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Sequential phases of cortical specification involve *Neurogenin*-dependent and -independent pathways

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Neocortical projection neurons, which segregate into six cortical layers according to their birthdate, have diverse morphologies, axonal projections and molecular profiles, yet they share a common cortical regional identity and glutamatergic neurotransmission phenotype. Here we demonstrate that distinct genetic programs operate at different stages of corticogenesis to specify the properties shared by all neocortical neurons. *Ngn1* and *Ngn2* are required to specify the cortical (regional), glutamatergic (neurotransmitter) and laminar (temporal) characters of early-born (lower-layer) neurons, while simultaneously repressing an alternative subcortical, GABAergic neuronal phenotype. Subsequently, later-born (upper-layer) cortical neurons are specified in an *Ngn*-independent manner, requiring instead the synergistic activities of *Pax6* and *Tlx*, which also control a binary choice between cortical/glutamatergic and subcortical/GABAergic fates. Our study thus reveals an unanticipated heterogeneity in the genetic mechanisms specifying the identity of neocortical projection neurons.

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Introduction

Neuronal diversity in the neocortex is striking, with the human neocortex subdivided into more than 40 tangential areas and six radial layers, each characterized by unique neuronal morphologies, cytoarchitectures, axonal projections and molecular identities (Job and Tan, 2003). During development, neocortical neurons are generated sequentially, with multipotent progenitors in the dorsal telencephalon initially giving rise to neurons in the cortical preplate, followed by lower-layer (layers V/VI) and finally upper-layer (layers II–IV) neurons of the cortical plate (Caviness, 1982). In addition to their unique properties, cortical projection neurons share several essential properties, including their regional identity, as defined by common gene-expression profiles in progenitors and neurons, and use of glutamate as an excitatory neurotransmitter. They can be distinguished from cortical interneurons, which are born and differentiate in the ventral telencephalon, reach the neocortex by tangential migration, express distinct ventral-specific regional markers and use GABA as a neurotransmitter (Parnavelas *et al*, 2000). A question that remains unanswered is whether a single genetic pathway specifies all common features of neocortical neurons, or whether distinct genetic programs are sequentially activated to specify the features that are both common and unique to projection neuron populations in each cortical layer.

The known molecular determinants of cortical identity include three homeodomain (HD) transcription factors, *Lhx2, Emx2* and *Pax6*, that act either alone or in combination to pattern the telencephalon and establish a cortical territory (Bulchand *et al*, 2001; Monuki *et al*, 2001; Muzio *et al*, 2002). *Pax6* and *Emx2* are also required to establish regional identities along the tangential axis of the neocortex, setting up territories that are thought to prefigure the formation of cortical areas (Bishop *et al*, 2000; Mallamaci *et al*, 2000). In contrast, the molecules involved in specifying laminar fates and a glutamatergic neurotransmitter phenotype remain virtually unexplored. Given the central role that *Pax6* plays in cortical development, it is of particular interest that *Pax6* directly activates *Ngn1* and *Ngn2*, two highly related basic–helix–loop–helix (bHLH) transcription factors (Scardigli *et al*, 2003). *Ngn*s have been implicated in multiple cell fate choices in the nervous system, including the selection of neural progenitors, specification of neuronal phenotype at the expense of glial cell fates, and choice of neuronal differentiation programs (Bertrand *et al*, 2002). In the telencephalon, *Ngn*s are specifically expressed in cortical and not subcortical progenitors, where they specify the regional identity of the earliest-born preplate neurons in the neocortex (Fode *et al*, 2000). Here we examine the function of *Ngn1*, *Ngn2*, *Pax6* and *Tlx* in specifying the cortical regional identity, glutamatergic neurotransmitter phenotype and laminar-specific properties of neurons in the cortical plate.

Results

Ngn1 and Ngn2 specify a glutamatergic, cortical phenotype and repress GABAergic, subcortical genes

We analyzed the role of *Ngn1* and *Ngn2* in specifying the identity of neocortical neurons. To evaluate specification defects resulting from *Ngn* mutations at the early stage of corticogenesis, we profiled and compared gene expression in wild-type versus *Ngn1* and *Ngn2* single-, and *Ngn1;Ngn2* double-mutant cortices at embryonic day (E) 13.5. Hybridization of total cortical cDNA to Affymetrix microarrays revealed a significant number of up- and downregulated genes in all genotypes, except in *Ngn1* mutants, which were not investigated further (Figure 1A).

Affymetrix data and RNA *in situ* hybridization for non-represented genes revealed no deregulation of genes normally expressed in dorsal telencephalic progenitors (*Gli3*, *Pax6*, *Emx2*, *Lhx2*, *Foxg1*, *Otx1*, *Tlx*, *COUP-TFI)* in *Ngn* mutants (Figure 1A, Supplementary Figure S1). In contrast, several transcription factors specifically expressed by cortical neurons (*Math2*, *Nscl1*, *NeuroD*, *NeuroD2*, *Tbr1*, *Tbr2)* were reduced in *Ngn2* mutant cortices, and more severely downregulated in *Ngn1;Ngn2* mutants (Figure 1A–D). *Vesicular glutamate transporter1* (*VGLUT1)* and *VGLUT2*, which load glutamate into synaptic vesicles (Fremeau *et al*, 2001), were also downregulated in *Ngn* mutants (Figure 1A). In E13.5 telencephalic sections, *VGLUT* genes were specifically expressed in dorsal neurons that use glutamate as a neurotransmitter, with VGLUT1 protein predominant in preplate (PP) and cortical plate (CP) neurons (Figure 1F), whereas *VGLUT2* was transiently expressed in differentiating cortical neurons in the subventricular zone (SVZ; Figure 1J). In *Ngn2* mutants, VGLUT1 protein and *VGLUT2* transcript levels were reduced in dorsomedial and not lateral cortical neurons, likely due to compensation by *Ngn1*, which persists in lateral domains (Fode *et al*, 2000), whereas defects extended throughout the cortex of *Ngn1;Ngn2* double mutants (Figure 1F–H and J–L). *Ngn*s are thus required to activate cortical- and glutamatergic-specific differentiation programs in early-born CP neurons, likely acting downstream of cortical patterning genes, which are normally expressed in *Ngn* mutants, and cannot compensate for the loss of *Ngn* activity.

