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RESEARCH ARTICLE

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

Jeonghoon Choi, Sungjin Park and Shanthini Sockanathan*

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 (Marín 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, later-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

PCTB1004, the Solomon Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205, USA.

*Author for correspondence (ssockan1@jhmi.edu)

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al., 2004) and RA derived from the meninges has been shown to be a primary contributor of cortical RA levels (Siegenthaler et al., 2009). Although recent work suggests that meningeal sources of RA control the asymmetric differentiation of radial glia during early cortical neurogenesis, relatively little is known of RA function at later stages of cortical development as a result of the early lethality of animals lacking the major RA-synthesizing enzyme Raldh2 (Aldh1a2 – Mouse Genome Informatics), and the paucity of useful Cre lines to conditionally inactivate Raldh2 at its site of expression in the meninges (Niederreither et al., 1999; Siegenthaler et al., 2009).

Here, we have taken an alternative approach to investigate the role of RA signaling in the developing cortex. We have ablated 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 (Ctnnb1 – 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 is 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

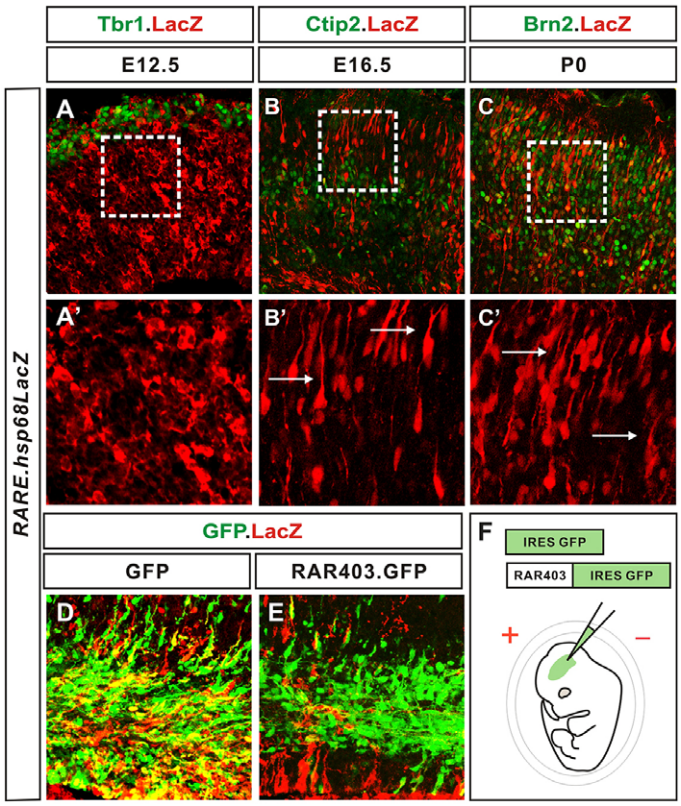

Fig. 1. RA signaling is active in cortical progenitors and migrating neurons. (A-E) Confocal micrographs of coronal sections of embryonic *RARE.hsp68.lacZ* mouse cortices. A′, B′ and C′ are close-ups of *lacZ*-expressing neurons in A-C. Arrows in B and C highlight migrating neurons with unipolar/bipolar morphologies. (D,E) Cortices that were *in utero* electroporated with constructs expressing green fluorescent protein (GFP) or RAR403.GFP at E14.5 and analyzed 3 days later show downregulation of *lacZ* expression in the presence of RAR403. (F) Schematic of control and RAR403.GFP constructs and their electroporation into mouse embryonic cortices *in utero*.

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 *RARE.hsp68lacZ* transgenic 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* 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

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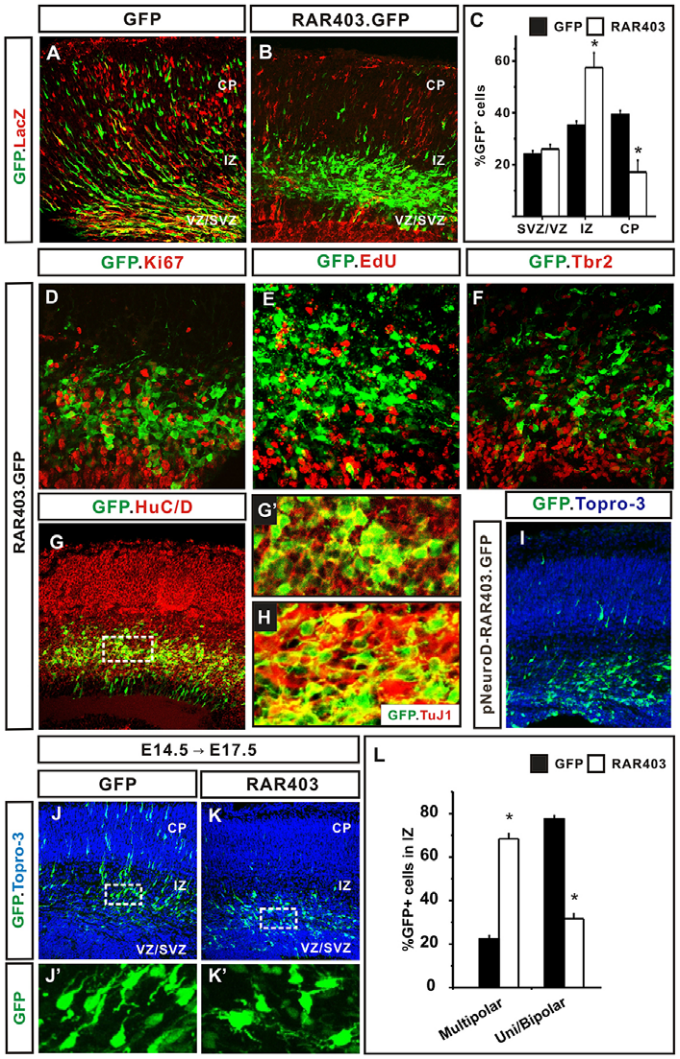

Fig. 2. RAR403 expression retards migration of cortical neurons. (A,B,D-K′) Confocal images of coronal sections of E17.5 mouse cortices that were electroporated at E14.5. G′, J′ and K′ are magnifications of the boxes outlined in G, J and K; H is of similar magnification to G′. (C) The percentage of GFP$^+$ 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-ethynyl-2¢-deoxyuridine (EdU) in numbers equivalent to control GFP$^+$ cells (GFP$^+$: 7.4±0.55%; RAR403.GFP$^+$: 7.1±0.56%, P=0.759). However, RAR403.GFP$^+$ cells were markedly increased in the lower IZ, with a concomitant decrease of RAR403.GFP$^+$ neurons in the CP (Fig. 2A-C). The RAR403.GFP$^+$ cells in the IZ lacked expression of Ki67, failed to incorporate EdU, did not co-express Tbr2, a marker of intermediate progenitor cells (IPCs), and colocalized with the neuronal markers HuC/D and TuJ1 (Tubb3 – Mouse Genome Informatics) (Fig. 2D-H; supplementary material Fig. S2A). Calculation of cell cycle exit indices showed that RAR403.GFP$^+$ cells exited the cell cycle at equivalent rates 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,J′,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 neurons specific for layers V-III (Ferland et al., 2003) (Fig. 3E,F,M). Instead, they co-expressed Brn2 (Pou3f2 – Mouse Genome Informatics) (Hevner et al., 2003) and Cux1 (Fig. 3D,G), a molecular profile characteristic of layer II neurons.

