# Mutually repressive interaction between Brn1/2 and Rorb contributes to the establishment of neocortical layer 2/3 and layer 4

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**Although several molecules have been shown to play important roles in subtype specification of neocortical neurons, the entire mechanism involved in the specification, in particular, of upper cortical plate (UCP) neurons still remains unclear. The UCP, which is responsible for intracortical connections in the neocortex, comprises histologically, functionally, and molecularly different layer 2/3 (L2/3) and L4. Here, we report the essential interactions between two types of transcription factors, Rorb (RAR-related orphan receptor beta) and Brn1/2 (Brain-1/Brain-2), for UCP specification. We found that Brn2 expression was detected in all upper layers in the immature UCP, but was subsequently restricted to L2/3, accompanied by up-regulation of Rorb in L4, suggesting demarcation of L2/3 and L4 during cortical maturation. Rorb indeed inhibited Brn2 expression and the expression of other L2/3 characteristics, revealed by ectopic expression and knockdown studies. Moreover, this inhibition occurred through direct binding of Rorb to the *Brn2* locus. Conversely, Brn1/2 also inhibited Rorb expression and the expression of several L4 characteristics. Together, these results suggest that a mutually repressive mechanism exists between Brn1/2 and Rorb expression and that the established expression of Brn1/2 and Rorb further specifies those neurons into L2/3 and L4, respectively, during UCP maturation.**

cerebral cortex | cell fate | transcription factor | layer formation

The mammalian neocortex consists of six anatomically distinct layers, each of which contains one or more subtype of neurons, and is characterized by a specific cell morphology, birth date, molecular identity, and connectivity to other regions of the central nervous system. The cortical plate (CP) can be categorized into two populations: lower and upper cortical plate (LCP and UCP). Neurons in the LCP, which consists of layer 5 (L5) and L6, send axons to subcortical targets such as the thalamus, pons, and spinal cord, whereas the UCP, which is known to be much thicker in primates than in rodents, is characterized by corticocortical (intracortical) connections (1–3). The UCP, in rodents, can be further divided into L2/3 and L4. Many L2/3 neurons show a typical pyramidal morphology and send axons to the contralateral cortex, whereas L4 neurons, showing a round shape and granular morphology, receive inputs from the thalamus and transmit the thalamic inputs to local cortical networks (4). Thus, although UCP neurons share at least some features (e.g., intracortical connections), there are apparent differences (e.g., long vs. local projections and cell morphologies). However, how these differences arise during development is poorly understood.

Previous studies have revealed the important roles of subtype- or lineage-specific transcription factors (TFs) in the specification of different neuronal subtypes (5–11). One of the intriguing features of how TFs specify neuronal subtypes is that a crucial TF for a given subtype sometimes suppresses other subtypes (3, 12). This mechanism might be fundamental, especially to obtain a couple of different subtypes with common characteristics. As mentioned earlier, the fact that L2/3 and L4 neurons are closely related yet different prompted us to investigate whether this molecular system plays a role in their differentiation.

In this study, we focused on two types of TFs: Rorb (RAR-related orphan receptor beta), and Brn1/Brn2 (Brain-1/Brain-2; also known as Pou3f3/Pou3f2; hereinafter, Brn1/2). Rorb belongs to the orphan nuclear receptor family and is preferentially expressed in L4 of the mature neocortex (13, 14). Brn1/2 are members of the class III POU (Pit1-Oct1/Oct2-UNC86) domain transcription factors and are preferentially expressed in L2/3 and L5 of the mature neocortex (15–17). Our detailed analysis of temporal expression profiles of Brn2 and Rorb suggested that UCP neurons initially possess common characteristics and then acquire distinct features. Furthermore, we studied repressive interactions between Rorb and Brn1/2 during the establishment of L2/3 and L4 in the developing neocortex.

## Results

**Expression Patterns of Brn2 and Rorb in the Developing CP.** Given that the possibility that UCP neurons initially arise with a common fate but acquire the specific characteristics of L2/3 and L4 during maturation, we sought to identify molecules that initially show uniform expression in the UCP, but become demarcated into L2/3 and L4 during maturation. Our analysis revealed Brn2 protein as one such molecule. We found the rather uniform expression of Brn2 in the UCP on embryonic day (E)16.5 and E18.5, using Cux1 (cut-like homeobox 1) expression as a reference of the entire UCP population (18) (Fig. 1*A* and Fig. S1*A*). Although a number of neurons in the LCP and intermediate zone also expressed Brn2, many of these neurons coexpressed Cux1 (arrows in Fig. 1*A*), suggesting they were migrating neurons destined for UCP neurons. In addition, some neurons were positive for Brn2 but negative for Cux1

### Significance

**The mammalian neocortex consists of six histologically distinct layers, called layer 1 (L1) to L6. Each layer contains several subtypes of neurons, which are characterized by specific cell morphology, gene expression profile, and connectivity to other regions of the central nervous system. As these neurons are mostly born from common progenitor cells, it is important to know how progenitor cells acquire a certain cell type during development. Here, we reveal the essential interactions between two types of transcription factors, Brn1/2 and Rorb, for subtype specification. Brn1/2 and Rorb are expressed in L2/3 and L4, respectively, in the mature neocortex. We found that Brn1/2 and Rorb repress each other and that this reciprocal repression is important for L2/3 and L4 specification.**

