell sorting^{13,15}, we electroporated ephrin-A2, A5, and the truncated forms of EphA7 and EphA4, together with the EYFP plasmid to determine which signaling is involved in columnar segregation. We found segregation of EYFP⁺ and EYFP⁻ neurons, but no effect on cell cycle progression and cell death, in the ephrin-A2 and A5 electroporated cortex (Supplementary Fig. 9, and data not shown). However, electroporation with the truncated EphAs, which can induce only reverse signaling as ligands, failed to induce segregation (Supplementary Fig. 9). These results indicate that forward signaling is necessary for tangential segregation, although we cannot rule out completely that reverse signaling might also be involved cooperatively.

To determine the mechanism of columnar segregation during radial migration, we analyzed the E15.5 neocortex electroporated at E12.5. No difference was observed in the random probability of gene transfer among progenitors in the VZ between control ($n = 16$) and EphA7 electroporated brains ($n = 23$) (Fig. 3b, c). In both groups, EYFP⁺ cells in the VZ and CP have radially elongated shapes without any evidence of tangential movement (Fig. 3a, b). In the IZ of all cases, most of the EYFP⁺ migrating neurons assumed a multipolar shape. They were more randomly distributed than EYFP⁺ progenitors in the VZ in controls (Fig. 3b, c), whereas EphA7 electroporated migrating neurons exhibited distinct segregation from EYFP⁻ neurons in the IZ, forming EYFP⁺ clusters (Fig. 3b, c). Live imaging observation confirmed that this segregation is mediated by tangential movement of neurons during the multipolar stage (Supplementary Movie 1, 2). These results indicate that neurons segregate from each other due to different levels of EphA/ephrin-A signaling that they receive during the multipolar phase of migration. Columnar grouping of EphA7-overexpressing neurons of multiclonal origins, through extensive tangential movement, exhibits the opposite phenotype to that observed in TKO, in which tangential dispersion of cortical neurons is impaired and columnar arrays of clonally related neurons are formed (Fig. 2a, b). Taken together, our analyses indicate that EphA/ephrin-A signaling regulates lateral (tangential) neuronal dispersion and intermingling during the multipolar stage of radial migration, and that this mechanism is required to generate cortical columns with appropriate cellular, molecular and anatomical components (see also Supplementary Discussion). Given these results, what is its biological significance? It is well established that progenitor cells have heterogeneous molecular properties, proliferative capacity and lineage commitment^{27,28,29}. We found that in wild-type, but not in TKO, an adjustment of neuron numbers, and an appropriate intermixing of neuronal subtypes can be achieved by lateral dispersion/intermixing of migrating neurons (Supplementary Fig. 10). These adaptive processes may be essential to achieve the modular function of cortical columns^{1,8,12}. Another possibility is that, given the preferential development of specific microcircuits within radially arrayed ontogenetic clones⁹, appropriate radial and tangential distribution of clonally related neurons may be essential for proper synaptic development and function of the radial units. The new role of EphA/ephrin-A signaling in assuring the homogeneous and continuous aspects of the neocortex demonstrated here contrasts with its previously known roles in restriction of cell movement and inhibiting cell intermingling during formation of boundaries in other systems^{13,15,16}. Although inappropriate neuronal positioning and abnormal columnar organization has been reported in post-mortem analysis of cortical tissue from subjects with psychiatric disorders, the molecular mechanisms of these abnormalities remain unknown. Our finding provides a novel perspective for investigating typical and atypical development that underlies higher order information processing in normal and pathophysiological states.