Mash1 is upregulated in *Ngn* mutant cortical progenitors, and was previously linked to the ectopic expression of subcortical genes *Dlx1* and *GAD1* in PP neurons (Fode *et al*, 2000). Gene profiling at E13.5 revealed a more extensive upregulation of subcortical genes in *Ngn2* and *Ngn1;Ngn2* mutants that included ventral telencephalic transcription factors (*Mash1*, *Dlx1*, *Dlx2*, *Dlx5*, *Brn4*), biosynthetic enzymes for GABA (*glutamic acid decarboxylase 1* (*GAD1)*, *GAD2*) and GABA transporters (*GABA transporter 1* (*GABA-T1*), *GABA and glycine transporter* (*GABA/glyT*)), suggesting that many, and possibly all, components of a subcortical, GABAergic differentiation program were ectopically activated in *Ngn* mutant cortical neurons (Figure 1A). Consistent with a switch of neurotransmitter phenotype, *GAD1* was ectopically expressed in the PP/CP and SVZ of *Ngn2* and *Ngn1;Ngn2* mutants (Figure 1N–P). The ectopic ventral-like neurons were misspecified neurons of cortical origin, and not subcortical neurons that had inappropriately migrated into the cortex, based on previous explant and migration studies (Fode *et al*, 2000; Chapouton *et al*, 2001). Moreover, *GAD1*

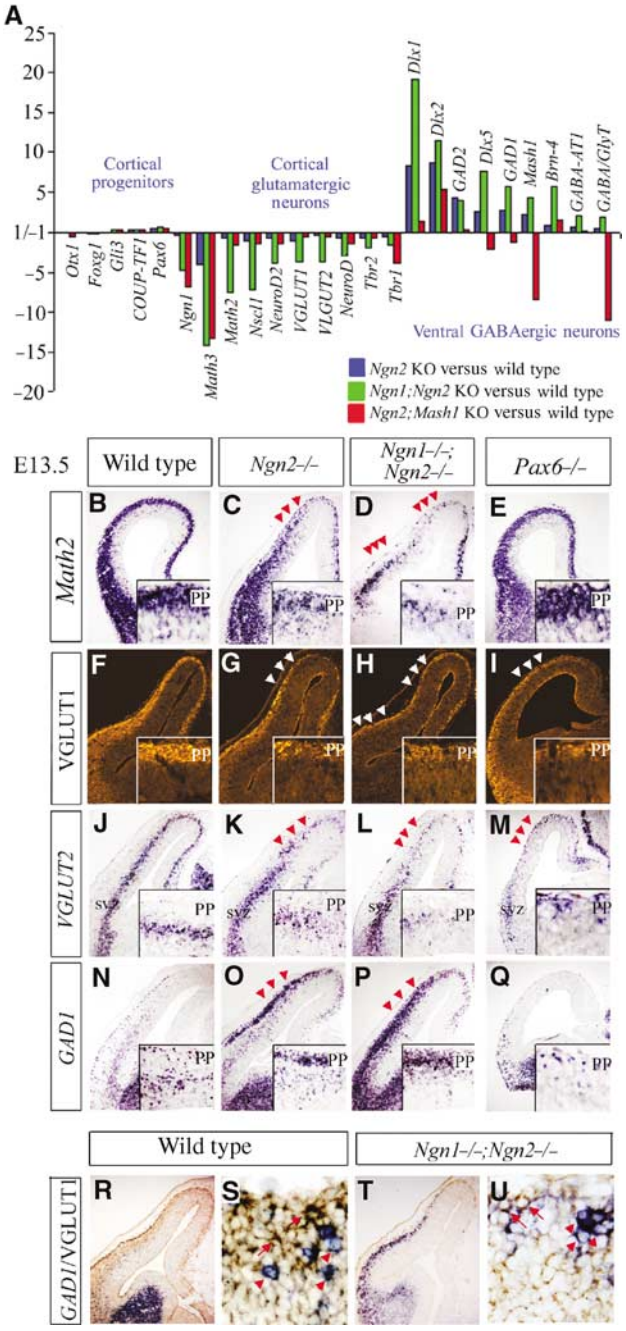

Figure 1 Gene profiling reveals a global shift of neuronal phenotype from cortical, glutamatergic to subcortical, GABAergic in E13.5 *Ngn* mutants. (**A**) Expression profiling in E13.5 cortices using Affymetrix microarrays, showing fold differences in gene expression, comparing *Ngn2* (blue bar), *Ngn1;Ngn2* (green bar) and *Ngn2;Mash1* (red bar) mutants to wild type. (**B–E**) Expression of *Math2* in PP and early-born CP neurons was reduced in *Ngn2* and *Ngn1;Ngn2* mutants (arrowheads, C, D). Expression of VGLUT1 protein in the PP (**F–I**) and *VGLUT2* transcripts (**J–M**) in the SVZ was reduced in *Ngn2*, *Ngn1;Ngn2* and *Pax6* mutants (arrowheads, G-I, **K–M**). (**N–Q**) *GAD1* was ectopically expressed in the PP/SVZ of *Ngn2* and *Ngn1;Ngn2* mutants (arrowheads, O, P). (**R–T**) Double staining of *GAD1* RNA (blue) and VGLUT1 protein (brown) in wild-type (R, S) and *Ngn1;Ngn2* (T, **U**) mutants showing that most cells express either the glutamatergic (arrows) or the GABAergic (arrowheads) marker. PP, preplate; SVZ, subventricular zone.

transcripts and VGLUT1 protein were for the most part detected in complementary sets of cortical neurons in both wild-type and *Ngn1;Ngn2* mutant cortices (Figure 1R–U),