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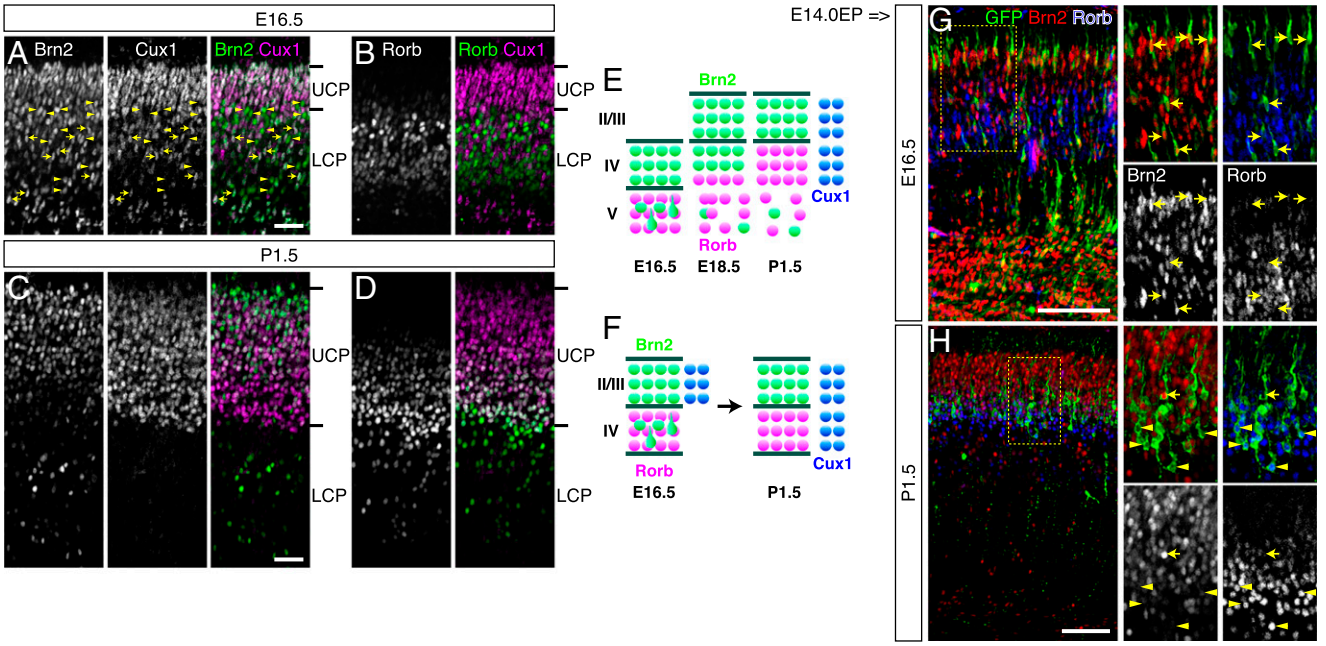


**Fig. 1.** Expression profiles of Brn2 and Rorb in the developing CP. (*A–D*) Immunohistochemical analyses for Brn2, Cux1, and Rorb were performed in the E16.5 (*A* and *B*) and P1.5 (*C* and *D*) neocortices. In *A* and *C*, Brn2 and Cux1 are shown in green and magenta, respectively. In *B* and *D*, Rorb and Cux1 are shown in green and magenta, respectively. The arrowheads and arrows show the cells positive for Brn2, but negative for Cux1, and positive for both, respectively. (*E* and *F*) Schematic representations of the expression patterns of Brn2, Cux1, and Rorb during the development of the CP. Two possibilities are considered: (*E*) that the immature UCP neurons at first share common characteristics, but with the progression of cortical maturation, acquire specific cell identities; or (*F*) that L2/3 and L4 exhibit distinct characteristics from the beginning, but the expression of Cux1 changes over time. (*G* and *H*) A GFP expression vector was electroporated into E14.0 brains, and then E16.5 (*G*) and P1.5 (*H*), brains were analyzed. The sections were immunostained for Brn2 (red), Rorb (blue), and GFP (green). The boxed regions are shown at higher magnification. The arrows and arrowheads indicate $Brn2^+/Rorb^-$ and $Brn2^-/Rorb^+$ cells, respectively. (Scale bars: 100 μm in *A* and *C*; 200 μm in *G* and *H*.)

beneath the immature UCP (arrowheads in Fig. 1*A*). These neurons were thought to be future L5 neurons, because Brn2 is also expressed in some L5 neurons in the mature CP (Fig. S1*C*) (16). The strong expression of Brn2 on postnatal day (P)1.5 and P3.5 became restricted to the upper half of the UCP, which corresponds to the developing L2/3, and only weak or no expression was observed in the lower half of the UCP, which corresponds to the developing L4 (Fig. 1*C* and Fig. S1*C*).