Methods Summary

Generation/genotyping of *ephrin-A* TKO was performed as described¹⁸. Wild-type C57/Bl6 and CD-1 mice were used as controls. Histology, immunohistochemistry and *in situ*

hybridization. were performed on cryosections or vibratome sections as described previously²¹. Radial glial cells were labeled by injecting saturated DiI (1,1'-dioctadecyl- 3,3,3', 3'-tetramethylindocarbocyanine perchlorate, Invitrogen) solution into the lateral ventricle of fixed P0 brains. CTPN and CPN were retrogradely labeled by injection of FluoroGold (Invitrogen) into the superior colliculus and red fluorescent microspheres (FluoSperes, Invitrogen) into the contralateral parietal associative/visual cortex, respectively. GFP retrovirus (a gift from F.H. Gage) was injected *in utero* into the lateral ventricle of the embryo at E12.5. The distance between migrating neurons and their parental radial glia at E14.5 was quantified in z-stacked confocal images using LSM Image Browser. *In utero* electroporation was performed at E12.5 – E15.5. Each EphA and ephrin-A expression plasmid was injected with pCAG-EYFP or pCAGGS-DsRed2. DTA expression plasmid was injected with pCAG-EYFP. The cortical area of each hemisphere was quantified using ImageJ software. Total numbers of cortical cells were estimated using the Isotropic Fractionator method. The thickness of Cutl1⁺ cortical layers was measured using ImageJ on coronal sections at the center of electroporated domains and the corresponding region in the contralateral hemisphere, and the ratios were calculated. Pregnant mice (E13.5) were labeled with BrdU for 30 min and 24 h, respectively, and labeling index (proportion of BrdU⁺ cells among Ki67⁺ cells) and cell cycle exit index (proportion of Ki67[−] cells among BrdU⁺ cells) were calculated. Apoptotic cell death was analyzed by counting TUNEL⁺ cells in 10,000 μm^2 in the neocortex. For quantification of the pixel intensity along the tangential axis of the neocortex, the regions were delimited from medial to lateral in the neocortex, straightened with ImageJ, and the plot profiles were made.

Methods

Animals

All experiments using animals were in accordance with the protocols approved by Yale University Institutional Animal Care and Use Committee. The *Ephrin-A2/A3/A5* triple knockout mouse was generated and genotyped as previously described^{18,30,31,32}. Wild-type mice were from the C57/Bl6 strain (Jackson). Wild-type CD-1 mice (Charles River) were also used in EphA/ephrin-A overexpression analysis. The day on which a vaginal plug was observed was designated as embryonic day 0.5.

In situ hybridization

In situ hybridization cryosections or whole-mount was performed as described^{33,34}. The probes for *EphA5*, *EphA7*, *ephrin-A5* and *cadherin-8* are described previously²¹. The probes for *Id2*, *Lmo3* and *Lmo4* are as described³⁵ (gifts from J.L.R. Rubenstein). The probes for *EphA4* (1014 bp mouse partial clone), *ephrin-A2* (mouse full-length clone) and *ephrin-A3* (~200 bp rat partial clone) were gifts from L. F. Kromer.

Histology and immunohistochemistry

Brains were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline overnight, and 60–70 μm coronal vibratome slices or 20 μm coronal cryosections were collected. Immunohistochemistry was performed as described previously²¹. The following primary antibodies were used: polyclonal anti-GFP (also recognize EYFP, 1:3000; Invitrogen, 1:250; Abcam), anti-DsRed (1:5000; BD Biosciences), anti-Cutl1 (1:500; Santa Cruz Biotechnology), anti-Sox5 (1:5000; Aviva Systems Biology), anti-Lmo4 (1:4000; Abcam), anti-Laminin (1:1000; Sigma-Aldrich), anti-NeuroD (1:300; Santa Cruz Biotechnology), and monoclonal anti-Ctip2 (1:700; Abcam), anti-Ki67 (1:100; Neomarker), anti-BrdU (1:100 Beckton and Dickinson), anti-RC2 (1:5; DSHB), anti-Reelin (1:1000; Millipore), anti-MAP2 (1:200; Sigma-Aldrich) antibodies. Sections were nuclear counterstained with TO-PRO3 or 4'-6-diamidino-2-phenylindole (DAPI, Invitrogen) when necessary. Cresyl violet staining was

performed following a standard protocol. All images were captured using a confocal LSM 510 NLO system or an Axioplan2 microscope (Carl Zeiss) equipped with epifluorescence.

Ligand binding and receptor binding histochemistry

Ligand and receptor binding histochemistry on were performed as described²¹ using recombinant human ephrin-A5/Fc and EphA7/Fc chimeric protein (R&D Systems), respectively.

