Activated retinoid receptors are required for the migration and fate maintenance of subsets of cortical neurons

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ABSTRACT
Layer-specific cortical neurons are essential components of local, intracortical and subcortical circuits and are specified by complex signaling pathways acting on cortical progenitors. However, whether extrinsic signals contribute to postmitotic cortical neuronal development is unclear. Here we show in mice that retinoic acid (RA) receptors are activated in newly born migrating cortical neurons indicative of endogenous RA in the cortex. Disruption of RA signaling in postmitotic neurons by dominant-negative retinoid receptor RAR403 expression specifically delays late-born cortical neuron migration in vivo. Moreover, prospective layer V-III neurons that express RAR403 fail to maintain their fates and instead acquire characteristics of layer II neurons. This latter phenotype is rescued by active forms of β-catenin at central and caudal but not rostral cortical regions. Taken together, these observations suggest that RA signaling pathways operate postmitotically to regulate the onset of radial migration and to consolidate regional differences in cortical neuronal identity.

KEY WORDS: Retinoid receptor, Radial migration, Laminar identity, Mouse

INTRODUCTION
The nervous system comprises diverse groups of neurons that are generated under tight spatial and temporal control to ensure that they are formed in the right numbers, migrate to their appropriate settling positions and make appropriate connections with relevant pre- and postsynaptic partners (Marin and Rubenstein, 2003; Molyneaux et al., 2007). Several extrinsic signaling molecules play key roles in many of these fundamental events. In particular, Shh, BMPs, Notch, Wnts and retinoic acid (RA) signals integrate to pattern progenitor cells, regulate their proliferation and control the progress of neuronal differentiation (Chenn and Walsh, 2002; Corbin et al., 2008; Ericson et al., 1998; Fuccillo et al., 2006; Maden, 2007). Studies in the developing spinal cord suggest that neuronal fates remain plastic in postmitotic motoneurons and that active signaling mechanisms operate to consolidate and refine terminal neuronal identities (Sockanathan and Jessell, 1998; Sockanathan et al., 2003). However, it is not clear if such regulation of terminal neuronal identity applies to neurons in other regions of the nervous system and if so, the types of signaling systems that are involved.

The cortex contains six major layers that consist of specialized groups of neurons that are molecularly distinct, possess characteristic dendritic fields, and exhibit unique axonal projection patterns (Molyneaux et al., 2007; Rakic, 2007). Layer-specific cortical neurons are born in broad sequential waves from progenitor cells within the ventricular zone (VZ) and subventricular zone (SVZ), with deep-layer neurons born first followed by neurons that occupy progressively superficial positions (Molyneaux et al., 2007). Once born, neurons undergo a morphological transition from multipolar to unipolar/bipolar states, in which a leading process precedes the radial migration of the cell body to its final laminar settling position (Noctor et al., 2004; Rakic, 1972; Tabata and Nakajima, 2003). Early-born neurons migrate short distances by somal translocation, in which neurons extend a long basal process followed by nucleokinesis and detachment from the VZ (Nadarajah and Parnavelas, 2002). By contrast, late-born neurons actively migrate for longer distances along radial glial processes (Nadarajah and Parnavelas, 2002; Rakic, 1972). These distinct migratory modes suggest that separate mechanisms might regulate the migration of deep versus superficial neurons; however, the signals that regulate these events remain unclear.