We next examined the expression profile of Rorb in detail, as this protein is well known to be specifically expressed in L4 neurons of the mature neocortex (13, 14). Interestingly, Rorb expression on E16.5 and E18.5 was mainly restricted to LCP neurons, as judged by almost no [on E16.5 (Fig. 1*B*)] or little [on E18.5 (Fig. S1*B*)] overlap with Cux1. On P1.5 and P3.5, strong expression of Rorb was detected in $Cux1^+$ neurons (Fig. 1*D* and Fig. S1*D*), similar to that in mature L4. These expression profiles support the notion that immature UCP neurons initially possess common characteristics, but go on to acquire specific identities as maturation of the cortex progresses (Fig. 1*E*).

**Shift from Brn2-Positive to Rorb-Positive in Future L4 Neurons.** The developmental changes of Rorb and Brn2 expression alternatively could be explained by the shift of Cux1 expression from L2/3 to L2–4 (Fig. 1*F*). In this model, future L4 neurons never express Brn2, as opposed to the former model. Future L4 neurons were identified by labeling E14.0 progenitors with green fluorescence protein (GFP), using an in utero electroporation system (19–21). On E16.5, a majority of the GFP-positive ($GFP^+$) cells expressed Brn2, but not Rorb (Fig. 1*G*). In contrast, on P1.5 and P3.5, a majority of the $GFP^+$ cells expressed Rorb but not Brn2 (Fig. 1*H* and Fig. S1*F*). E18.5 CP appeared to be in a transition stage, as both $Brn2^+/Rorb^-$ and $Brn2^-/Rorb^+$ were frequently found (Fig. S1*E*). This result, that future L4 neurons once express Brn2 and then express Rorb with reduction of Brn2 (Fig. 1*E*), suggests there could be interplay between these TFs.

**Rorb Suppresses Expression of Brn2, as Well as L2/3 Characteristics.** The reciprocal expression pattern of Brn2 and Rorb in the mature neocortex raises at least two possibilities if there is any regulatory relationship; the first is that Rorb expression is initially induced in L4, which then inhibits Brn2 expression in these neurons. The other is that, if Brn2 inhibits Rorb expression, down-regulation of Brn2 in L4 would induce Rorb expression in these neurons.

To test the former possibility, we first examined the effect of ectopically expressed Rorb on Brn2 expression. Because persistent expression of Rorb from neural progenitors inhibited cell migration, as reported previously (22), we used a neuron-specific Tα1 (α1-tubulin) promoter-driven expression system. Ectopic Rorb expression after neuronal differentiation, in fact, did not cause migration defect. We found that ectopic Rorb expression reduced the percentage of $Brn2^+$ neurons in L2/3 (Fig. 2 *A–C*), suggesting that Rorb potentially inhibits Brn2 expression.

To further investigate this inhibitory effect on Brn2, we knocked down the expression of endogenous Rorb by an RNA interference (RNAi) technique with short hairpin RNAs (shRNAs) in future L4 neurons. Immunohistochemistry for Rorb confirmed the efficiency of the constructed two shRNAs (hereinafter, Rorbsh#1 and Rorbsh#2) (Fig. S2 *A* and *B*). We then examined the expression of Brn2 in the Rorb-knockdown neurons and found a marked increase in the percentage of $Brn2^+$ cells compared with controls (Fig. 2 *G–J* and Fig. S2 *D–G*). The fairly specific expression of Rorb in maturing L4 also raised the possibility that Rorb might regulate other subtype-specific characteristics. Immunohistochemistry for several markers showed that Rorb knockdown indeed increased the percentage of neurons positive for Tbr1 (T-box brain gene 1), a L2/3 and L6 marker (23) (Fig. 2 *K–N* and Fig. S2 *H–K*). Conversely, it decreased the percentage of neurons positive for Cux1, which is strongly expressed in L4 but only moderately in L2/3 (24) (Fig. S2*C*), although this effect was weak, especially in the case of Rorbsh#1 (Fig. S2 *L–O*). Importantly, these changes of marker expressions by Rorb knockdown were rescued well with the