RAR403.GFP$^+$ cells exit the cell cycle at similar rates as GFP$^+$ cells but at P5, RAR403.GFP$^+$EdU$^+$ cells born at the same time as GFP$^-$EdU$^+$ cells occupy more superficial positions (Fig. 3H-J; supplementary material Fig. S3G). Similar results were obtained when RAR403.GFP was expressed from the NeuroD (Neurod1 – Mouse Genome Informatics) promoter (Yokota et al., 2007), suggesting that these phenotypes arise as a consequence of disrupting RA signaling in postmitotic neurons (supplementary material Fig. S3A-C). Strikingly, RAR403.GFP$^+$ neurons that were clustered within the ventral IZ displayed robust expression of the

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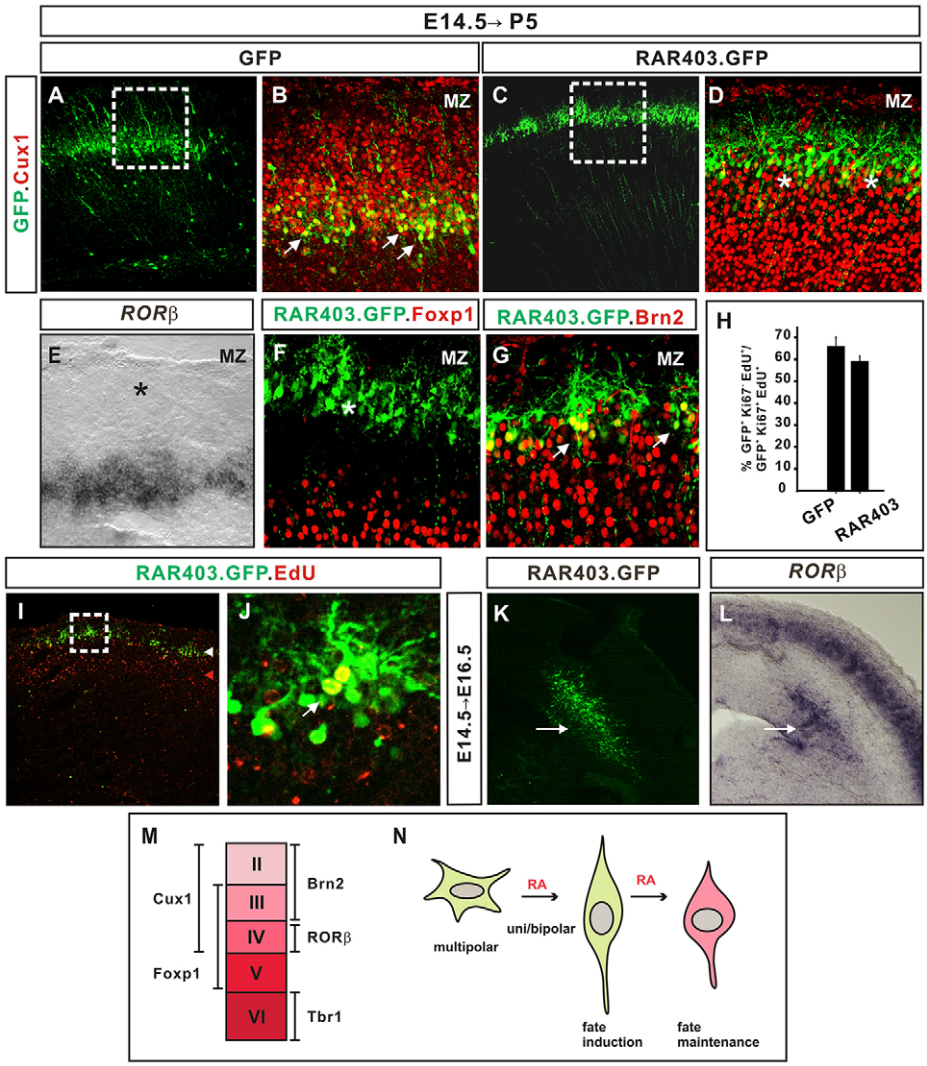

Fig. 3. RAR403 expression alters the fates of cortical neurons. (A-D,F,G,I,J) Confocal images of mouse embryonic cortices *in utero* electroporated at E14.5 and analyzed at P5. Boxed areas in A and C are magnified in B and D, respectively. Arrows in B mark GFP$^+$ neurons that express Cux1; the asterisks in D mark regions containing RAR403.GFP$^+$ neurons with weak to no Cux1 expression. (E) *In situ* hybridization of coronal sections of mouse embryonic cortex electroporated at E14.5 and analyzed at P5. The asterisk marks a region containing RAR403.GFP$^+$ neurons in E and F. The arrows in G mark RAR403.GFP$^+$ neurons that co-express Brn2. (H) Cell cycle exit indices of control GFP and RAR403.GFP$^+$ neurons at E16.5, mean ± s.e.m.; P=0.1824; n=4 embryos. The boxed area in I is magnified in J. In I, the white arrowhead marks the location of RAR403.GFP$^+$ neurons, and the red arrowhead marks the location of EdU$^+$ neurons born at the time of EdU injection. The arrow in J marks EdU$^+$ RAR403.GFP$^+$ neurons that are localized directly under the marginal zone. (K,L) Neighboring sections of embryonic mouse cortices electroporated with RAR403.GFP at E14.5 and analyzed at E16.5. Arrows mark sites of retarded RAR403.GFP$^+$ neurons. (M) Schematic showing layer-specific marker expression. (N) Model of RA function in cortical neuron migration and fate maintenance. MZ, marginal zone.

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. S4D,E). By contrast, embryos electroporated with RAR403.GFP at E15.5 accumulated within the ventral IZ at positions similar to that observed when RAR403 was expressed in cortices at E14.5 (Fig. 4C,F, Fig. 2; supplementary material Fig. S4F). No changes in cell death were observed between cortices electroporated with GFP or RAR403.GFP at different time points (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

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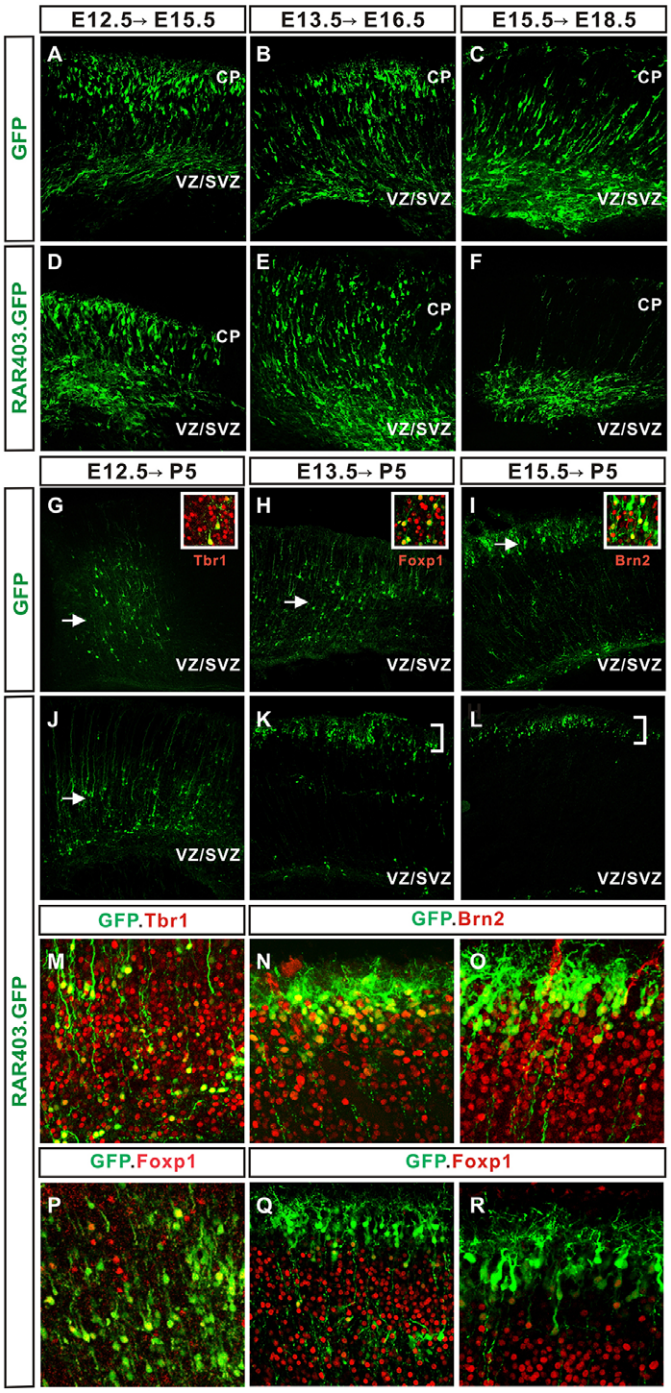