Cortical neurons acquire their correct identities in the absence of appropriate cell body migration (Rice and Curran, 2001), implying that neuronal fate specification and radial migration are independent and thus controlled by different mechanisms. Neuronal fate is determined by the timing of progenitor cell cycle exit, and the competence of progenitors to generate deep-layer neurons decreases over time (Luskin et al., 1988; McConnell and Kaznowski, 1991; Desai and McConnell, 2000; Frantz and McConnell, 1996). In vitro studies reveal that cortical neurons differentiate in an ordered fashion, with deep-layer neurons born first followed by more superficial ones, consistent with intrinsic clock-like mechanisms of differentiation (Gaspard et al., 2008; Qian et al., 2000; Shen et al., 2006); however, extrinsic factors can influence the time of cell cycle exit and are thus important regulators of cortical neuronal diversity (Mizutani and Saito, 2005; Rodriguez et al., 2012). Nevertheless, the control of progenitor cell cycle exit is unlikely to be the sole factor that defines the fates of cortical neurons. Recent studies suggest that superficial fates are already programmed in subsets of early progenitors at the time of deep layer neuronal differentiation (Franco et al., 2012), and that layer IV neurons retain a degree of plasticity (De la Rossa et al., 2013). These observations raise the possibility that cortical neuronal fates require consolidation after cell cycle exit, potentially by extrinsic signals that refine terminal postmitotic neuronal identity, connectivity and function. Although the identities of such signals are not known, one potential candidate is RA, which regulates multiple aspects of neuronal development, including morphology, migration and fate (Maden, 2007; Fu et al., 2010; Corcoran et al., 2002; Sockanathan and Jessell, 1998; Sockanathan et al., 2003; Zhuang et al., 2009). Activated RA receptors (RARs) are present at the time of cortical neuronal differentiation and migration (Luo et
was expressed by neurons with unipolar/bipolar morphology that is superficial cortical layers, during the birth and migration of pyramidal neurons that populate SVZ progenitors (supplementary material Fig. S1). In addition, little to no expression was detected in VZ and SVZ progenitor cells. At embryonic day (E) 12.5, when deep-layer cortical neurons are being born, presence of activated RARs (Rossant et al., 1991). At embryonic stages of RA signaling in the cortex by in utero electroporation of RAR403, a dominant-negative RAR that has been successfully employed to disrupt endogenous RA signaling pathways in avian and mouse models (Damm et al., 1993; Rajaii et al., 2008; Hägglund et al., 2006; Sockanathan et al., 2003). We find that RAR403 expression delays the migration of subsets of cortical neurons, and in addition causes late-born neurons to lose their initial identities and acquire positional and molecular profiles characteristic of layer II neurons. The effects of RAR403 on neuronal fates are reversed by stabilized β-catenin (Ctnmb1 – Mouse Genome Informatics) expression, but this is restricted to specific axial levels. Taken together, our observations identify RA signaling pathways as key regulators of radial migration and cortical neuronal diversity, and furthermore uncover two new principles in the regulation of cortical development: (1) that the migration of deep-layer and superficial cortical neurons are regulated by distinct signaling pathways; and (2) that layer V-III cortical neuronal fates are plastic and require active consolidation of their final identities.

RESULTS
Activated RA receptors are detected in migrating cortical neurons
To determine the endogenous sites of RA signaling in the developing cortex, we analyzed the reporter mouse line RARE.hsp68lacZ, which expresses β-galactosidase (lacZ) in the presence of activated RARs (Rossant et al., 1991). At embryonic day (E) 12.5, when deep-layer cortical neurons are being born, lacZ was expressed in a mosaic pattern in VZ and SVZ progenitor cells. However, little to no lacZ expression was detected in postmitotic Tbr1+ neurons in the cortical plate (CP) (Fig. 1A,A'). At later stages, during the birth and migration of pyramidal neurons that populate superficial cortical layers, lacZ continued to be expressed in VZ and SVZ progenitors (supplementary material Fig. S1). In addition, lacZ was expressed by neurons with unipolar/bipolar morphology that are characteristic for neurons undergoing radial migration (Fig. 1B–C'). Consistent with observations at E12.5, the majority of neurons that had detached from the radial glia, settled within appropriate cortical laminae, and expressed layer-specific markers, did not express the lacZ reporter (Fig. 1A–C). Thus, the major sites of activated RARs indicative of endogenous RA signaling are in cortical progenitors and in migrating newly born cortical neurons. These observations suggest that RA signaling might be required at different stages of cortical development, specifically during the differentiation and radial migration of cortical neurons.