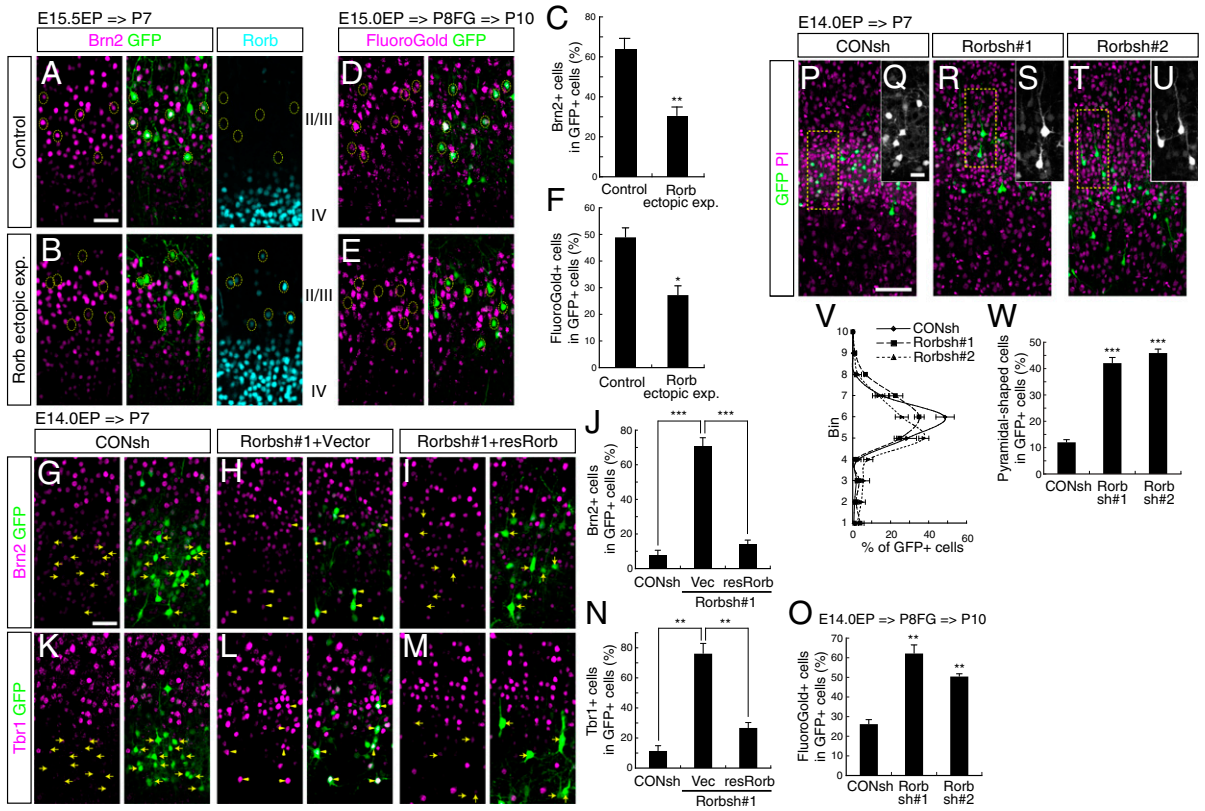


**Fig. 2.** Rorb suppresses Brn2 expression and L2/3 characteristics. (*A–F*) Control or Rorb expression vector together with a GFP vector was electroporated into E15 brains, and then P7 (*A–C*) or P10 (*D–F*) brains were analyzed. In *D–F*, FluoroGold solution was injected to the contralateral side of the electroporated hemisphere at P8. The sections were immunostained for Brn2 (magenta), GFP (green), and Rorb (cyan). FluoroGold fluorescence was shown in magenta in *D* and *E*. *C* and *F* show quantification. (*G–N*) shRNA vectors and a rescue vector (resRorb) were electroporated into E14.0 brains as indicated, and then P7 brains were analyzed. The sections were immunostained for Brn2 (*G–I*) and Tbr1 (*K–M*). The arrows and arrowheads indicate a maker-negative and positive cells, respectively. *J* and *N* show quantification. (*O*) CONsh, Rorbsh#1, or Rorbsh#2 vector was electroporated into E14.0 brains. FluoroGold solution was injected at P8, and then P10 brains were analyzed for quantification. (*P–U*) Experiments were performed as described in *G*, except that the brain sections were counterstained with propidium iodide (PI, magenta). The boxed regions are shown at higher magnification. (*V*) Quantitative data of cell positioning from *P*, *R*, and *T* are presented. (*W*) Quantitative data of cell morphology are presented. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$. (Scale bars: 100 μm in *A*, *D*, and *G*; 200 μm in *P*; 50 μm in *Q*.)

expression of a shRNA-resistant variant of Rorb (Fig. 2 *I*, *J*, *M*, and *N*, and Fig. S2*S*).

To further characterize the Rorb-expressed neurons in L2/3, we examined axonal projection patterns by retrograde labeling with a fluorescent dye FluoroGold that was injected into the contralateral cortex. We found that ectopic Rorb expression reduced the percentage of callosal projection neurons in L2/3, from which neurons normally project to the contralateral side through the corpus callosum (Fig. 2 *D–F*). Conversely, Rorb knockdown increased callosal projections in the E14.0-born neurons, which normally project short distances to make local networks (Fig. 2*O* and Fig. S2 *P–R*). These results suggest that Rorb would regulate not only molecular properties but also the projection profile of neurons.

We also noticed alteration of cell positioning by Rorb knockdown. The distribution of control cells that had been electroporated on E14.0 was highly restricted to L4, whereas the Rorb-knockdown neurons were distributed more broadly (Fig. 2 *P*, *R*, *T*, and *V*). Although the disrupted cell positioning could have been caused by impaired neuronal migration in the embryonic stages, it was not affected by Rorb knockdown (Fig. S3). This abnormal distribution of the Rorb-knockdown neurons seemed to be rescued with shRNA-resistant Rorb only when the neurons expressed moderate levels of Rorb (Fig. S2*T*, arrowheads), whereas excessive Rorb appeared to cause disruption of cell positioning (Fig. S2*T*, arrows), suggesting that regulation of the Rorb protein levels and/or its expression timing might also be important. These results suggest that Rorb is required for correct positioning of postmigratory L4 neurons, which is consistent with a previous report showing that ectopic Rorb expression induced the formation of ectopic cortical "barrels" containing tightly packed L4 neurons (22).