Fig. 4. RAR403 disrupts the migration and fates of late-born cortical neurons. (A-F) Confocal images of coronal sections of mouse cortices electroporated at E12.5 (A,D), E13.5 (B,E) or E15.5 (C,F) and analyzed 3 days later. (G-R) Confocal images of coronal sections of mouse cortices electroporated at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

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 (Δ90β-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 Δ90β-catenin, suggesting that β-catenin does not mediate the RA-dependent initiation of radial migration (Fig. 5A,B).

We next examined if Δ90β-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 Δ90β-catenin or RAR403.GFP alone, or RAR403.GFP and Δ90β-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 Δ90β-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 Δ90β-catenin now expressed Foxp1, in contrast to neurons expressing RAR403.GFP$^+$ alone (Fig. 5H,I, Fig. 3F). The rescue of RAR403.GFP$^+$ neurons by Δ90β-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 Δ90β-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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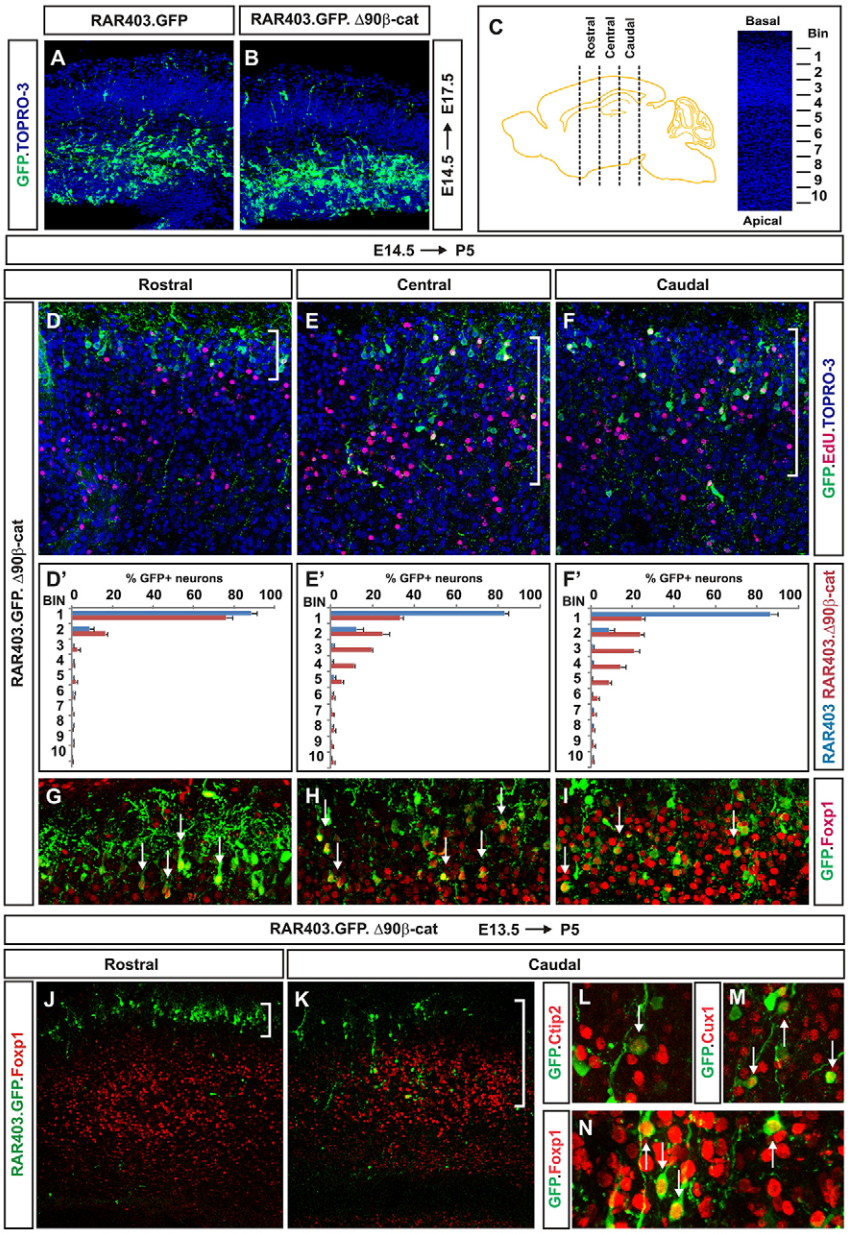

Fig. 5. Fate changes imposed by RAR403are rescued by β-catenin in central and caudal areas. (A,B;D-N) Confocal images of coronal sections of mouse cortices co-electroporated with constructs expressing RAR403.GFP and Δ90-β-catenin. (C) Schematic diagram showing rostral, central and caudal areas analyzed in β-catenin rescue experiments. The image shows apicobasal division of cortex into ten bins. Bars (D-F,J,K) denote the extent of RAR403.GFP$^+$ neuron distribution radially within the cortex. (D′-F′) Quantitation of RAR403.GFP$^+$ neurons in the absence (blue) and presence (red) of β-catenin; mean ± s.e.m.; *n*=3-5 pups, ~300 cells/pup; **P*<0.05 for Bin 2 (rostral), Bins 1-5 (central), Bins 1-6 (caudal). Arrows in G-I mark RAR403.GFP$^+$ cells with weak (G) and strong expression of Foxp1 (H-I); arrows in L-N show co-expression of RAR403.GFP with layer V-III markers.

catenin protein levels 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 (Hnf1a – 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-catY654E, which is a phosphomimetic mutant that markedly decreases β-catenin-cadherin interaction; and β-catY654F, which fails to be phosphorylated and has a strong affinity for cadherins (Valenta et al., 2012). RAR403.GFP constructs were co-electroporated with either pTα-β-catY654E or pTα-β-catY654F in