Ablation of RA signaling by RAR403 delays initiation of cortical radial migration
To determine the role of endogenous RA signaling in the developing cortex, we expressed the dominant-negative RA receptor RAR403 in E14.5 embryonic cortices by in utero electroporation. RAR403 is a version of human RARα that lacks the AF2 domain required for ligand-dependent activation and has been demonstrated to effectively abolish RA signaling in vitro and in vivo (Damm et al., 1993; Hägglund et al., 2006; Rajaii et al., 2008; Sockanathan et al., 2003). We electroporated bicistronic constructs that contained internal ribosomal entry site-green fluorescent protein (IRES-GFP) sequences downstream of RAR403 to facilitate the identification of electroporated cells by GFP expression (Fig. 1F). Electroporation of control GFP constructs into the cortices of E14.5 wild-type embryos showed many GFP+ cells that co-expressed lacZ, indicating that they were still responsive to RA signaling (Fig. 1D). By contrast, little to no co-expression of lacZ and GFP was evident in RARE.hsp68lacZ transgenic embryos electroporated with RAR403.GFP (Fig. 1E). These observations confirm that RAR403 expression is sufficient to disrupt endogenous RA signaling in the developing cortex.

We next electroporated E14.5 RARE.hsp68lacZ cortices with control or RAR403.GFP plasmids and examined the distribution of electroporated cells 3 days later. In control animals, a small number of GFP+ or GFP+/lacZ+ cells were found within the VZ/SVZ, but the majority of electroporated cells consisted of newly born neurons...
Fig. 2. RAR403 expression retards migration of cortical neurons. (A,B,D-K) Confocal images of coronal sections of E17.5 mouse cortices that were electroporated at E14.5. G; J and K are magnifications of the boxes outlined in G, J and K; H is of similar magnification to G. (C) The percentage of GFP+ cells in different cortical regions. Mean ± s.e.m.; for VZ/SVZ, P=0.2851; IZ, *P=0.0044; CP, **P=0.0010; n=4-5 embryos. (L) The percentage of GFP+ cells in IZ with multipolar or unipolar/bipolar morphologies, mean ± s.e.m.; for multipolar *P=0.0005; unipolar/bipolar, **P=0.0005; n=4 embryos.

migrating through the intermediate zone (IZ) and the CP (Fig. 2A,C). A similar percentage of RAR403.GFP+ cells were detected within the VZ/SVZ, and these cells expressed Ki67 (Mki67 – Mouse Genome Informatics), a marker of proliferating progenitors (GFP+: 8.0±0.3%; RAR403.GFP+: 7.8±0.8%, P=0.829), and incorporated the S-phase nucleotide analog 5-ethyl-2¢-deoxyuridine (EdU) in numbers equivalent to control GFP+ cells (Fig. 3H). Thus, the majority of RAR403.GFP+ cells located within the IZ and CP are not progenitor cells but have exited the cell cycle at normal rates to become postmitotic neurons. The accumulation of RAR403.GFP+ neurons in the IZ was recapitulated when RAR403.GFP expression was restricted to postmitotic neurons using neuron-specific promoters (Yokota et al., 2007) (Fig. 2I). These observations suggest that the disruption of RA signaling pathways in newly born neurons causes them to stall within the ventral IZ and fail to migrate to the CP (Fig. 2C).

Analysis of GFP and vimentin expression shows no obvious disruptions in radial glial morphology and endfeet, ruling out the possibility that RAR403.GFP expression disrupts the radial glial scaffold (supplementary material Fig. S2B,C). Newly born neurons transition from a multipolar to a unipolar/bipolar morphology to initiate radial migration (Rakic, 1972). Analysis of electroporated neurons in the IZ of E17.5 cortices after electroporation at E14.5 showed that ~75% of neurons expressing GFP alone were unipolar or bipolar, with their leading processes oriented towards the pial surface (Fig. 2J,L). By contrast, the majority of RAR403.GFP+ neurons retained a multipolar morphology that lacked directionality (Fig. 2K,L). Thus, the ablation of RA signaling in newly born neurons results in deficits in the transition from multipolar to unipolar/bipolar states, causing disruptions in the initiation of radial migration.