Another phenotypic feature that we noticed was the change in cell morphology. Control L4 neurons showed a round and granular morphology, with dendrites extending in all directions (stellate cells) (25), whereas the Rorb-knockdown neurons showed a pyramidal shape with an apical dendrite (Fig. 2 *Q*, *S*, *U*, and *W*), similar to typical pyramidal neurons, including those in L2/3.

Together, these results suggest that Rorb regulates the differentiation of L4 neurons by inhibiting Brn2 expression, as well as several other L2/3 characteristics.

**Rorb Protein Directly Binds to the Consensus Sites Near the *Brn2* Gene in Vivo.** We next asked whether Rorb directly inhibited Brn2 expression. We used the ECR Browser (26) to identify conserved genomic regions between mice and humans, which often include gene regulatory elements. In addition, we searched for putative ROR family binding sites around the *Brn2* gene, using the rVista program (27), which is interconnected with the ECR Browser and is capable of identifying conserved consensus sites of TFs. We found two putative ROR binding sites in a highly conserved region about 8 kb upstream of the *Brn2* transcription start site (Fig. 3*A*). To examine the binding of Rorb protein to this region, we performed chromatin immunoprecipitation (ChIP) analysis, using chromatins from P3.5 neocortices. ChIP with a Rorb antibody resulted in marked enrichment of this region compared with that using control IgG (Fig. 3*B*), suggesting that Rorb binds to this region to repress Brn2 expression in L4. We therefore performed luciferase assays using reporter constructs

containing the identified ROR binding sites to test the effect on transcription. Overexpression of Rorb was found to repress the luciferase activity of each reporter (BS1-luc, BS-2-luc) in Neuro2a cells (Fig. 3*C*).

To investigate the requirement of Rorb in suppression of these elements in vivo, we next introduced the reporter constructs into L4 neurons by in utero electroporation together with a Rorb knockdown vector. The Rorb knockdown increased the activity of these reporters (Fig. 3*D*), suggesting that endogenous Rorb plays a repressive role on these elements, which would lead to suppression of Brn2 expression. Taken together, these results suggest that Rorb negatively regulates *Brn2* transcription via direct binding to the conserved consensus sites.

**Brn1/2 Suppress Rorb Expression and L4 Characteristics.** We next examined the other possibility underlying the reciprocal expression of Brn2 and Rorb, that of Brn2 inhibiting Rorb expression. In this scenario, a reduction of Brn2 in L4 first takes place, resulting in induction of Rorb. We first looked at the inhibitory effect of ectopically expressed Brn2 on Rorb expression. Brn2 expression in L4 neurons was found to inhibit the expression of Rorb (Fig. 4 *A*–*C*). Interestingly, Brn2 expression also changed the morphology and positioning of the transfected cells. Many of the Brn2-expressing cells were positioned in L2/3, showed a pyramidal morphology, and were negative for Rorb (the circled cells in Fig. 4*B*). Because malpositioning of future L4 neurons into L2/3 by itself could cause them to lose Rorb expression (28), we also examined the Brn2-expressing cells remaining in L4. Many of these cells were negative for Rorb as well (arrows in Fig. 4*B*), suggesting that Brn2 inhibits Rorb expression, irrespective of cell positioning.

To further investigate the inhibitory effect of Brn2 on Rorb expression, we knocked down the expression of endogenous Brn2 together with Brn1, a closely related gene expressed similarly to Brn2 (17, 29, 30). Indeed, either Brn2 or Brn1 knockdown showed little effect, although knockdown worked well (Fig. S4 *A*–*D*). Brn1/2 double knockdown, instead, increased markedly the percentage of Rorb$^+$ cells compared with controls (Fig. 4 *G*, *H*, and *J*). In addition, immunohistochemistry for other subtype-specific markers showed that Brn1/2 knockdown decreased the number of Tbr1$^+$ cells (Fig. 4 *K*, *L*, and *N*), although it did not affect Cux1 expression (Fig. S4 *E*–*G*). These results suggest that Brn1/2 knockdown changes some, but not all, of the characteristics of the transfected L2/3 neurons to those of L4 neurons. Importantly, these changes of marker expressions by Brn1/2 knockdown were well rescued by expression of a shRNA-resistant variant of Brn2 (Fig. 4 *I*, *J*, *M*, and *N*, and Fig. S4*J*).

Axonal projection patterns were also examined, and ectopic Brn2 expression was found to be sufficient for the future L4 neurons to project callosally to the contralateral cortex (Fig. 4 *D*–*F*). Moreover, Brn1/2 knockdown decreased callosal projections by the E15.0-born (L2/3) neurons, which are normally callosal projection neurons (Fig. 4*O* and Fig. S4 *H* and *I*).

We also examined the cell positioning of the Brn1/2-knockdown neurons born at E15.0. Control cells were mainly located in L2/3, whereas the Brn1/2-knockdown neurons were mainly located in L4 (Fig. 4 *P*, *R*, and *T*). This was not caused by perturbed radial migration because the migration of the knockdown neurons in the embryonic stages was normal (Fig. S4 *K* and *L*). These results suggest that Brn1/2 regulate the correct positioning of post-migratory L2/3 neurons, presumably by inhibiting the expression of the factor or factors that control the positioning of L4 neurons.