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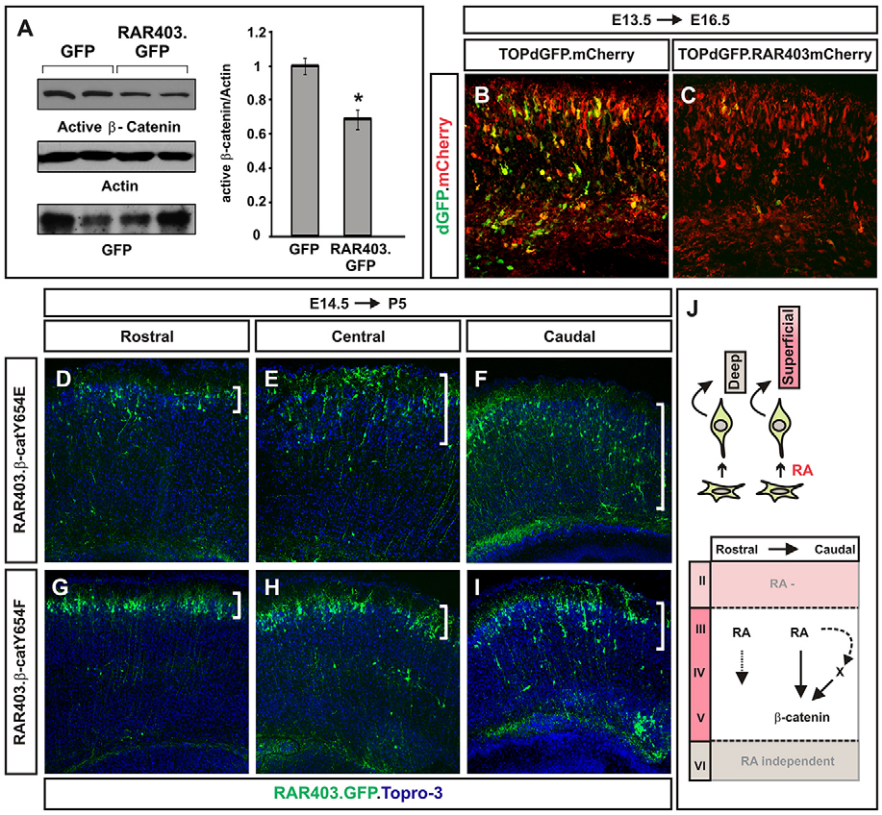

Fig. 6. RAR403 suppresses β-catenin signaling in caudal cortical areas. (A) Representative western blot of dissected cortical areas that were electroporated with plasmids expressing GFP or RAR403.GFP. Densitometric analysis of β-catenin levels normalized to actin. Similar results were obtained when normalized to GFP expression. Mean ± s.e.m., **P*=0.001559, *n*=5-6 electroporated embryos. (B-I) Confocal images of coronal sections of mouse cortices electroporated at E13.5 or E14.5 and analyzed at E16.5 (B,C) and P5 (D-I). Bars in D-I mark the radial distribution of RAR403.GFP$^+$ neurons in electroporated cortices. (J) Model showing that RA is required for the multipolar to unipolar/bipolar transition of late-born neurons before their migration to superficial cortical layers. Moreover, RA signaling is required to maintain the fates of cortical neurons that span layers V-III. Neurons in layer VI are generated independently of RA signaling, whereas the ablation of RA signaling in postmitotic neurons causes them to accumulate in positions directly below the marginal zone in layer II. At more caudal areas of the cortex, RA maintains levels of endogenous β-catenin, which mediates events necessary for terminal fate acquisition and position of layer V-III cortical neurons.

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-catY654E showed a similar pattern of rescue to that of constitutively active Δ90β-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-catY654F showed weaker effects than β-catY654E 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 in upper cortical layers (Fig. 6J). Additionally, once the fates of prospective layer V-III cortical neurons have been initiated, RA signaling pathways maintain and possibly consolidate their final identities and 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.

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RA signals and fate consolidation

Neuronal fate in the cortex is intimately connected with the time of cell cycle exit (Molyneaux 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 neurons 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 and expressed markers corresponding to layer II cortical neurons. Thus, two distinct zones emerge within the cortex: a deep-layer zone containing cortical plate neurons that are formed independently of RA; and a superficial sub-MZ zone that is populated by neurons that lack activated RA signaling exemplified by RAR403^{+} neurons (Fig. 6J). Our results suggest the existence of a third zone (Neuro$^{RA\text{-}dep}$) that lies in between these two extremes, where ongoing RA signaling is necessary for maintaining layer V-III neuronal fates that were initiated in newly born cortical neurons. How might RA maintain the fates of layer V-III neurons? Given that all RAR403^{+} neurons born after E13.5 expressed Brn2 and clustered directly beneath the MZ, one possibility is that RA signals might normally suppress superficial layer II fates in prospective layer V-III neurons. However, *in utero* electroporation of a constitutively active VP16RAR in E15.5 cortices failed to suppress the expression of Cux1 and Brn2 in prospective layer II neurons, suggesting that forced activation of RA signaling is not sufficient to inhibit layer II fates (our unpublished observations).

Our analysis suggests that one pathway by which RA signaling preserves the fates of Neuro$^{RA\text{-}dep}$ neurons occurs through the maintenance of endogenous β-catenin levels. Strikingly, this interplay between RA and β-catenin appears restricted to central and caudal regions of the cortex, and is consistent with the rostrocaudal pattern of activated β-catenin signaling visualized by the reporter TOPdGFP (Chenn, 2008; Hirabayashi et al., 2004) (supplementary material Fig. S7). Our results suggest that RA signals maintain normal levels of cellular β-catenin protein and that this process occurs in postmitotic neurons, which contrasts with the known roles for β-catenin in cortical progenitors where it controls their differentiation into cortical neurons (Mutch et al., 2009; Munji et al., 2011; Fang et al., 2013). Interestingly, Cre-mediated excision of β-catenin causes cortical neurons to adopt more superficial fates. Although this phenotype was attributed to β-catenin activity in progenitor cells, it is possible that β-catenin function in postmitotic neurons could also contribute to this effect (Mutch et al., 2009). We find that RA stabilization of β-catenin does not occur at the transcriptional level, or by increasing its association with cadherins. Activated Wnt signaling stabilizes β-catenin by inhibiting the function of the Axin/Apc/Gsk3β destruction complex. It is possible that RA integrates with this pathway to maintain β-catenin stability, either by potentiating Wnt signaling or by influencing the function of the destruction complex by Wnt-independent mechanisms (Valenta et al., 2012). We note that RA signaling maintains cortical neuronal identity and position in rostral cortical areas but that this function does not appear to be mediated by β-catenin. Whether RA acts alone in this case or interacts with other signaling pathways requires further investigation; possible candidates include FGFs, which are known to be important for patterning rostral cortical areas (Hoch et al., 2009). Consistent with this idea, we find that levels of phosphoERK, an indicator of activated FGF signaling, are decreased in rostral cortical areas when RAR403 is expressed (our unpublished observations).

Our study suggests that RA signaling pathways in postmitotic neurons consolidate and possibly refine cortical neuronal identities and properties in different rostrocaudal regions of the cortex. This extends current models that propose signaling molecules specify cortical area identity within cortical progenitors and that this information is inherited by their neuronal progeny. These combined observations suggest that the generation of functionally and positionally distinct cortical areas is a complex process that requires the coordination of intrinsic and extrinsic factors in progenitors and postmitotic neurons.

MATERIALS AND METHODS

Animals

RARE-hsp68lacZ mice (Rossant et al., 1991) were maintained as described (Rajaii et al., 2008). Timed pregnant CD-1 mice were purchased from Charles River Laboratories. All animal procedures were carried out according to Johns Hopkins University IACUC guidelines.

In utero electroporation

Procedures were performed as described (Rodriguez et al., 2012). Briefly, timed pregnant dams were anesthetized by intraperitoneal (i.p.) injection of avertin before injection of 1µl of DNA (1.5 µg/µl) into the lateral ventricle of the embryos. Electric pulses of 35 V for 50 milliseconds were delivered five times with 950-millisecond intervals. Mice were sacrificed at each time point required and embryonic or postnatal brains were analyzed. For the analysis at P5, a pulse of EdU (50 mg/kg) was injected i.p. into pregnant mice 2 hours before electroporation (Stancik et al., 2010). Detection of EdU was performed according to the manufacturer's protocol (Invitrogen).