RAR403.GFP+ neurons occupy superficial positions in the postnatal cortex

To determine if the disruption of RA signaling leads to a delay or a permanent block of cortical radial migration we electroporated E14.5 cortices with plasmids expressing RAR403.GFP and examined the distribution of RAR403.GFP+ cells after 4 days instead of 3. RAR403.GFP+ neurons now had morphologies characteristic of migrating neurons, were broadly distributed within the IZ and could be detected within the CP (supplementary material Fig. S3D), suggesting that RAR403 expression delays the initiation of neuronal migration. At postnatal day (P) 5, control GFP+ cells that were electroporated at E14.5 were distributed in layer IV and expressed Cux1, a marker of layer IV-II neurons, consistent with E14.5 being the peak period of layer IV neuronal differentiation (Molyneaux et al., 2007; Nieto et al., 2004) (Fig. 3A,B,M). Analysis of RAR403.GFP+ cells that had been electroporated at E14.5 showed that they retained expression of RAR403 at P5 (supplementary material Fig. S3E,F); however, RAR403.GFP+ neurons occupied abnormal superficial positions that were directly below the marginal zone (MZ) (Fig. 3C,D). Moreover, they failed to express Rorβ (Rorb – Mouse Genome Informatics), a marker of layer IV neurons (Molyneaux et al., 2007) or Foxp1, which marks permanent block of cortical radial migration we electroporated E14.5 cortices with plasmids expressing RAR403.GFP and examined the distribution of RAR403.GFP+ cells after 4 days instead of 3. RAR403.GFP+ neurons now had morphologies characteristic of migrating neurons, were broadly distributed within the IZ and could be detected within the CP (supplementary material Fig. S3D), suggesting that RAR403 expression delays the initiation of neuronal migration. At postnatal day (P) 5, control GFP+ cells that were electroporated at E14.5 were distributed in layer IV and expressed Cux1, a marker of layer IV-II neurons, consistent with E14.5 being the peak period of layer IV neuronal differentiation (Molyneaux et al., 2007; Nieto et al., 2004) (Fig. 3A,B,M). Analysis of RAR403.GFP+ cells that had been electroporated at E14.5 showed that they retained expression of RAR403 at P5 (supplementary material Fig. S3E,F); however, RAR403.GFP+ neurons occupied abnormal superficial positions that were directly below the marginal zone (MZ) (Fig. 3C,D). Moreover, they failed to express Rorβ (Rorb – Mouse Genome Informatics), a marker of layer IV neurons (Molyneaux et al., 2007) or Foxp1, which marks
layer IV marker Rorβ 2 days after electroporation (Fig. 3K,L). The expression of Rorβ was transient, as no Rorβ transcripts could be detected 24 hours later (data not shown). These observations suggest that newly born cortical neurons that express RAR403 induce fate specification programs appropriate for their time of birth; however, as a consequence of disrupted RA signaling, RAR403.GFP+ neurons fail to maintain their appropriate fates and instead adopt laminar positions and gene expression patterns consistent with that of layer II neurons (Fig. 3N).

**RAR403 disrupts the migration and fates of late-born cortical neurons**

To determine if RA signaling regulates the migratory properties and fates of all or subsets of cortical neurons, we electroporated plasmids expressing GFP or RAR403.GFP at time points immediately preceding the peak periods of layer VI, V and II/III neuronal generation at E12.5, E13.5 and E15.5, respectively. No obvious changes between the distribution of RAR403.GFP+ or GFP+ neurons were detected throughout the cortex in embryos that had been electroporated at E12.5 or E13.5 and analyzed 3 days later (Fig. 4A,B,D,E; supplementary material Fig. S4A-C). These observations suggest that RA signaling pathways are required in late-born neurons to initiate their migration to superficial cortical layers but that RA signaling is not necessary for the migration of early-born neurons to deep layers of the cortex. These data suggest that separate programs exist to control the radial migration of different subtypes of cortical neurons.