With respect to the cell morphology, control neurons in L2/3 showed a pyramidal morphology with an apical dendrite, whereas the Brn1/2-knockdown neurons showed a stellate morphology (Fig. 4 *Q*, *S*, and *U*) similar to that of L4 neurons. Taken together, these results suggest that Brn1/2 regulate the differentiation of L2/3 neurons by inhibiting Rorb expression, as well as several other L4 characteristics.

**Brn1/2-Knockdown Phenotypes Are Dependent on Rorb Deregulation.** To examine whether the phenotypes caused by Brn1/2 knockdown depend on up-regulation of Rorb, we introduced both Brn1/2- and Rorb-knockdown vectors into the E15.0-born neurons. Immunohistochemistry for Tbr1 revealed that Brn1/2&Rorb double knockdown restored the percentage of Tbr1$^+$ cells (Fig. S5 *A*–*C*), cell positioning (Fig. S5 *D*, *F*, and *H*), and cell morphology (Fig. S5 *E*, *G*, and *I*). Collectively, these data indicate that the deregulation of Rorb is required for the acquisition of L4 characteristics in the Brn1/2-knockdown neurons and that Brn1/2 restricts the latent differentiation program to L4 by repressing Rorb expression in L2/3 neurons in the normal condition.

We also tried to examine the effect of double knockdown in future L4 neurons. However, Brn1/2 knockdown itself caused various differentiation phenotypes, including abnormal expression of Tbr1 and disrupted cell positioning (Fig. S5 *J*–*L*), preventing us from performing further analyses. However, these results, in another aspect, suggest that the initial expression of Brn1/2 in the L4 lineage is required for correct differentiation and that Brn1/2 have multiple functions in a context-dependent manner in the specification of UCP neurons.

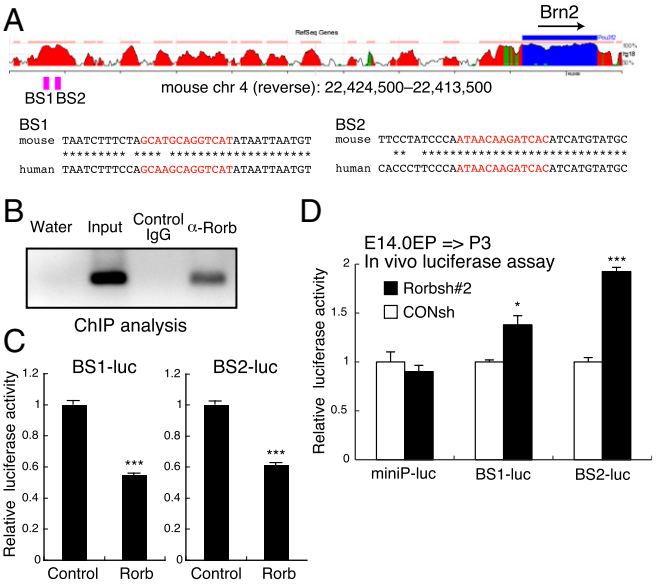


**Fig. 3.** Rorb directly binds to an upstream region of the *Brn2* gene and negatively regulates the elements. (*A*) Schematic representation of the mouse *Pou3f2* (*Brn2*) locus and conservation with the human genome. Two binding sites (BS) of Rorb on this locus were predicted by rVista. (*B*) ChIP-PCR analysis in cortical neurons obtained from the P3.5 neocortex. DNA immunoprecipitated with control mouse IgG or anti-Rorb antibody was amplified by PCR, using primers flanking the Rorb binding sites. (*C*) Luciferase assay showing that Rorb inhibits the firefly luciferase activity. Neuro2a cells were transfected with reporter plasmids containing either of the Rorb binding sites (BS1-luc or BS2-luc), together with an empty vector (Control) or Rorb expression vector. (*D*) E14.0 cortices were electroporated with the luciferase vectors together with CONsh or Rorbsh#2. The P3 brains were lysed and subjected to luciferase analysis. $*P < 0.05$, $***P < 0.0005$.

## Discussion

The high complexity of neuronal subtypes in the mammalian neocortex implies the existence of a huge variety of underlying mechanisms to generate a full set of neuronal subtypes. Here, we studied the fate specification mechanisms of the UCP during cortical development. The UCP, comprising L2/3 and L4, has been demarcated traditionally by histological features and more recently by the molecular properties, as is the case for other cortical layers. We found that the immature UCP is not yet fully differentiated and shares common characteristics; Brn2, expressed in mature L2/3, is uniformly expressed in immature L2–4, and Rorb, expressed in mature L4, is not expressed in the immature UCP (Fig. 1*E*). We further demonstrated that the reciprocal expressions of Brn2 and Rorb in L2/3 and L4 were established by their mutually repressive interactions in