Plasmids

pCAGGS-IRES-EGFP, pCAGGS-hRAR403-IRES-EGFP were as described (Sockanathan et al., 2003). pNeuroD and pTα vectors were kindly provided by Dr Anton and Dr Kawauchi, respectively (Kawauchi et al., 2010; Yokota

et al., 2007). Mutant forms of β-catenin (Δ90, Y654E and Y654F) were generated by PCR and cloned into pTα vector. pCAGGS-IRES-mCherry was provided by Dr Kolodkin (Johns Hopkins University, MD, USA) and RAR403 was subcloned to generate pCAGGS-RAR403-IRES-mCherry. 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-Brn2 (sc28594, 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 (Arnel, 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 (sc13024, Santa Cruz, 1:500), rabbit anti-Foxp1 (ab16645, Abcam, 1:2000), rabbit anti-DsRed (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-IRES-GFP (Control) or pCAGGS-RAR403-IRES-GFP 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 $GFP^+Ki67^-EdU^+/GFP^+Ki67^+EdU^+$.

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 RORβ, the 3′ coding and untranslated region (780 bp) was cloned and *in vitro* transcribed.

Western blots

pCAGGS-IRES-GFP (Control) or pCAGGS-RAR403-IRES-GFP constructs were electroporated at E13.5. The cortical area that expresses GFP was dissected out under the fluorescence dissection microscope and lysed [2% sodium dodecyl sulfate (SDS) in PBS]. Protein amounts were quantified using BCA Kit (Pierce) and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). β-Catenin levels were normalized to actin or GFP using ImageJ (NIH). Antibodies used were mouse anti-active β-catenin (05-665, Millipore, 1:2000), mouse anti-actin (MAB1501R, Millipore, 1:5000) and rabbit anti-GFP (A-11122, Invitrogen, 1:500).

Statistical analysis

All statistical analyses used unpaired two-tailed Student's *t*-test.

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Competing interests

The authors declare no competing financial interests.

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.

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Supplementary material

Supplementary material available online at
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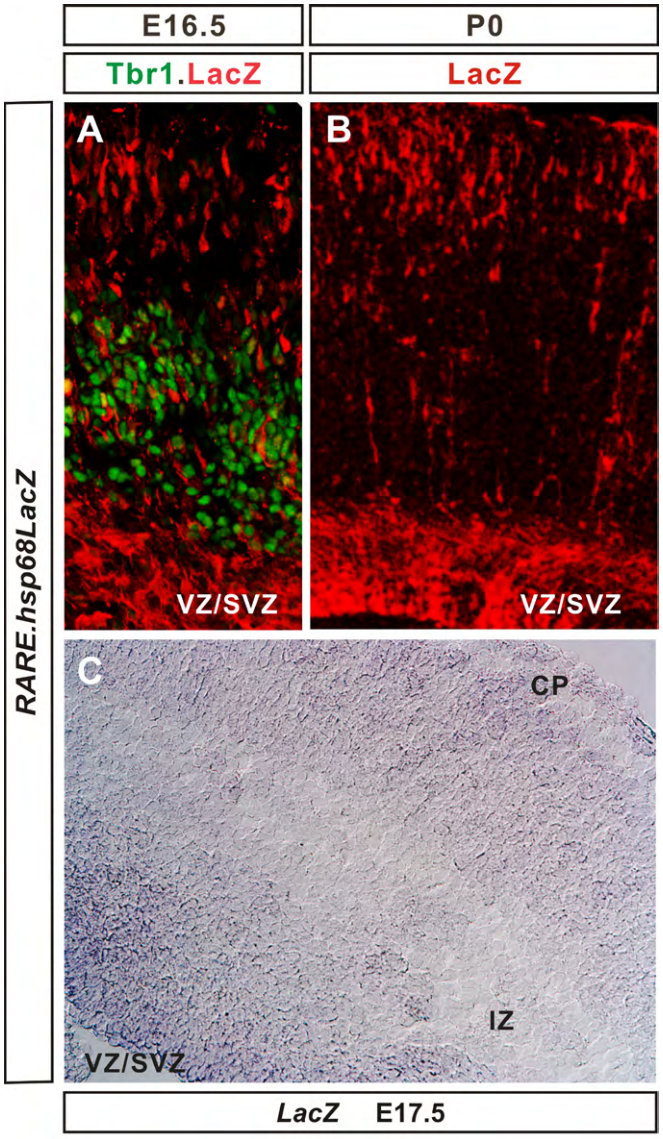

Figure S1. Activated RARs are present within cortical progenitors.
(A, B) Confocal images of coronal sections of *RARE.hsp68LacZ* mouse cortices. LacZ expression is evident within Ventricular zone/ Subventricular zone (VZ/SVZ) progenitor cells at embryonic and early postnatal stages. (C) In situ hybridization showing distribution of LacZ transcripts at E17.5. Higher LacZ expression is observed in VZ/SVZ regions with scattered expression in the intermediate zone (IZ) and cortical plate (CP).

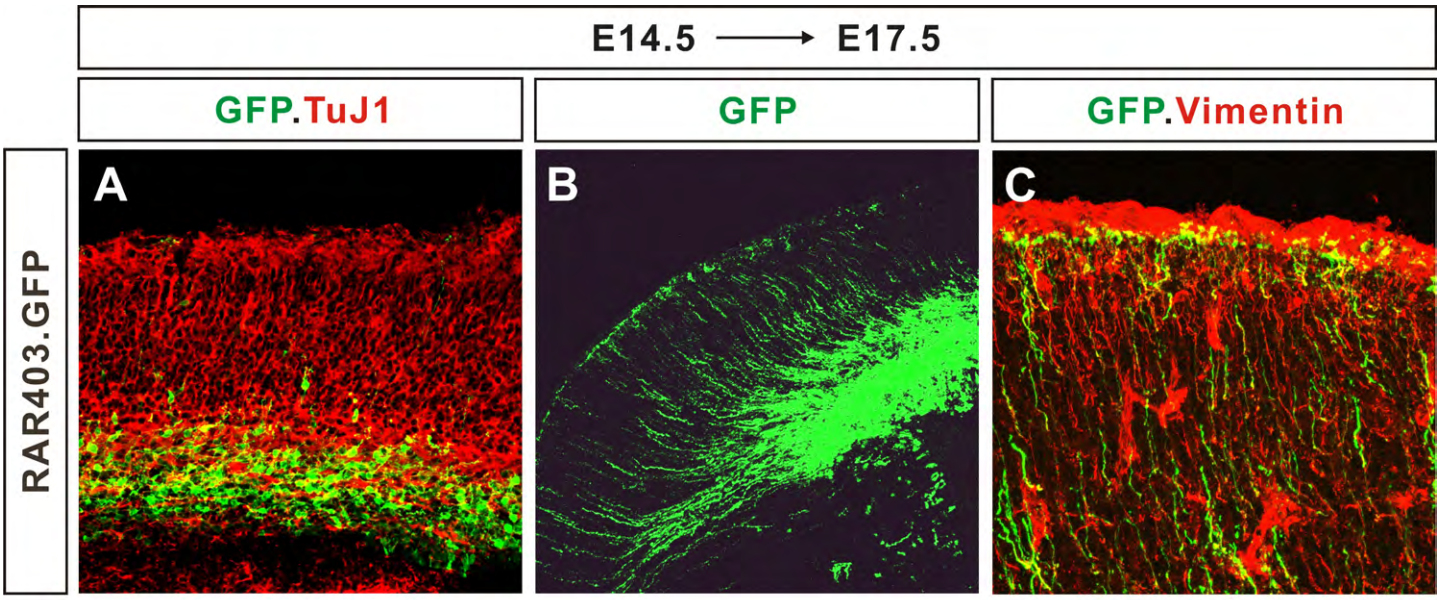

Figure S2. RAR403 disrupts radial migration in postmitotic neurons without affecting radial glial scaffolds.
(A-C) Confocal images of coronal sections of mouse cortices electroporated with constructs expressing RAR403.GFP at E14.5 and analyzed at E17.5. In (A) RAR403.GFP$^+$ cells express the neuronal marker TuJ1. (B, C) GFP staining shows that cells expressing RAR403.GFP extend normal processes to contact the pial surface and that neighboring vimentin$^+$ radial glia show grossly normal formation of the radial scaffold.