We next examined if RA signals regulate the final fates of different neuronal populations in the cortex. We electroporated mouse embryos with plasmids expressing RAR403.GFP or GFP alone at E12.5, E13.5 and E15.5, and analyzed the positions and molecular profiles of RAR403.GFP+ and GFP+ neurons at P5. Control GFP+ neurons showed appropriate cell body positions and molecular marker expression characteristic for neurons born within the timeframe of electroporation; thus, electroporation at E12.5 generated Tbr1+GFP+ neurons situated in layer VI, whereas electroporation at E13.5 or E15.5 resulted in Foxp1+GFP+ and Brn2+GFP+ neurons located in layers V and II/III, respectively (Fig. 4G-I). At P5, embryos that were electroporated with RAR403.GFP at E12.5 showed the same positions and marker expression as controls, reinforcing the notion that RA signaling pathways do not regulate layer VI neuronal development.
RAR403.GFP+ neurons from embryos that were electroporated at E15.5 expressed Brn2; however, they were located in a tight superficial band adjacent to the MZ that contrasted with controls, suggesting that RAR403 expression had disrupted their laminar position and fate (Fig. 4L,O,R). The disruption of RA signaling in neurons that are born on or after E13.5 results in their acquisition of cell body positions and gene expression patterns consistent with sub-MZ layer II neurons.

**β-Catenin expression partially rescues RAR403 cortical phenotypes**

Using a candidate gene approach to define the events downstream of RA signaling, we focused on β-catenin, given its multiple roles in neuronal differentiation and function (Nusse, 1999). To test if RA mediates its effects on neuronal migration and fate maintenance through β-catenin, we co-expressed a stabilized, constitutively active form of β-catenin (90β-catenin) with RAR403.GFP in E14.5 mouse cortices and examined the position of RAR403.GFP+ neurons 3 days later. We restricted β-catenin expression to postmitotic neurons (Kawauchi et al., 2010; Yokota et al., 2007; Barth et al., 1997) in order to bypass possible confounding effects of β-catenin stabilization on progenitor proliferation, IPC generation and differentiation (Chenn and Walsh, 2002; Mutch et al., 2009; Munji et al., 2011; Fang et al., 2013). The migration of RAR403.GFP+ neurons was retarded within the IZ in the absence or presence of 90β-catenin, suggesting that β-catenin does not mediate the RA-dependent initiation of radial migration (Fig. 5A,B).

We next examined if 90β-catenin could rescue the changes in neuronal cell body position and fate that we observed in RAR403.GFP+ neurons at P5. We delivered a pulse of EdU to label cells in S-phase, and electroporated plasmids that express 90β-catenin or RAR403.GFP alone, or RAR403.GFP and 90β-catenin into E14.5 mouse cortices 2 hours later. Cortices were dissected at P5, and the distribution of electroporated cells within ten bins assigned across the apicobasal axis was examined (Fig. 5C; supplementary material Fig. S5A,B). Co-expression of RAR403.GFP and 90β-catenin resulted in many RAR403.GFP+ cells that overlapped the same bins as control EdU+ neurons that were born during the same time frame (Fig. 5E-F). Further, many of the RAR403.GFP+ neurons that were co-electroporated with 90β-catenin now expressed Foxp1, in contrast to neurons expressing RAR403.GFP+ alone (Fig. 5H,I, Fig. 3F). The rescue of RAR403.GFP+ neurons by 90β-catenin in terms of cell body position and fate was most pronounced in central and caudal regions of the cortex, with relatively modest effects detected at more rostral cortical regions (Fig. 5C-I; supplementary material Fig. S5). Similar results in terms of cell body position were observed upon co-expression of RAR403.GFP and 90β-catenin at E13.5, and the expression of appropriate layer-specific markers such as Ctip2 (Bcl11b – Mouse Genome Informatics), Cux1 and Foxp1 was restored (Fig. 5J-N). These observations suggest that RA signaling pathways maintain the position and fates of cortical neurons in central and caudal cortical areas through β-catenin function.

**RA regulates cortical neuronal fates by maintaining β-catenin levels**

Cellular β-catenin levels are regulated by mechanisms that control its stability or its distribution into cell junctions by cadherin association (Nusse, 1999). To determine if RAR403 affects β-

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**Fig. 4. RAR403 disrupts the migration and fates of late-born cortical neurons.** (A-F) Confocal images of coronal sections of mouse cortices electroporated at E12.5 (A,D), E13.5 (B,E) or E15.5 (C,F) and analyzed 3 days later. (G-R) Confocal images of coronal sections of mouse cortices electroporated at E12.5 (G,J,M,P), E13.5 (H,K,N,Q) or E15.5 (I,L,O,R) and analyzed at P6. Arrows and bars mark the location of GFP+ or RAR403.GFP+ neurons. Images inset in G-I reflect the expression of layer-specific markers analyzed at P5. Arrows and bars mark the location of GFP+ or RAR403.GFP+ neurons. Bars in K and L highlight approximate areas magnified in M-R, showing analysis of layer-specific marker expression in RAR403.GFP+ neurons.