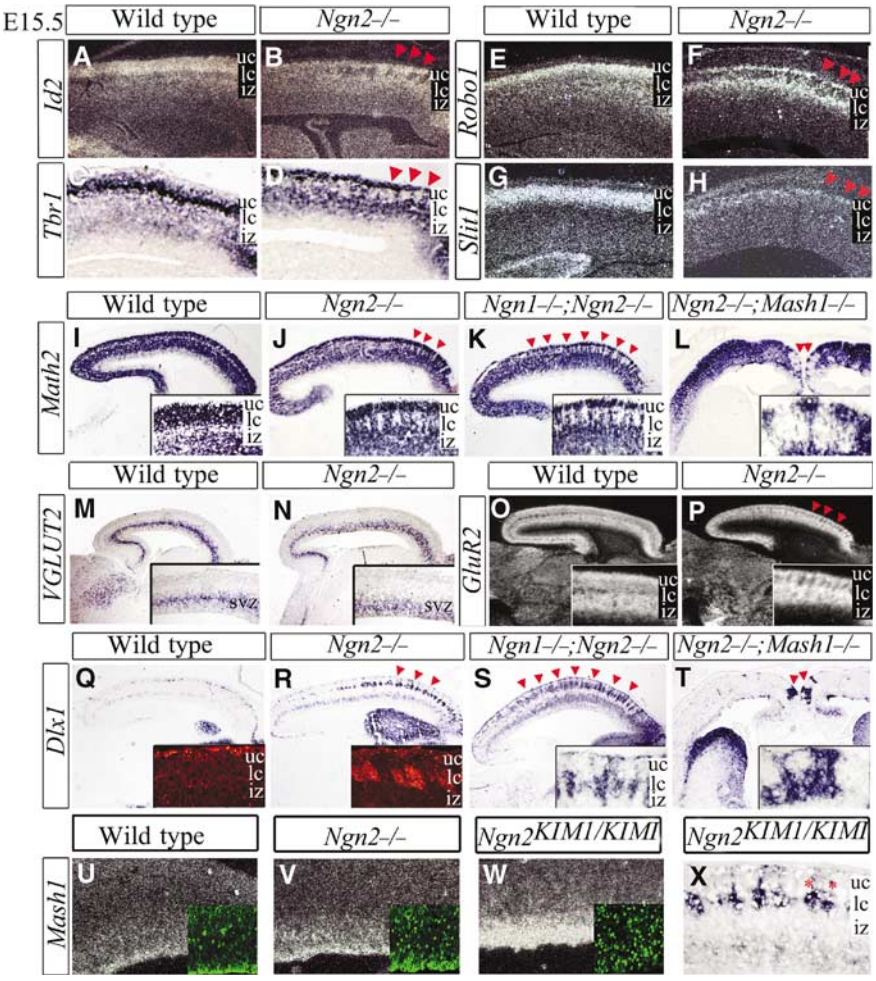

Figure 2 Change in regional identity and neurotransmitter phenotype of early-born CP neurons in E15.5 *Ngn* mutants. E15.5 expression of cortical neuronal markers *Id2* (**A, B**), *Tbr1* (**C, D**), *Robo1* (**E, F**), *Slit1* (**G, H**) and *Math2* (**I–L**), showing a correctly specified upper layer in *Ngn2* and *Ngn1;Ngn2* mutants above distinct gaps in lower CP expression (arrowheads, B, D, F, H, J, K) and in the medial cortex of *Ngn2;Mash1* mutants (arrowheads, L). (**M–P**) The glutamatergic marker *VGLUT2* was appropriately expressed in newly born neurons in the SVZ of wild-type (M) and *Ngn2* mutants (N), whereas *GluR2* was expressed in the IZ and CP of wild-type cortices (O), but displayed distinct gaps in the lower CP of *Ngn2* mutants (arrowheads, P). (**Q–T**) *Dlx1* transcripts and protein (insets) were detected in marginal zone and SVZ interneurons, and were ectopic in lower CP clusters in *Ngn2* and *Ngn1;Ngn2* mutants (arrowheads, R, S), and in medial clusters in *Ngn2;Mash1* mutants (arrowheads, T). (**U–W**) *Mash1* transcript and protein (insets) levels were moderately and strongly upregulated in *Ngn2* (V) and $Ngn2^{KIM1}$ mutants (W), respectively. (**X**) Ectopic *Dlx1*+ clusters were in the deep CP in $Ngn2^{KIM1}$ mutants (asterisks). uc, upper CP; lc, lower CP; iz, intermediate zone; svz, subventricular zone.

suggesting that cortical neurons choose between a glutamatergic and GABAergic phenotype.

To determine the extent to which ectopic *Mash1* expression was responsible for specification defects in *Ngn* mutant cortices, we profiled gene expression in E13.5 *Ngn2;Mash1* double mutants. A similar reduction in transcription of cortical-specific neuronal markers was observed in *Ngn2* and *Ngn2;Mash1* mutants, with the exception of *Math3* (Figure 1A; compare *Ngn2* versus WT (blue bars) against *Ngn2;Mash1* versus WT (red bars)). In *Ngn2;Mash1* mutants, the loss of *Math2* transcripts was restricted to rostromedial domains (Figure 2L), where *Ngn1* was no longer expressed. Thus, the loss of neurons with a cortical character in *Ngn* mutants occurs independently of the upregulation of *Mash1*, suggesting that the *Ngn*s directly activate a cortical, glutamatergic differentiation pathway.

Dlx1, *Dlx2* and *Brn4* remained elevated to varying extents in *Ngn2;Mash1* cortices as compared to wild type (Figure 1A), with the ectopic expression of ventral markers restricted to rostromedial domains (Figure 2T). *Ngn*s therefore repress ventral-specific genes in at least a partially *Mash1*-independent manner. In contrast, ectopic expression of *Dlx5*, *GAD1*, *GAD2*, *GABA-T1* and *GABA/glyT* in *Ngn2* mutant cortices was mostly or strictly dependent on the presence of *Mash1* (Figure 1A), suggesting that ectopic activation of these genes was largely due to derepression of *Mash1*. *Ngn1/2* thus specify regional and neurotransmitter phenotypes via multiple mechanisms, including activation of dorsal, cortical-specific genetic pathway(s), and repression of ventral telencephalic programs that are both *Mash1* dependent and independent (Figure 8).

Ngns are required to specify early- and not later-born CP neurons

We extended our analysis of *Ngn* mutants to mid-corticogenesis (E15.5), when lower-layer neurons have differentiated

and are migrating to the CP. As at earlier stages, telencephalic patterning genes (*Emx2*, *Lhx2*, *Tlx*, *Pax6*) were normally expressed in *Ngn2* and *Ngn1;Ngn2* mutants, suggesting a correct regional identity of progenitors (data not shown). To assess neuronal identities, we analyzed the expression of CP-specific markers *Id2*, *Robo1*, *Slit1*, *Math2* and *Tbr1* (Figure 2A–K). In the rostral cortex of *Ngn2* mutants, where Ngn1 expression was selectively lost, and throughout the cortex of *Ngn1;Ngn2* mutants, gaps in expression of all cortical markers were observed in the deep CP (Figure 2B, D, F, H, J and K). The loss of cortical-specific gene expression was not due to a loss of neurons, as pan-neuronal markers such as *SCG10* were unperturbed (data not shown), and cell death, as assessed by TUNEL, was not elevated in E15.5 *Ngn* mutants (Supplementary Figure S2).

Strikingly, gaps in cortical-specific gene expression were not observed in the superficial CP or intermediate zone (IZ) of *Ngn* mutants, zones that contain more recently generated CP neurons, suggesting that only early-born CP neurons are misspecified. Consistent with this, CP neurons differentiating at E15.5 in *Ngn2* and *Ngn1;Ngn2* mutants acquired their correct glutamatergic phenotype, as assessed by normal levels of *VGLUT2*, which transiently labels glutamatergic neurons migrating through the SVZ (Figure 2M–N). Transcripts for the glutamate receptor *GluR2* were also maintained in the IZ and upper CP of E15.5 *Ngn2* mutants, and were only lost in clusters of lower-layer CP neurons (Figure 2O and P). In a complementary manner, ectopic expression of *Dlx1* (Figure 2Q–S) and *GAD1* (data not shown) was confined to large aggregates in the lower CP of *Ngn* mutants (Figure 2A–K), and was excluded from the more recently generated, superficial layer of the CP. Thus, in contrast with early-born CP neurons, neurons differentiating during mid-corticogenesis acquire a normal identity in the absence of *Ngn* function.