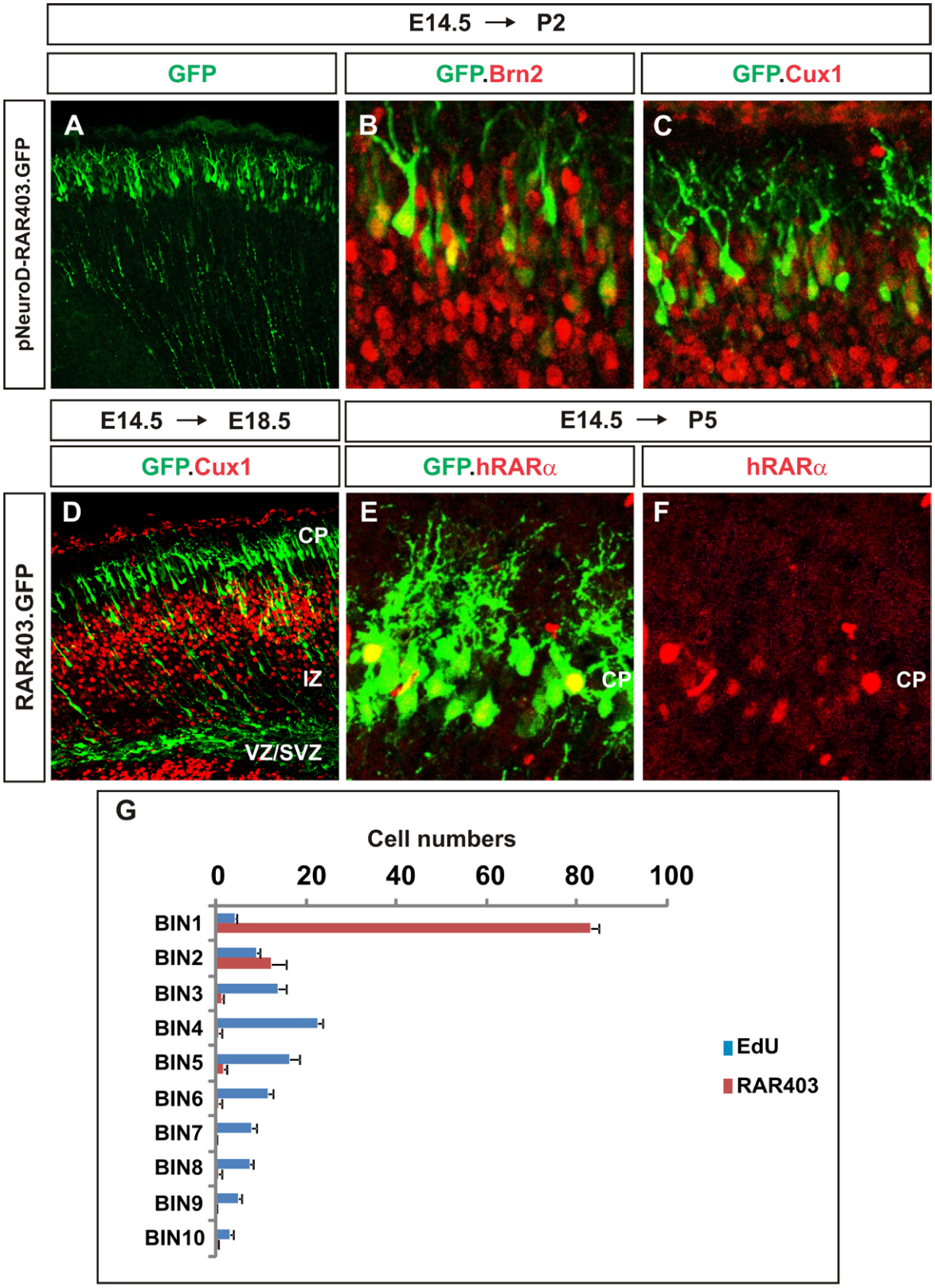

Figure S3. RAR403.GFP expressing neurons adopt layer II cortical neuronal fates.
(A-C) Images showing that expression of RAR403.GFP in postmitotic neurons using the NeuroD promoter mimic the phenotype of cells that initiate expression of RAR403.GFP in progenitors when CAGGS promoter constructs are used. (D) Electroporation of RAR403.GFP at E14.5 retards initial migration; however, RAR403.GFP$^+$ cells exhibit radial migration 4 days after electroporation. (E, F) Cells electroporated with RAR403.GFP at E14.5 maintain expression of RAR403 at P5 as visualized by antibodies directed against hRARα. CP: cortical plate; VZ: ventricular zone; SVZ: subventricular zone; IZ: intermediate zone. (G) The dorsal-ventral extent of the cortex was divided into 10 bins as described in Figure 5C. Graphs quantify the numbers in each bin of EdU$^+$ cells on the contralateral unelectroporated cortex (blue) compared with RAR403.GFP$^+$ cells (red). RAR403$^+$ cells are present in the most superficial bins in contrast to unelectroporated EdU$^+$ neurons. Mean ± SEM. n = 6-8 animals.