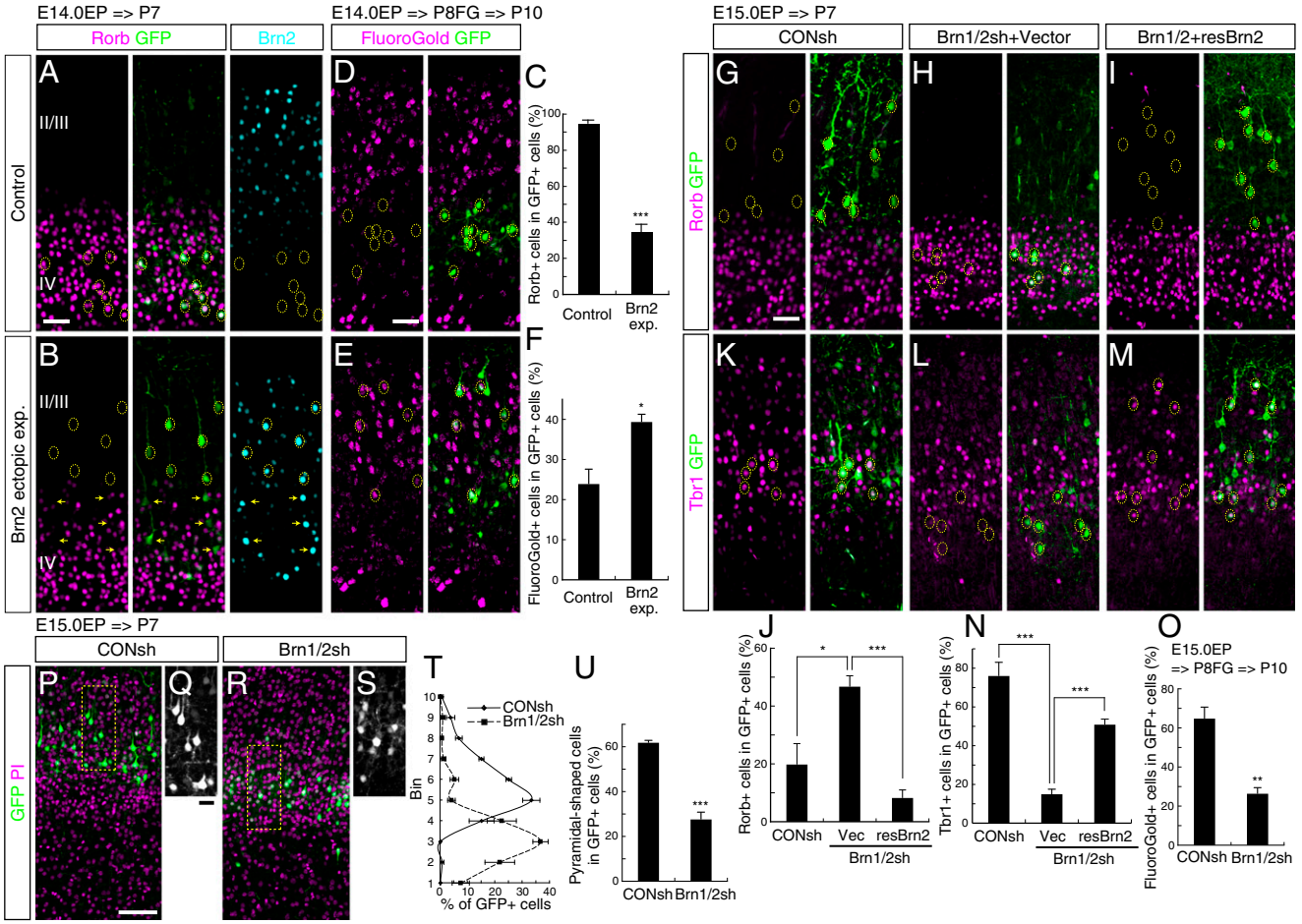


**Fig. 4.** Brn1/2 suppress Rorb expression and L4 characteristics. (*A–F*) Control or Brn2 expression vector together with a GFP vector was electroporated into E14.0 brains, and then P7 (*A–C*) or P10 (*D–F*) brains were analyzed. In *D–F*, FluoroGold solution was injected to the contralateral side of the electroporated hemisphere at P8. The sections were immunostained for Rorb (magenta), GFP (green), and Brn2 (cyan). FluoroGold fluorescence was shown in magenta in *D* and *E*. *C* and *F* show quantification. (*G–N*) shRNA vectors and a rescue vector (resBrn2) was electroporated into E15.0 brains, and then P7 brains were analyzed. The sections were immunostained for Rorb (*G–I*) and Tbr1 (*K–M*). *J* and *N* show quantification. (*O*) CONsh or Brn1/2sh vectors were electroporated into E15.0 brains. FluoroGold solution was injected at P8, and then P10 brains were analyzed for quantification. (*P–S*) Experiments were performed as described in *G*, except that the brain sections were counterstained with PI (magenta). The boxed regions are shown at higher magnification. (*T*) Quantitative data of cell positioning from *Q* and *S* are presented. (*U*) Quantitative data of cell morphology are presented. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$. (Scale bars: 100 μm in *A*, *D*, and *G*; 200 μm in *P*; 50 μm in *Q*.)

postnatal stages. Furthermore, the knockdown experiments showed that each of Brn1/2 and Rorb is also required for correct differentiation of the subtype. The misspecified neurons by RNAi, to our surprise, acquired the cell fate of the other subtype, including subtype-specific markers, cell positioning, and cell morphology. We propose that mutually repressive interactions of Brn1/2 and Rorb lead to the establishment of L2/3 and L4 in the UCP (Fig. S5*M*). Such mutually repressive interactions have also been observed in other fate specifications (3, 12), suggesting broader use of this type of interactions for cortical subtype specification.