Changing Mash1 responsiveness of cortical progenitors

The misspecification of early- and not later-born CP neurons in *Ngn* mutants was surprising, given that *Ngn* expression persists throughout corticogenesis (data not shown). Since derepression of *Mash1* contributes to cortical misspecification in *Ngn* mutants at E13.5 (Fode *et al*, 2000), we speculated that *Mash1* might not be sufficiently upregulated at later stages to alter the differentiation of cortical progenitors. We tested this using $Ngn2^{KIM1}$ homozygous mutant embryos in which *Ngn2* coding sequences were replaced with *Mash1* (Fode *et al*, 2000; Parras *et al*, 2002). At E15.5, *Mash1* transcript and protein levels were low in wild-type cortical progenitors, slightly elevated in *Ngn2* mutants and very significantly elevated in $Ngn2^{KIM1}$ mutants (Figure 2U–W). However, *Dlx1* was ectopically expressed only in small clusters deep within the rostral CP of $Ngn2^{KIM1}$ mutants (Figure 2X). Thus, increasing *Mash1* expression levels was not sufficient to respecify cortical neurons born after E14.5, suggesting that both the dependency of cortical neurons on *Ngn1/2* and the responsiveness of cortical progenitors to ectopic *Mash1* change over time.

A subset of Ngn mutant early-born neurons contribute to deep cortical layers while others segregate out of the CP

To further examine the fate of early-born, misspecified neurons, we analyzed *Ngn* mutant cortices at E18.5, a stage when neurogenesis is complete, although CP neurons are still migrating to their final destination. Strikingly, the cortical-specific marker *Math2* was for the most part uniformly expressed throughout the CP in E18.5 *Ngn2* and *Ngn1;Ngn2* mutant cortices (Figure 3A–D), in contrast to defects at E15.5 (Figure 2I–K). Moreover, in E18.5 *Ngn* mutants, misspecified *Dlx1*+ (Figure 3E–J) and *GAD1*+ (data not shown) neurons segregated out of the CP, aggregating instead in small clusters in the germinal zone (GZ), rostral marginal zone (MZ) and flanking the IZ/lower CP border. Thus, by E18.5, the *Ngn* mutant CP was comprised almost exclusively of neurons with their correct regional identities.

To determine whether any early-born cortical neurons remained in the CP of *Ngn* mutants, neuronal lamination was assessed with histological stains at P0, revealing that lower layers were indeed present in *Ngn2* and *Ngn1;Ngn2* mutants, but were significantly thinner in double mutants (Figure 4A–F). To determine whether histologically identifiable lower layers were composed of neurons born at the correct time, we used birthdating (Caviness, 1982; Caviness *et al*, 1995). BrdU was administered at different embryonic stages (Supplementary Figure S3), followed by an assessment at P0 of the laminar position of darkly labeled nuclei, marking neurons born at the time of BrdU injection (black bars, Figure 4G), and lightly labeled nuclei, representing neurons derived from progenitors that had undergone additional cell divisions (gray bars, Figure 4G). Labeling at E12 revealed a peak accumulation of strong BrdU+ nuclei in layer VI of wild-type cortices (top row, Figure 4G). In *Ngn* mutants, there was an increased number of heavily labeled neurons blocked in the GZ/IZ, and in the MZ of *Ngn1;Ngn2* mutants (top row, Figure 4G), corresponding to sites of aggregation of misspecified *Dlx1*+/*GAD1*+ neurons (Figure 3F–J), and confirming that many early-born, misspecified neurons were not integrated in the CP. However, not all *Ngn* mutant neurons born at E12 migrated aberrantly, as a significant number of BrdU+ neurons were correctly positioned in layer VI, likely corresponding to the subset of lower-layer CP neurons with a correct regional identity and neurotransmitter phenotype at E15.5 (Figure 2A–P).

A BrdU pulse at E14 primarily marked the genesis of layer IV and V neurons, but in *Ngn2* and *Ngn1;Ngn2* mutants there was a clear decline in the number of darkly stained nuclei in the lower CP, and instead an accumulation of cells in the GZ (middle row, Figure 4G). This suggested that a subset of CP neurons destined for layers IV–V aggregated in abnormally deep positions in *Ngn2* and *Ngn1;Ngn2* mutant CPs. In contrast, the laminar position of upper-layer neurons was largely unperturbed in *Ngn* mutants, as assessed by birthdating at E16, which in mutant as well as control brains resulted in a clear bias towards darkly stained nuclei localizing to upper layers II–IV (bottom row, Figure 4G). Specification defects resulting in abnormal laminar localization in *Ngn2* and *Ngn1;Ngn2* mutants were thus largely restricted to cortical neurons born between E12 and E14, but a subset of early-born neurons acquired a correct regional identity and populated deep cortical layers.

Disruptions in laminar specification restricted to lower layers in Ngn mutants

The correct specification and layering of a subset of lower-layer neurons in the *Ngn* mutant CP allowed us to examine