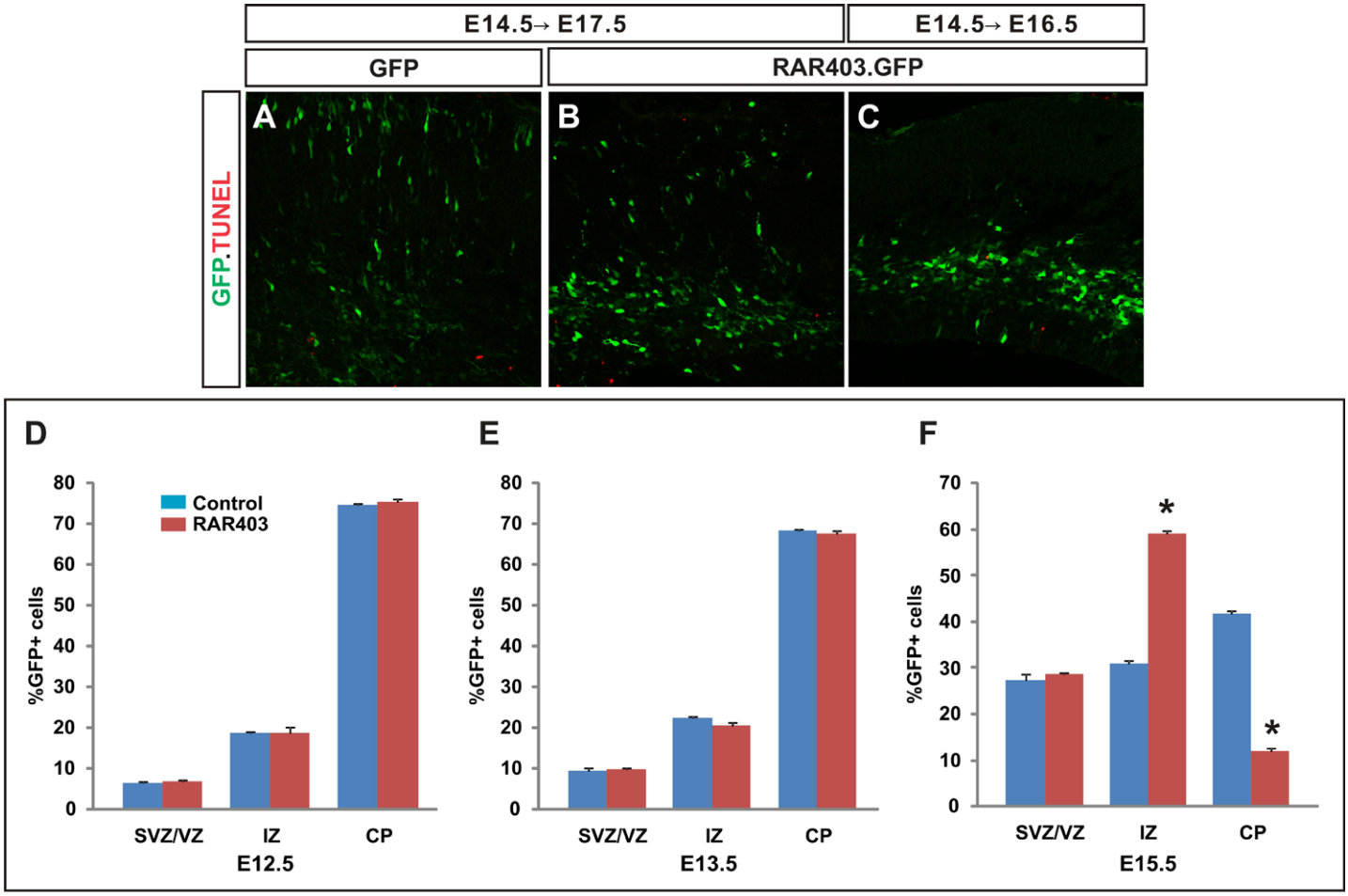

Figure S4. RAR403 alters neuronal migration of specific subsets of cortical neurons and does not cause cell death.
(A-C) Sections of mouse cortices show that comparable amounts of TUNEL positive cells are evident between control and RAR403. GFP electroporated conditions. (D-F) Graphs quantifying the distribution of GFP+ cells in the ventricular/subventricular zones VZ/SVZ, intermediate zone (IZ) and cortical plate (CP) when cortices are electroporated at E12.5, E13.5 or E15.5 and analyzed 3 days later. Mean ± SEM. n = 6-8 animals. In (D) and (E), $p > 0.05$; (F) SVZ/VZ $p=0.3730$; IZ $*p=5.939 \times 10^{-6}$; CP $*p=8.734 \times 10^{-6}$.

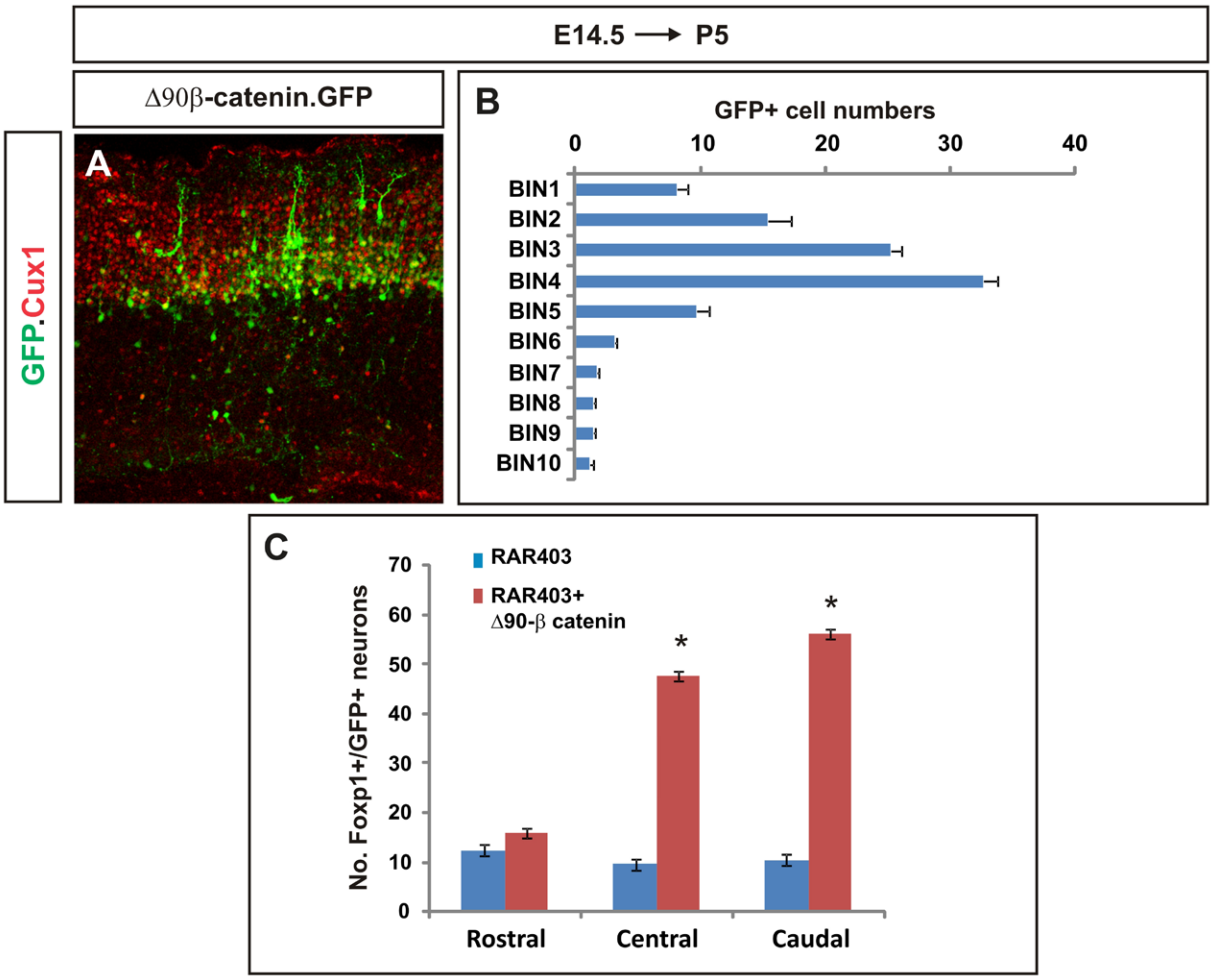

Figure S5. Δ90β-catenin rescues the fates of RAR403.GFP^{+} neurons.
(A) Representative section of mouse cortex electroporated with Δ90 β-catenin. No obvious changes in cell body position or fate are observed. (B) Graphs quantifying the number of neurons located in Bins distributed along the dorsal-ventral axis according to Figure 5C; Δ90 β-catenin GFP^{+} neurons occupy similar positions for control neurons born at the time of electroporation (see Figure S3). (C) Graph quantifying the numbers of GFP^{+} neurons expressing Foxp1 in sections of rostral, central and caudal cortices electroporated with RAR403 alone or RAR403^{+}Δ90-β catenin. Mean ± SEM, n=4 animals; p Rostral=0.089 ; *p Central=$6.54\text{x}10^{-5}$; *p Caudal=$3.667\text{x}10^{-5}$.