(Fig. 4J,M,P). By contrast, embryos that were electroporated at E13.5 with RAR403.GFP were aberrantly located at superficial positions adjacent to the MZ, expressed the layer II marker Brn2 and did not express the layer V-III marker Foxp1 (Fig. 4K,N,Q). As neurons expressing RAR403.GFP at E13.5 did not show obvious impediments in radial migration (Fig. 4E), this observation suggests that the roles of RA signaling in the initiation of radial migration and in fate consolidation are independent. RAR403.GFP+ neurons from embryos that were electroporated at E15.5 expressed Brn2; however, they were located in a tight superficial band adjacent to the MZ that contrasted with controls, suggesting that RAR403 expression had disrupted their laminar position and fate (Fig. 4L,O,R). Thus the disruption of RA signaling in neurons that are born on or after E13.5 results in their acquisition of cell body positions and gene expression patterns consistent with sub-MZ layer II neurons.
In the cortex, we expressed RAR403.GFP or GFP alone in mouse cortices by in utero electroporation, dissected out the electroporated areas and examined levels of active β-catenin by western blot (van Noort et al., 2002). Quantification of β-catenin levels normalized to actin or GFP showed that the expression of RAR403.GFP consistently reduced levels of endogenous β-catenin compared with when GFP alone is expressed (Fig. 6A). Analysis of β-catenin transcript levels in electroporated cells by quantitative polymerase chain reaction (qPCR) showed no changes in β-catenin mRNA levels (data not shown). These observations suggest that RA signaling normally maintains appropriate levels of endogenous β-catenin protein. Stabilization of β-catenin results in β-catenin nuclear localization and association with transcription factors such as Tcf-1 (Hnf1α – Mouse Genome Informatics)/Lef1. To determine if RA stabilization of β-catenin influences activity of endogenous Tcf-1/Lef1, we co-electroporated mCherry or RAR403.mcherry expression constructs with TOPdGFP reporters that contain Tcf-1/Lef1 binding sites upstream of destabilized (d) GFP (Dorsky et al., 2002). Embryos electroporated at E13.5 and examined 3 days later showed a marked reduction of dGFP expression, although the efficiency of electroporation was equivalent, as visualized by mCherry expression (Fig. 6B,C). Thus, RAR403 decreases endogenous Tcf-1/Lef1 protein function, presumably as a consequence of decreasing endogenous levels of β-catenin.

β-Catenin can be stabilized through two main mechanisms: first, by activated canonical Wnt signaling that inhibits β-catenin degradation by a destruction complex containing APC, Axin and GSK-3β, and second, by Wnt-independent sequestration of β-catenin by association with cadherins at cell junctions (Valenta et al., 2012). To determine which form of β-catenin is required for RA-dependent consolidation of neuronal fate, we compared the functions of β-catenin by association with cadherins at cell junctions (Valenta et al., 2012). To determine which form of β-catenin is required for RA-dependent consolidation of neuronal fate, we compared the functions of β-catenin by association with cadherins at cell junctions (Valenta et al., 2012). RAR403.GFP constructs were co-electroporated with either pTα-β-catenin or pTα-β-catenin in
E14.5 cortices and the position and molecular profile of layer-specific expression of RAR403.GFP+ cells were analyzed at P5. Co-expression of RAR403.GFP with pTα-β-catenin showed a similar pattern of rescue to that of constitutively active Δ90-β-catenin (Fig. 5, Fig. 6D-F; supplementary material Fig. S6). In both cases, many RAR403.GFP+ neurons in central and caudal cortical regions expressed the marker Foxp1, and were distributed throughout deep and superficial cortical laminae (Fig. 6E; supplementary material Fig. S6). Little to no rescue of the RAR403 phenotype was observed in rostral cortical areas (Fig. 6D). By contrast, pTα-β-catenin showed weaker effects than β-catenin in rescuing the neuronal position and terminal fates of RAR403.GFP+ neurons in rostral, central and caudal cortical areas (Fig. 6G-I; supplementary material Fig. S6). These observations suggest that the form of β-catenin that is not associated with cadherins is responsible for the RA-dependent maintenance of cortical neuronal fates.