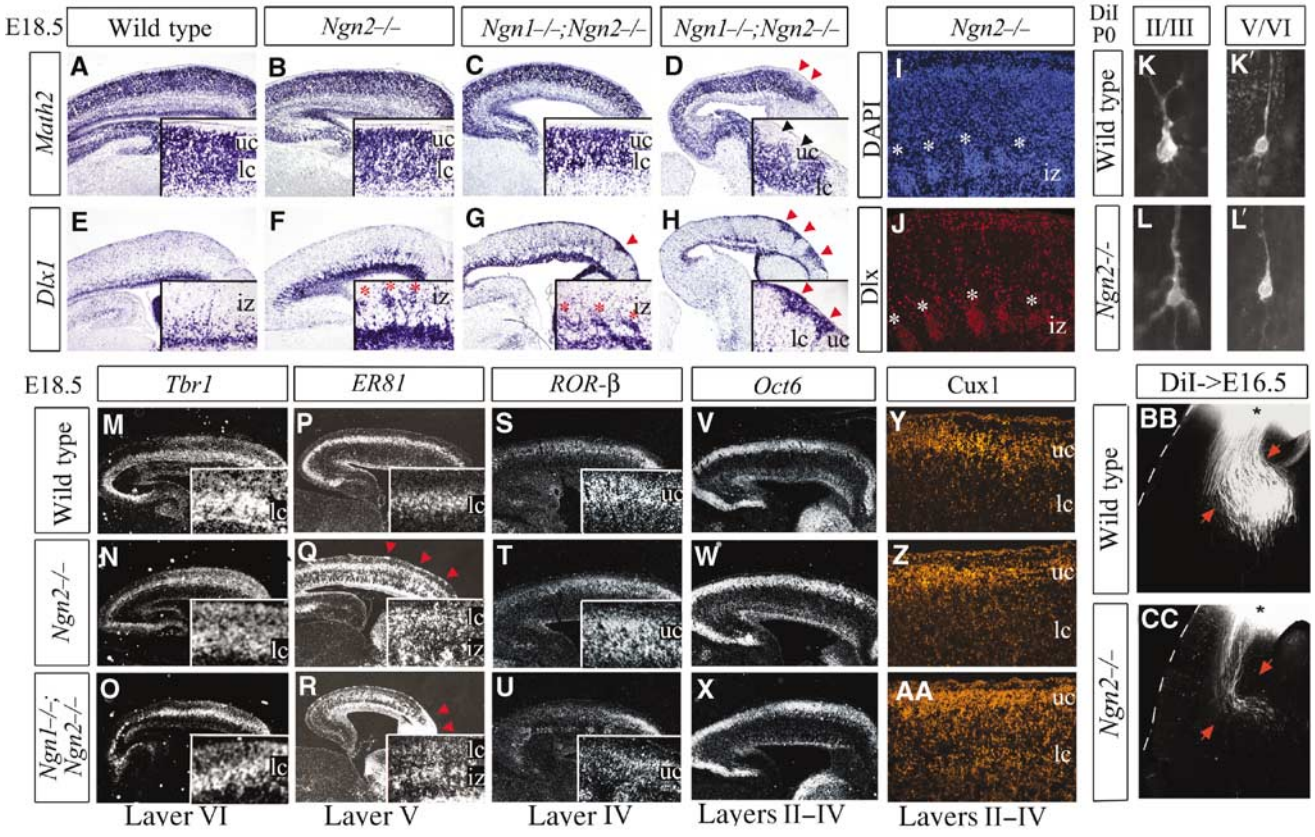

Figure 3 Misspecification of lower-layer CP neurons in E18.5 *Ngn* mutants. (**A–D**) *Math2* was uniformly expressed in the CP and IZ of wild-type (A), *Ngn2* (B) and *Ngn1;Ngn2* (C) mutants, with the exception of small superficial gaps in *Ngn1;Ngn2* mutants (uc, arrowheads, D). (**E–H**) *Dlx1* was expressed in cortical interneurons in the GZ, MZ and CP (E), and in ectopic clusters in the IZ (asterisks) and superficial CP (arrowheads) of *Ngn2* (F) and *Ngn1;Ngn2* (G, H) mutants. (**I**) DAPI and (**J**) Dlx immunostaining showed that abnormal cellular aggregates (asterisks) beneath the CP were Dlx+. (**K–L**) Retrograde labeling of P0 cortical neurons in layers II/III (K, L) and V/VI (**K′, L′**) revealed similar immature neuronal morphologies in wild-type (**K, K′**) and *Ngn2* mutant (**L, L′**) cortices, with sparsely branched apical and basal dendritic processes (also see Supplementary Figure S4). (**M–O**) Layer VI expression of *Tbr1* was reduced in the rostral CP in *Ngn2* mutants (N), and throughout the *Ngn1;Ngn2* mutant cortex (O). (**P–R**) *ER81* expression was reduced and disorganized (arrowheads) in rostral layer V in *Ngn2* mutants (Q), and throughout the *Ngn1;Ngn2* mutant layer V (R). (**S–U**) Layer IV expression of *ROR*β was normal in *Ngn2* (T) and *Ngn1;Ngn2* (U) mutants. Ectopic *ROR*β (U) and *ER81* (Q, R) were observed in clusters in the IZ and MZ of *Ngn2* and *Ngn1;Ngn2* mutants, correlating with migration defects of a subset of early-born neurons. *Oct6* transcripts (**V–X**) and Cux1 protein (**Y, Z, AA**) were expressed in layers II–IV in all genotypes. (**BB**, **CC**) Anterograde tracing revealed fewer corticofugal projections in E16.5 *Ngn2* mutants (arrows, CC). uc, upper CP; lc, lower CP; iz, intermediate zone.

whether these neurons had their correct laminar phenotypes based on molecular markers. At E18.5, *Tbr1* was expressed at high levels in layer VI in wild-type cortices, but at diminished levels in the rostral cortex of *Ngn2* mutants and throughout the cortex of *Ngn1;Ngn2* double mutants (Figure 3M–O). *ER81*, which is expressed in layer V, was lost in small rostral gaps in layer V of *Ngn2* mutants, and was more globally disturbed throughout layer V in *Ngn1;Ngn2* mutants (Figure 3P–R). In contrast, layer II–IV markers *RORβ* (Figure 3S–U), *Oct6* (Figure 3V–X) and Cux1 (Figure 3Y, Z and AA) labeled correctly positioned neurons in all genotypes, indicating that upper-layer neurons were correctly specified. Anterograde tracing of descending corticofugal projections of layer V/VI (Koester and O'Leary, 1993) revealed strongly reduced axonal numbers in *Ngn2* mutant versus wild-type cortices at E16.5, which were consistent with selective specification defects of lower-layer neurons (Figure 3BB and CC). Lower-layer neurons in the *Ngn* mutant CP thus present both molecular and axonal projection defects.

Neuronal migration in the cortex is not complete until postnatal day (P) 7, such that unequivocal conclusions about laminar phenotypes in *Ngn* mutants could not be made from embryonic analyses. We thus examined laminar specification in the rare *Ngn2* single-mutant pups that survived the first postnatal week, but could not analyze laminar identities in the complete absence of *Ngn* activity because all *Ngn1;Ngn2* mutants died at birth. At P5, the overall size of the *Ngn2* mutant neocortex was smaller, but all the six cortical layers were clearly identified by cell morphology (Figure 5O–P). Analysis of molecular markers revealed striking defects in layer VI, with expression of *Slit1* (Figure 5A and B), *Tbr1* (Figure 5C and D) and *Id2* (Figure 5M and N) strongly reduced in the rostromedial CP (where Ngn1 is lost) of P5–P7 *Ngn2* mutants. Layer V defects were more modest, likely due to persistence of *Ngn1*, although the distribution of both *Robo1* (Figure 5E and F) and *ER-81* (Figure 5G and H) transcripts was clearly disorganized in the rostral cortex of P7 *Ngn2* mutants. In contrast, layer IV expression of *ROR-β* (Figure 5I and J) and layer II/III expression of *Oct6* (Figure 5K and L) and *Id2* (Figure 5M and N) appeared normal in postnatal *Ngn2* mutants. We performed several additional tests to confirm that, in *Ngn2* mutants, lower-layer neurons