Dil, Fluorogold and fluorescent microsphere labeling

To label radial glial cells and their endfeet, saturated DiI solution (in ethanol) was injected into the lateral ventricle of P0 wild-type and TKO brains fixed with 4% PFA. After 48 hr of incubation at 37 °C, coronal vibratome slices were made at 100 µm thickness and photographed. CTPN and CPN in visual cortex were retrogradely labeled by pressure-injection of Fluorogold (Invitrogen) into the superior colliculus and red fluorescent microspheres (FluoSperes, Invitrogen) into the contralateral parietal associative/visual cortex, respectively, of P2 pups (to minimize the effect of activity-dependent reorganization). Coronal cryosections were made at P4 and images were captured using an Axioplan2 microscope (Carl Zeiss).

In utero retrovirus injection analysis

GFP-retrovirus (a gift from F.H. Gage) was produced and injected *in utero* into E12.5 embryos as previously described³⁶. Brains were collected at E14.5 or P0, fixed in 4% PFA for overnight and thick vibratome sections (70 µm) were made for GFP immunohistochemistry. To quantify the distance between migrating neurons and their parental radial glia, z-stack images for each slice were taken using a confocal LSM 510 NLO system (Carl Zeiss), and the distance was measured using LSM Image Browser (Carl Zeiss). The investigator was blind to the genotype for analysis.

Expression plasmids for electroporation

EphA7 expression plasmid (pCAGGS-EphA7-IRES-Venus), control plasmid (pCAGGS-IRES-EGFP), and pCAGGS-DsRed2 were described previously²¹. pCAG-EYFP and pCAG-DTA were provided by T. Saito³⁷ and K. Kohno³⁸, respectively. Full length cDNA of mouse EphA4 (a gift from N.Y. Ip), ephrin-A2, and ephrin-A5 (gifts from L.F. Kromer) were inserted into pCAGGS-IRES-EGFP as their expression plasmids. A truncated form of EphA4 (EphA4-T) lacking its intracellular domain (at Val⁵⁹⁷) was designed as previously described³⁹, and EphA7 truncated form (EphA7-T) was similarly designed (truncated at Ile⁶⁰⁹). cDNA fragments were amplified by Polymerase Chain Reactions using full-length expression plasmids as templates, and the following primers: EphA4-T 5'-AGGAGCAGCGTTGGCACC-3' (forward) and 5'-CACCTAAGTTCTAACACCTTGATT-3' (reverse), EphA7-T 5'-CCATGGTTGTTCAAACCTCGGTA-3' (forward), and 5'-AATCTAGGTTTTGGTGCCTGGA-3' (reverse), and the fragments were inserted into pCAGGS-IRES-EGFP.

In utero electroporation

In utero electroporation was performed as previously described³⁷ at E12.5 – E15.5. Each EphA and ephrin-A expression plasmid (2 mg/ml) was injected with pCAG-EYFP (0.5 mg/ml) or pCAGGS-DsRed2 (1 mg/ml). In some cases, BrdU (50 mg/kg body weight) was intraperitoneally injected immediately or 24 hrs after surgery for cell proliferation and cell cycle exit analyses as described below. DTA expression plasmid (1 mg/ml) was injected with pCAG-EYFP (2 mg/ml). We observed a massive increase of TUNEL⁺ cells in the electroporated domains of both wild-type and TKO within 24 hr (*n* = 3 and 2, respectively,

data not shown). This striking increase of dying cells was restricted to the VZ and IZ, but was not present in the CP at either E14.5 (data not shown) or E15.5 (Fig.2d, $n = 6$ [wild-type] and 4 [TKO]). However, the increase of cell death was no longer evident by P0 (data not shown). Whole brain images were captured using Stemi SV 11 Apo stereomicroscope (Carl Zeiss) equipped with fluorescent source.