**DISCUSSION**

The developing cortex contains significant amounts of RA, and recent work indicates that its primary source derives from Raldh2 expression in the meninges (Luo et al., 2004; Siegenthaler et al., 2009). Our study suggests that activated RA signaling pathways play functionally distinct roles in newly generated cortical neurons that are destined to populate different cortical layers (Fig. 6J). Initially, activated RA receptors are required for later-born neurons to switch from a multipolar to unipolar/bipolar state, which enables their migration along radial glial processes to their final settling positions. At more caudal areas of the cortex, this process involves β-catenin stabilization and the subsequent preservation of its function along the rostrocaudal axis (Fig. 6J). Thus, the coordinate action of RA and β-catenin function is important for the acquisition of final neuronal fates at central and caudal cortical areas, raising the possibility that these factors play key roles in specifying regional differences in cortical cytoarchitecture and function.

**RA regulates the initiation of superficial neuronal migration**

RAR403 expression delays the migration of superficial neurons but does not compromise early-born neuronal migration, suggesting that RA signaling regulates the initiation of glial-assisted neuronal migration but not somal translocation (Nadarajah and Parnavelas, 2002). This concept suggests that different migratory modes are regulated by separate pathways, an idea that is consistent with mouse mutant phenotypes such as Cdk5 nulls, which have disorganized superficial cortical layering but normal layer VI formation (Gilmore et al., 1998), and mice lacking Brn1/2 that show delayed migration of superficial neurons (Sugitani et al., 2002). Our results suggest that the transition of neurons from a multipolar to unipolar/bipolar morphology is an early event that is differentially regulated during somal translocation or glial-mediated locomotion, as this process is perturbed upon RAR403 expression. Several proteins are required for the multipolar to unipolar/bipolar switch in cortical neurons; these include transcription factors such as Ngn2 (Neurog2 – Mouse Genome Informatics), cell polarity protein Par1 (Mark2 – Mouse Genome Informatics) and the Rho GTPase Rnd2 (Hand et al., 2005; Heng et al., 2008; Sapir et al., 2008). It is not clear if these proteins function selectively for early- or late-born neuronal migration, but it will be of interest to determine if they function downstream of RA signaling or if novel targets of RAR activation exhibit this distinction.
RA signals and fate consolidation

Neuronal fate in the cortex is intimately connected with the time of cell cycle exit (Molynieux et al., 2007), suggesting that events directly preceding or at the time of cell cycle exit are crucial for specifying the terminal identities of cortical neurons. Genetic lineage-tracing studies now suggest that the fates of superficial neurons are specified much earlier and are encoded in cortical progenitors at the time of deep-layer neuronal generation (Franco et al., 2012). Thus, the regulation of cortical neuronal fate is complex, and involves mechanisms that operate in actively cycling progenitors and during the terminal S phase before cell cycle exit. We show here that final cortical neuronal identity is achieved by an ongoing process that initiates in progenitors and continues in postmitotic neurons. Neurons expressing RAR403 initiate fate-specification programs appropriate for their birthdate; however, they fail to maintain their appropriate fates and instead adopt laminar positions and transcriptional profiles that resemble layer II neurons that abut the MZ. Of note, this phenomenon pertains to newborns born after E13.5 and does not apply to layer VI neurons, which show normal positions and fates even in the presence of RAR403. These observations suggest that the fates of most cortical neurons are plastic and that ongoing RA signaling pathways are required to consolidate and perhaps refine the fate-specification programs initiated at the time of cell cycle exit. The continual plasticity of postmitotic neurons and the importance of RA has a precedent in another system – namely, in developing spinal motoneurons (Sockanathan and Jessell, 1998; Sockanathan et al., 2003). Here, divisional identities within the limb-innervating lateral motor column (LMC) are imposed in late-born postmitotic motoneurons by RA. Thus, at least in the cortex and spinal cord, the acquisition of final neuronal identities is a multistep process that occurs in progenitors and in postmitotic neurons. It is conceivable that this strategy to specify and refine terminal neuronal fate is more general and is utilized to regulate neuronal diversity in other regions of the central and peripheral nervous system.