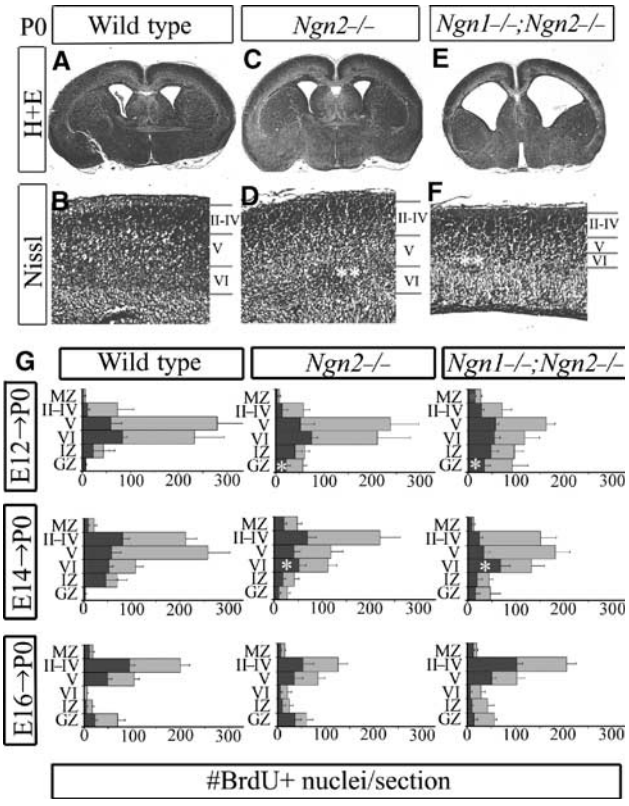

Figure 4 Aberrant location of lower-layer CP neurons in *Ngn* mutants. (**A–F**) Histological analyses at P0 revealed neurogenesis defects primarily in lower layers of *Ngn1;Ngn2* mutants (asterisks, F), a disorganization of layer VI in *Ngn2* mutants (asterisks, D), but a correctly laminated CP in both genotypes. (**G**) Graphical representations of BrdU birthdating studies showing the distribution of P0 cortical neurons labeled with BrdU at different times (E12, E14, E16). Cortices were divided into six bins corresponding to MZ, layers II–IV, layer V, layer VI, IZ and GZ. Darkly labeled nuclei (black bar) and lightly labeled nuclei (gray bars) in each bin were counted. Asterisks label the increased number of cells generated at E12 in the *Ngn2* and *Ngn1;Ngn2* mutant GZ, and the skewed distribution of neurons labeled at E14 in *Ngn2* and *Ngn1;Ngn2* mutants. MZ, marginal zone; GZ, germinal zone.

did not lose gene expression due to apoptosis, and that upper-layer neurons had their correct molecular and cellular properties (i.e. barrel field formation in layer IV, synaptic zinc in layer II/III axon terminals, upper-layer contribution to callosal projections; Figure 5Q and R; Supplementary Figure S5).

Pax6 specifies a cortical, glutamatergic phenotype and represses subcortical phenotypes at mid-corticogenesis

Given that *Ngn1/2* were not required to specify neuronal phenotypes in mid–late corticogenesis, we hypothesized that an *Ngn*-independent genetic program operated during a second phase of neuronal fate specification. Previous studies had suggested that mutations in *Pax6* and the orphan nuclear receptor *Tlx* specifically affected development of upper cortical layers, but molecular analyses were limited (Tarabykin *et al*, 2001; Land and Monaghan, 2003). These two factors also cooperate genetically to establish the cortical–subcortical boundary (Stenman *et al*, 2003), prompting us to investigate whether genetic interactions between *Pax6* and *Tlx* were required to specify regional, neurotransmitter and/or laminar phenotypes of upper-layer cortical neurons.

During early corticogenesis (E12.5–E13.5), *Ngn2* was expressed in *Pax6* mutant cortical progenitors, except in a small lateral territory (data not shown; Toresson *et al*, 2000; Yun *et al*, 2001), allowing us to assess how *Pax6* functions independently of the *Ngn*s. The regional identity of *Pax6* mutant cortical neurons was correctly specified at E13.5, as assessed by *Math2* expression (Figure 1E) and *Tbr1* (Stoykova *et al*, 2000). Despite this, VGLUT1 and *VGLUT2* expression was strongly reduced in *Pax6* mutants (Figure 1I and M), indicating that these neurons were not glutamatergic. *Pax6* mutant cortical neurons were also not GABAergic, as *GAD1* was not ectopically expressed (Figure 1Q), likely due to *Ngn*-mediated repression of GABAergic pathways at this stage (Figure 1A). Thus, *Pax6* is not required to specify a cortical (regional) identity or suppress GABAergic differentiation pathways in early-born neurons, but does participate in the specification of a glutamatergic phenotype.

We next assessed cortical and glutamatergic identities in *Pax6* and *Tlx* mutants at E15.5. No specification defects were observed in cortical progenitors or neurons in *Tlx* single mutants at this stage (data not shown). There was a clear decrease in *Ngn2* transcript levels in the rostral GZ of *Pax6* and *Pax6;Tlx* mutants, but other cortical-specific progenitor markers were normally expressed (Figure 6B and C; Stoykova *et al*, 2000; Toresson *et al*, 2000; Muzio *et al*, 2002). Expression of cortical neuronal markers *Math2* and *Tbr1* was also clearly reduced in newly generated neurons migrating through the IZ of *Pax6* and *Pax6;Tlx* mutants, and *VGLUT2* expression was reduced in the SVZ of *Pax6* and *Pax6;Tlx* mutants (Figure 6D–L). Thus, specification of both a cortical regional identity and glutamatergic neurotransmission phenotype was perturbed in neurons generated during mid-corticogenesis in *Pax6* and *Pax6;Tlx* mutants, in contrast to *Ngn* mutants where neuronal specification defects were rescued by E15.5.

Many *Pax6* mutant CP neurons born from E14 onwards do not migrate appropriately, becoming trapped in an expanded SVZ (Caric *et al*, 1997). Neurons trapped in the SVZ of E15.5 *Pax6* and *Pax6;Tlx* mutants were misspecified and ectopically expressed *Dlx1* (Figure 6N–O) and *GAD1* (data not shown), suggesting that neurons born during mid-corticogenesis acquired a subcortical phenotype, in striking contrast to the normal regional identity of neurons generated at E13.5. Thus, *Pax6* is required to specify a cortical identity and glutamatergic neurotransmitter phenotype in CP neurons generated in mid-corticogenesis, while simultaneously repressing a ventral, GABAergic fate. Although *Pax6* regulates *Ngn* expression at E15.5, the requirement for *Pax6* in specifying cortical identities must be independent of *Ngn* regulation, since CP neurons born during mid-corticogenesis are correctly specified in *Ngn* mutants.

Pax6 and Tlx cooperate in the specification of late-born cortical neurons

To assess the specification of late-born cortical neurons, we examined E18.5 *Pax6* and *Tlx* single- and double-mutant cortices. In *Pax6* and *Pax6;Tlx* mutants, *Math2* labeled a thinner CP, and the IZ was almost completely devoid of *Math2* transcripts, suggesting that many CP neurons generated during mid–late corticogenesis did not acquire their appropriate cortical identity or migrate into the CP (Figure 7A–D). In *Tlx* mutants, small gaps in *Math2* expression were