Interestingly, in the double-knockdown experiments, we observed that the future L2/3 neurons without the Rorb–Brn1/2 interplay could differentiate into L2/3-like neurons with the expression of a subtype-specific marker, cell positioning, and morphology (Fig. S5 *A–I*). These results suggest not only the requirement of Rorb for Brn1/2-knockdown-induced phenotypes but also that Brn1/2 function is mainly to repress Rorb expression and not required for L2/3 specification per se and that differentiation into L2/3 neurons is highly robust and is the default state behind the Rorb–Brn1/2 interplay.

How is the reciprocal expression pattern of Rorb and Brn1/2 regulated at molecular levels? We demonstrated that Rorb binds to the upstream region of the *Brn2* gene, thereby inhibiting *Brn2* expression. Conversely, we found a putative binding site of Brn2 in the upstream region of the *Rorb* gene, although Brn2 could not inhibit the transcriptional activity mediated by this site (K.O. and K.N., unpublished data). A recent study showed that Brn2 increased the expression of Rorb in the embryoid body (31) as opposed to the effect observed in cortical neurons, suggestive of a context-dependent action of Brn2. There may be a context-dependent downstream effector or effectors of Brn2 that inhibit *Rorb* expression. Tbr1 could be such an effector in the UCP, as its expression was markedly increased by Brn2 (Fig. S4*M*).

Brn1/2 are expressed from neural progenitors to mature neurons. Earlier knockout mouse studies showed that Brn1/2 had multiple roles such as in proliferation, migration, and cell fate specification (29, 30), leaving it an open question whether Brn1/2 play a role in subtype specification in maturing neurons, especially in L2/3, where Brn1/2 are eventually expressed. Recently, POU-III transcription factors including Brn1/2 were reported to play an important role to specify upper layer fate in neural progenitors (17). Our knockdown experiments targeting Brn1/2, however, did not appear to affect progenitor and migrating cells but only could inhibit the late function of Brn1/2, presumably because of late onset of RNAi effects. Collectively, it is conceivable that Brn1/2 initially determine upper layer fate in progenitor cells and further specify L2/3 identity when they are expressed persistently in neurons.

The mechanism or mechanisms that make the border between L2/3 and L4 remain to be determined. Because the maturation of L4 neurons couples with the arrival of thalamocortical axons (TCAs) into the CP (4), TCAs would be an attractive candidate. Previous studies showed that synaptic activities from TCAs to cortical neurons were not essential for the initial specification of L4 neurons but only required for their maturation (e.g., barrel formation) (32–34). In addition, absence of TCAs themselves in the CP leads to misspecification of L4 identity (28, 35). It is thus possible that soluble factors or membrane proteins on TCAs, rather than their synaptic activities, may regulate the expression of Rorb or Brn1/2. Once the balance between Rorb and Brn1/2 is altered, the repressive interaction between them would work as a positive feedback loop; for example, when Rorb is increased, Brn1/2 are decreased, leading to further up-regulation of Rorb. This may contribute to the establishment of the clear border between L4 and L2/3. Other mechanisms might also be involved for this demarcation. One candidate is Cux1, which is highly expressed in L4 and could bind to the *Rorb* promoter through the putative binding sites predicted in silico (www.sabiosciences.com/chipqpcrsearch.php?app=TFBS).

In the developing spinal cord, neural progenitors are first specified into different dorsal–ventral progenitor domains, and the particular combination of TFs expressed by a progenitor domain regulates the postmitotic identity of generated neurons (36–38). The primordium of the neocortex is not parcellated and is relatively even, although there exist some molecular gradients, suggesting that other mechanisms than progenitor parcellation must be involved. One mechanism is sequential changes of TF expression in progenitors. Another is postmitotic regulation of fate specification, which was studied here. Although this mechanism is also seen in the spinal cord (37, 38), its role might be greatly expanded along the development and evolution of the neocortex that contains a larger spectrum of neuronal subtypes.

## Materials and Methods

Pregnant ICR (Institute for Cancer Research) mice were purchased from Japan SLC. The morning of vaginal plug detection was designated as E0.5. The day of birth, usually E19.5, was designated as P0.5. All animal experiments were performed in accordance with Institutional Guidelines on Animal Experimentation at Keio University. Details of plasmids, in utero electroporation, immunohistochemistry, FluoroGold injection, luciferase assay, quantitative analysis of the brain slices, and ChIP assay are provided in *SI Materials and Methods*.

**Statistical Analysis.** The statistical data were represented as mean ± SEM. Statistical analysis was performed using the two-tailed Welch's *t* test. Differences between groups were considered to be significant at $P < 0.05$.

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