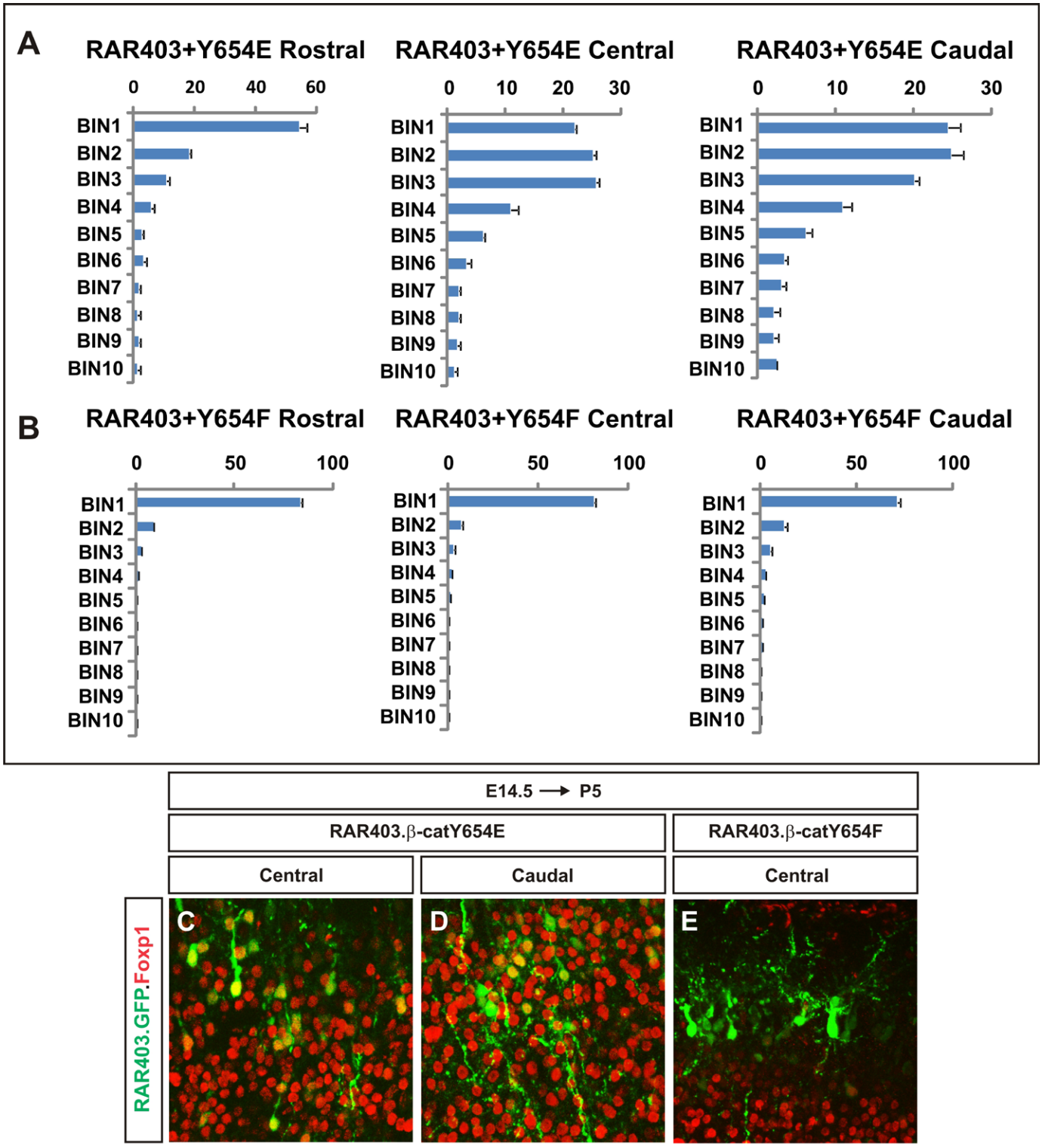

Figure S6. Cortical fate disruption elicited by RAR403.GFP expression is rescued by β-catenin.
(A, B) Analysis of cell body position of RAR403.GFP+ neurons coexpressed with β-catY654E or β-catY654F. Graphs show quantification of the number of neurons located in Bins distributed along the dorsal-ventral axis according to Figure 5C. Mean ± SEM; n= 6-8 animals. β-catY654E partially rescues the cell body position of RAR403 expressing neurons but β-catY654F does not. (C-E) Confocal images of coronal sections of mouse cortices electroporated at E14.5. Analyses at P5 shows that neurons coelectroporated with RAR403 and β-catY654E express Foxp1 and are distributed throughout lower layers of the central and caudal cortices; however, this is not the case when RAR403 is coelectroporated with β-catY654F

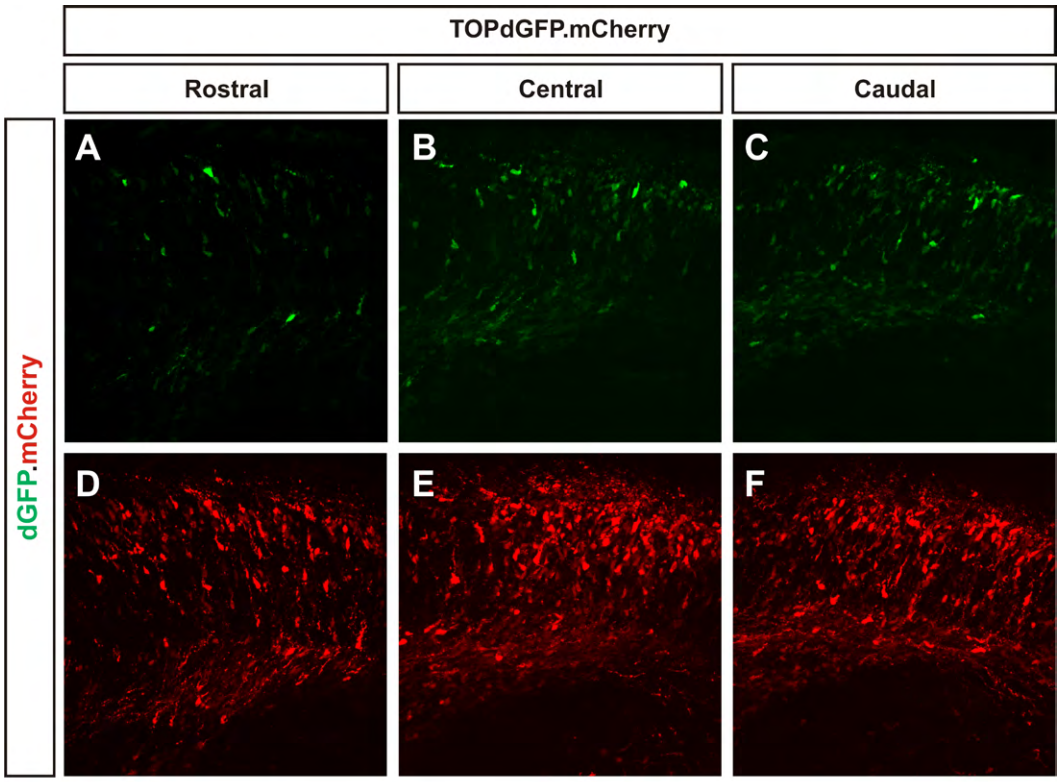

Figure S7. The TOPdGFP reporter gene is activated at central and caudal cortical regions.
(A-F) Representative sections of rostral, central and caudal cortices electroporated with TOPGFP and mCherry show GFP expression at central and caudal regions, consistent with region-specific activation of β-catenin.