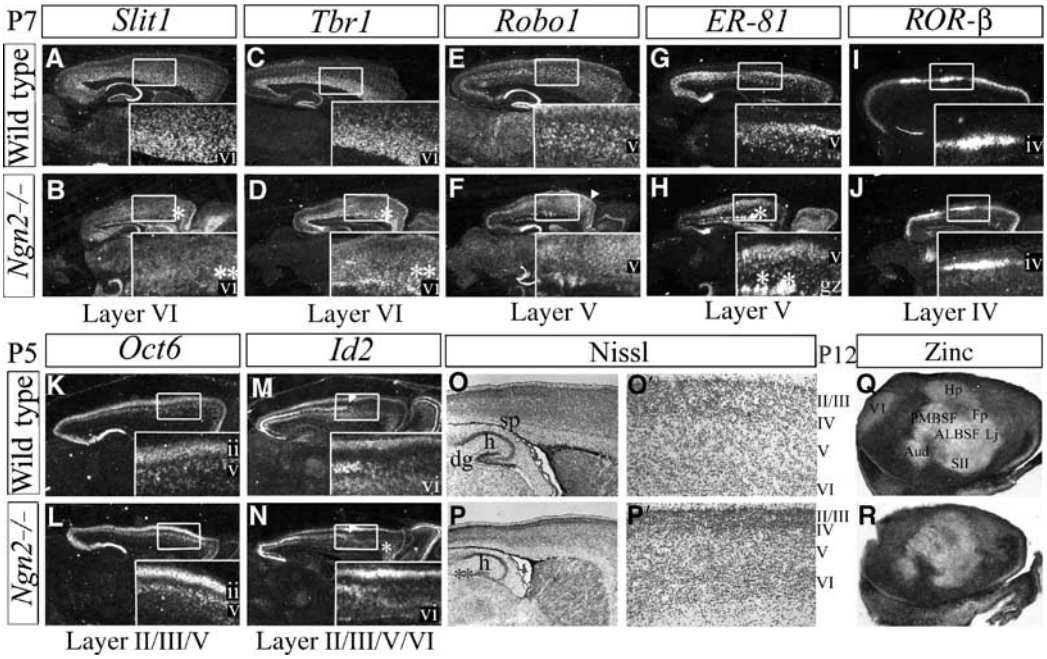

Figure 5 Postnatal laminar defects restricted to deep CP layers in *Ngn2* mutants. At P7, layer VI expression of *Slit1* (**A, B**) and *Tbr1* (**C, D**) was severely reduced in the rostral cortex of *Ngn2* mutants (asterisks, B, D). Layer V expression of *Robo1* (**E, F**) and *ER81* (**G, H**) demarcated a thinner, disorganized layer, and ectopic expression in the IZ of *Ngn2* mutants (asterisks, H). Layer IV expression of *ROR*β at P7 (**I, J**), layer II/III/V expression of *Oct6* at P5 (**K, L**) and layer II/III expression of *Id2* (rostral limit marked by arrowhead) at P5 (**M, N**) were unaffected in *Ngn2* mutants (J, L, N). *Id2* expression was lost in *Ngn2* mutant layer VI (asterisks, N). (**O, P**) Nissl staining of P5 wild-type (O, **O′**) and *Ngn2* mutant (P, **P′**) cortices revealed no defects in cortical layering, but neurogenesis defects were evident in the SP (*) and dentate gyrus (**; P) of *Ngn2* mutants. (**Q, R**) P12 cortices stained for synaptic zinc, a marker of axon terminals of glutamatergic projection neurons in layers II/III and VI, were normally excluded by layer IV in P12 *Ngn2* mutants (R). gz, germinal zone; h, hippocampus; dg, dentate gyrus; sp, subplate; ALBSF, antero-lateral barrel subfield; PMBSF, posterio-medial barrel subfield; Hp, hindpaw; Fp, forepaw; Lj, lower jaw; SII, secondary somatosensory cortex; Aud, primary auditory cortex; V1, primary visual cortex.

observed in the GZ, suggesting that a small subset of late-born CP neurons were not correctly specified (Figure 7B). The misspecified neurons instead acquired a subcortical identity as *Dlx1* (Figure 7E–H) and *GAD1* (data not shown) were expressed in a complementary manner, in small ectopic patches in the *Tlx* mutant GZ and large ectopic bands spanning the GZ/IZ in *Pax6* and *Pax6;Tlx* mutants. Thus, *Tlx* and *Pax6* both act at late stages of corticogenesis to promote a cortical identity and suppress a subcortical GABAergic phenotype.

We next examined laminar identities of CP neurons in *Pax6* and *Tlx* mutants. In contrast to *Ngn* mutants, no defects were detected in lower layers V and VI, with *Tbr1* expressed in a distinct layer VI (Figure 7I–L) and *ER81* in layer V (Figure 7M–P) in all genotypes. In contrast, the layer IV marker *ROR*β was not detectable in the rostral cortex of *Pax6;Tlx* mutants, while it was expressed in *Pax6* and *Tlx* single mutants (Figure 7Q–T). Similarly, two markers of layers II–IV, *Oct6* (Figure 7U–X) and Cux1 (Figure 7Y–BB), were completely absent in the upper layers of the rostral cortex of *Pax6;Tlx* double mutants, but were detectable, although at reduced levels, in *Tlx* and *Pax6* single mutants. Thus, *Pax6* and *Tlx* must both be mutated to lose upper-layer marker expression, suggesting that they cooperate genetically to specify the identity of late-born neurons in the cortex.

Discussion

We have identified several genetic determinants that operate in a biphasic manner to specify the regional (cortical), neurotransmission (glutamatergic) and laminar identity of neocortical neurons. Early in cortical development, *Ngn1* and *Ngn2* function redundantly to specify regional and glutamatergic phenotypes common to all neocortical projection neurons, and participate in specifying the laminar identity of deep-layer neurons (Figure 8). In mid–late corticogenesis, as *Ngn* function becomes dispensable, *Pax6* and *Tlx* begin to function both independently and synergistically to specify cortical identity and neurotransmitter choice by later-born neurons in upper layers (Figure 8). Interestingly, both the *Ngn*s and *Pax6/Tlx* are required not only to activate cortical-specific traits but also to repress an alternative subcortical, GABAergic differentiation program. Taken together, these data reveal an unexpected degree of heterogeneity in the genetic mechanisms underlying neuronal specification during corticogenesis, and highlight the highly coordinated manner in which common and diverse aspects of neuronal phenotype are specified.