Organotypic slices and time-lapse imaging

Cortical slices were prepared as previously described⁴⁰ from the E15.5 embryos electroporated at E13.5 ($n = 6$ each for control- and EphA7-electroporation). The slices were transferred to a RC25 recording chamber (Warner Instruments) maintained at 36°C and perfused with fresh culture media. Time-lapse imaging of EYFP-labeled cells around the VZ-IZ was performed following the previously described procedure⁴¹. Time series were collected every 15 min for ~15 h per slice through 20× objective on a confocal LSM 510 NLO system fitted with a motorized stage, and edited with Adobe Premier Pro.

Quantitative analyses of cortical size, Cutl1⁺ layer thickness, cell proliferation, cell cycle exit and apoptotic cell death

Investigator was blind to the genotype or electroporated gene. The cortical area of each hemisphere was quantified using ImageJ from images of the whole brain taken as dorsal view using Stemi SV 11 Apo stereomicroscope (Carl Zeiss), and averaged for wild-type and TKO. Total brain mass was weighed after fixation in 4% PFA overnight, and averaged for each genotype. The thickness of Cutl1⁺ cortical layers was measured using ImageJ on coronal sections at the center of electroporated domains and the relative thickness was calculated against the corresponding region in the non-electroporated hemisphere, and averaged for each genotype. A few sections that showed an apparent TKO phenotype of abnormal cortical thickness around the domain of measurement on either hemisphere were excluded from quantification. For proliferation and cell cycle exit analyses, pregnant mice were injected intraperitoneally with BrdU (50 mg/kg body weight) at E13.5, and embryos were collected after 30 min and 24 h, respectively. Brain sections were immunostained for Ki67 and BrdU, and BrdU labeling index (proportion of BrdU⁺ cells among Ki67⁺ cells) and cell cycle exit index (proportion of Ki67⁻ cells among BrdU⁺ cells) were calculated from the counted cell numbers as previously described⁴². For quantification of apoptotic cell death, TUNEL assay was performed using ApopTag *in situ* apoptosis detection kit (Millipore) following the manufacture's protocol, and TUNEL⁺ cells in 10,000 μm^2 was calculated from counted cell numbers throughout the neocortex.

Quantification of the fluorescent pixel intensity along the tangential axis of the neocortex

The fluorescent pixel intensities of EphA7/ephrin-A5-Fc binding histochemistry in the IZ (Supplementary Fig. 1), Ctip2 immunohistochemistry in the layer 5 (Fig. 1), and EYFP⁺ electroporated cells in the VZ and IZ (Fig. 3), along the medial-lateral axis of the neocortex, were quantified using a previously described method⁴³ with modification. Regions for quantification were delimited from medial to lateral in the neocortex, straightened with ImageJ software ('straighten' plugin), and the plot profiles of the fluorescent intensity was made as gray values (y) against the tangential distance (x). To present the distribution of EphA/ephrin-A-Fc binding and EYFP⁺ electroporated cells, the pixel intensity was normalized and expressed as a percentage to the total value measured inside the region of interest. Pixel intensity of the Ctip2 labeling was not normalized between the wild-type and TKO cortex for comparison of their total values. Tangential distance was subgrouped into ~30 bins for better presentation for EphA/ephrin-A-Fc binding and Ctip2 labeling.

Isotropic Fractionator

Because of the highly anisotropic cerebral cortex of TKO, total numbers of cells in a cortical hemisphere were estimated using the Isotropic Fractionator method as described⁴⁴ instead of using stereological method. Briefly, a cortical hemisphere was dissected from each wild-type and TKO brain at P0 and a suspension of nuclei was obtained through mechanical dissociation in 40 mM sodium citrate and 1% Triton X-100 using a tissue homogenizer. The homogenate was collected by centrifugation and suspended in PBS containing 1% DAPI, and the nuclear density was determined using a hemocytometer under fluorescence microscope to estimate the total cell number in the original tissue. For each genotype, 6 pups from 3 dams were used. The coefficients of variation (CV) were < 0.1 for both wild-type and TKO, indicating the reliability of the method. The investigator was blind to the genotype for analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Abnormal tangential organization of the cortical plate in the TKO neocortex. a, CTIP2 immunohistochemistry on wild-type (WT) and TKO brains. b, Higher magnification images of the boxed area in a. The arrow and arrowhead indicate the domains where abnormally more and less neurons are labelled, respectively. c, Quantification of the grey values of fluorescent CTIP2 labelling from the left panels in a along the medial-lateral axis (yellow arrows). Total labelling was similar in wild-type and TKO (39,361 and 39,036 in pixels, respectively). d, Irregular tangential distribution of Sox51 neurons, associated with abnormal thickening and thinning of the cortical plate of TKO (arrow and arrowheads). e, Cutl1 and CTIP2 labelling shows an increase and decrease of labelled neuronal subpopulations (white arrows and