RA signals and the patterning of cortical neurons

Electroporation of RAR403 at E12.5 did not alter the cell fate or laminar position of layer VI neurons, suggesting that neurons that are first to exit the cell cycle are formed normally in the absence of activated RA. By contrast, RAR403+ cortical neurons that were born after this time point were consistently located directly beneath the MZ, one possibility is that RA signals might directly beneath the MZ, one possibility is that RA signals might
et al., 2007). Mutant forms of β-catenin (Δ90, Y654E and Y654F) were generated by PCR and cloned into pTur vector. pCAGGS-IREScMerry was provided by Dr Kolodkin (Johns Hopkins University, MD, USA) and RAR403 was subcloned to generate pCAGGS-RAR403-IREscMerry. TOPdGFp was provided by Dr Nathans (Johns Hopkins University, MD, USA).

**Immunofluorescence**

Immunofluorescence experiments were performed as described (Rao and Sockanathan, 2005). Confocal images were acquired with a Zeiss LSM 5 PASCAL microscope. Primary antibodies used were as follows: rabbit anti-Tbr1 (AB10554, Millipore, 1:1000), rabbit anti-Bmi2 (sc26894, Santa Cruz, 1:400), chick anti-GFP (GFP-1010, Aveslab, 1 μg/μl), rabbit anti-GFP (A-11122, Invitrogen, 1:500), goat anti-β-gal (Armel, 1:3000), rabbit anti-Ki67 (ab15580, Abcam, 1:1000), rabbit anti-Tbr2 (ab23345, Abcam, 1:2000), mouse anti-Hu C/D (A-21271, Invitrogen, 1:100), chick anti-vimentin (AB5733, Chemicon, 1:1000), mouse anti-Tuj1 (MMS435P, Covance, 1:1000), rabbit anti-Cux1 (sc1024, Santa Cruz, 1:500), rabbit anti-Foxp1 (ab16645, Abcam, 1:2000), rabbit anti-DeSir (632496, Clontech, 1:1500), mouse anti-hRARα (NB200-322, Novus Biological, 1:500). Nuclei were stained with Topro-3 iodide (1:40,000).

**Neuronal counts**

For migration analysis, the number of GFP+ cells from three to four serial sections of an embryo was counted with ImageJ software. Approximately 200 cells were counted per section. These numbers were averaged and regarded as n=1 (per embryo). For morphology analysis, cells that have one leading process toward the pial surface and a lagging process toward the VZ were counted as unipolar/bipolar cells. In this case ~70-80 cells per section were counted, three to four sections per embryo. Cells that had more than two processes in radial directions were defined as multipolar cells.

**Cell cycle exit analysis**

pCAGGS-IREScGFp (Control) or pCAGGS-RAR403-IREScGFp constructs were electroporated at E14.5, and EdU was injected i.p. at E15.5. Mice were sacrificed at E16.5. The cell cycle exit index was calculated as GFPKi67 EdU/GFPKi67 EdU.

**In situ hybridization**

In situ hybridization experiments were carried out as described previously (Rao and Sockanathan, 2005). Brightfield images were captured on a Zeiss Axioskop2 microscope. To generate antisense probes for RORα, RORβ and neurite outgrowth in the adult mouse spinal cord in vitro. J. Cell Sci. 115, 3779-3786.

**Funding**

This work was funded by grants from the National Research Foundation of Korea [NRF-2009-352-C00115 to J.C.]; and by an Independent Investigator Award from the Brain Research and Behavior Foundation (to S.S.).

**References**


**Author contributions**

J.C., S.P. and S.S. conceived and designed the study; J.C. and S.P. performed the experiments; J.C., S.P. and S.S. analyzed the data. S.S. wrote the manuscript.