arrowhead, respectively) in adjacent cortical domains in TKO. The increase and decrease was not always synchronized (yellow arrowhead). f, Lmo4 and CTIP2 staining shows distinct patterns of increased/decreased labelled neurons in TKO (arrow and arrowheads). g, The numbers of labelled CTPN and CPN were both highly variable among neighbouring columnar domains in TKO (arrow and arrowhead). Scale bars, 500 μ m (a) and 200 μ m (b–g).

Figure 2.

Impaired lateral dispersion of cortical neurons in TKO. **a**, GFP-labelled (green) clonal neurons. The column width of the clonal neurons (brackets) was smaller in TKO. Nuclei were counterstained (blue). **b**, Magnified view of clonally related neurons. The right panel for wild-type shows the most extreme columnar case in wild-type. In the TKO cortex, straight alignments of GFP-labelled neurons (between arrows) were found. **c**, Left: representative image of GFP-labelled neurons along their parental radial glial fibre (RGF; arrowheads). CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone. Middle: box-plot of the distance between labelled migrating neurons and the parental RGF. The box represents the median and the 25th and 75th percentiles. The top and bottom whiskers denote the 5th and 95th percentiles.

Plus signs denote the mean distances. The distance was significantly shorter in TKO. P50.0014 (Mann–Whitney U test, n532 from 14 TKO brains, and 21 from 10 wild-type brains). Right: cumulative percentage plot from the same data. P,0.001 (Kolmogorov–Smirnov test). d, Discontinuity of the band of NeuroD-labelled migrating neurons in the TKO cortex (arrowheads) by DTA electroporation. Similar levels of apoptotic cell death were observed in wild-type and TKO cortices as shown with adjacent slices. Note that a few surviving EYFP1 neurons can demarcate the electroporated fields (between dotted lines). Fibrous labelling in the upper intermediate zone in the NeuroD staining of TKO is a nonspecific background. e, Interruption of the NeuroD-labelled domain (arrows) in the TKO cortex, which received DTA electroporation only in a very narrow region. f, The thickness of Cutl11 cortical layers at the DTA electroporated domains (brackets) was decreased in TKO. Relative thickness of Cutl11 layers at the electroporated domains against the control hemisphere was significantly decreased in TKO. Data are mean $\pm$ s.e.m. (n520 sections from five brains per genotype), P50.0006 (Student's t-test). Scale bars, 100 μ m (a), 50 μ m (b, c) and 200 μ m (d–f).

Figure 3.

EphA overexpression leads to tangential sorting of cortical neurons. a, Distribution of EYFP-labelled neurons (green or black) during development in the cortex in which control or EphA7 expression plasmid was delivered using electroporation (EP) with EYFP plasmid. Scale bars, 600 mm (for E18.8), 50 mm (for E15.5) and 200 mm (for P4). b, E15.5 cortex electroporated with control or EphA7 expression plasmid at E12.5. Right panels are higher magnification views around the ventricular-intermediate zone, showing multipolar shaped migrating neurons. EYFP1 EphA7- electroporated neurons start to segregate from EYFP2 neurons in the intermediate zone (arrowheads). Scale bars, 100 mm (left panels) and 50 mm (right panels). c, Fluorescence grey values were quantified from the images in b from left to right (medial to

lateral in the cortex) in the ventricular zone and the intermediate zone as indicated by blue and red arrows in b. Plot profiles indicate the clustering of labelled EphA7-electroporated neurons in the intermediate zone (arrowheads in b and c).