Distinct genetic programs specify features common to early- and late-born cortical neurons

Despite their inherent diversity, neocortical projection neurons share several essential characteristics, including their regional identity and use of glutamate as a neurotransmitter. *A priori*, we anticipated that such uniformity would arise through the activation of a common genetic cascade. On the contrary, we found that distinct genetic programs specify shared regional and neurotransmitter properties during early and late stages of corticogenesis. In early stages, *Ngn*

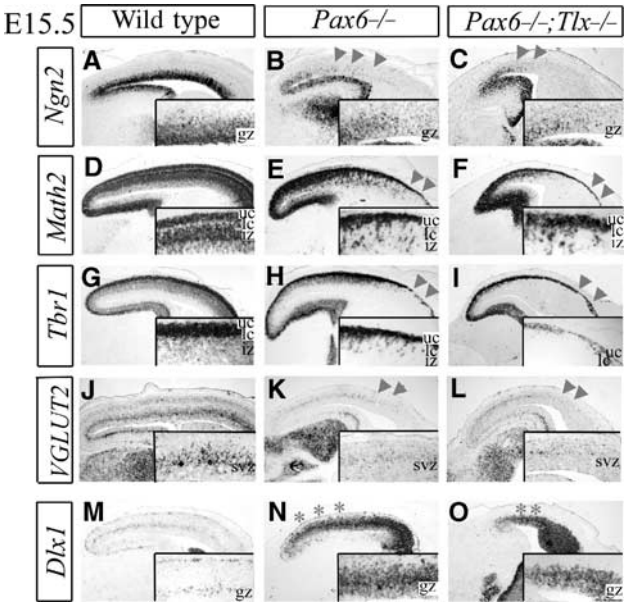

Figure 6 Defects in neuronal specification at mid-corticogenesis (E15.5) in *Pax6;Tlx* mutants. The rostral CP of *Pax6* and *Pax6;Tlx* mutants was visibly thinner, and there was an overall reduction in the size of the *Pax6;Tlx* cortex. (**A–C**) GZ expression of *Ngn2* was reduced in the rostral GZ of *Pax6* (arrowheads, B) and *Pax6;Tlx* mutants (arrowheads, C). (**D–I**) *Math2* (D–F) and *Tbr1* (G–I) were expressed in the IZ and CP, and were reduced throughout the IZ and rostral CP of *Pax6* (arrowheads, E, H) and *Pax6;Tlx* mutants (arrowheads, F, I). (**J–L**) *VGLUT2* expression in the SVZ was reduced in *Pax6* (arrowheads, K) and *Pax6;Tlx* (arrowheads, L) mutants. (**M–O**) *Dlx1* was expressed in migrating interneurons in the MZ and SVZ (M), and a massive, ectopic upregulation of *Dlx1* was detected in the SVZ/IZ of *Pax6* (asterisks, N) and *Pax6;Tlx* mutants (asterisks, O). gz, germinal zone; uc, upper CP; lc, lower CP; iz, intermediate zone; svz, subventricular zone.

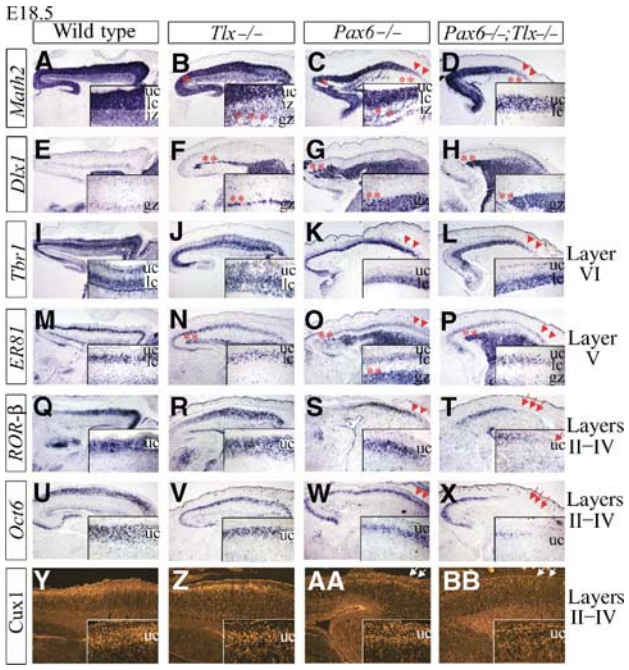

Figure 7 *Pax6* and *Tlx* cooperate during late corticogenesis (E18.5) to specify neuronal phenotypes. (**A–D**) *Math2* expression in the CP and IZ was lost in small clusters in *Tlx* mutant IZ, and in large regions of the IZ in *Pax6* and *Pax6;Tlx* mutants (asterisks, insets, B–D), but was maintained in the upper CP (arrowheads, C, D). (**E–H**) *Dlx1* was expressed in migrating cortical interneurons in the GZ, MZ and CP (E), in small ectopic clusters in the GZ of *Tlx* mutants, and in a grossly expanded GZ in *Pax6* and *Pax6;Tlx* mutants (asterisks, F–H). (**I–L**) Layer VI expression of *Tbr1* was maintained in all genotypes (arrowheads, K, L). (**M–P**) *ER81* was expressed in layer V in all genotypes (arrowheads, O, P) and ectopically expressed in the IZ of *Tlx* mutants, and SVZ/IZ of *Pax6* and *Pax6;Tlx* mutants (asterisks, N–P). (**Q–T**) Layer IV expression of *ROR*-β was lost in rostral CP of *Pax6;Tlx* mutants (arrows, T), but maintained in *Pax6* mutants (arrowheads, S). Layers II–IV expression of *Oct6* (**U–X**) and Cux1 (**Y-BB**) was detected, albeit at decreased levels, in *Pax6* mutants (arrowheads, W; arrows, **AA**), but was not expressed in rostral CP of *Pax6;Tlx* double mutants (arrows, X, BB). gz, germinal zone; uc, upper CP; lc, lower CP; iz, intermediate zone.

activity is absolutely required to induce a cortical differentiation program in a subset of deep-layer neurons. The ability of the *Ngn*s to function in cortical progenitors to specify the properties of postmitotic neurons is consistent with previous results that suggest that neuronal identities are specified at the progenitor cell stage in the neocortex (Job and Tan, 2003). However, given that some early-born neurons do acquire their correct regional and neurotransmitter identities in *Ngn* mutants, additional regulatory genes may normally act in parallel with the *Ngn*s to specify lower-layer phenotypes. Furthermore, *Ngn2* is not sufficient to specify a dorsal cortical identity in subcortical progenitors, suggesting that *Ngn*s must interact with as yet unidentified cofactors to activate cortical-specific programs of gene transcription (Parras *et al*, 2002).

In contrast to their limited roles early, *Pax6* and *Tlx* are absolutely required to activate cortical- and glutamatergic-specific differentiation programs in CP neurons during mid–late corticogenesis. We hypothesize that *Emx2* also functions during mid–late corticogenesis, primarily in the caudal cortex, where neuronal specification defects are less severe in *Pax6* and *Pax6;Tlx* mutants. Consistent with this, the cortex is converted to striatal-like tissue in the absence of both *Pax6* and *Emx2* (Muzio *et al*, 2002). Importantly, although *Ngn*s are downstream of *Pax6* and *Emx2* in mid–late cortical development, as shown by the loss of *Ngn1* and *Ngn2* transcripts in the *Pax6;Emx2* mutant pallium, and the direct transcriptional activation of *Ngn2* by *Pax6* in the cortex (Stoykova *et al*, 2000; Muzio *et al*, 2002; Scardigli *et al*, 2003; Stenman *et al*, 2003), they are not mediating the specification function of these genes at these stages.

A binary switch between cortical/glutamatergic and subcortical/GABAergic phenotypes

We uncovered a binary switch between glutamatergic and GABAergic phenotypes, which is regulated by distinct genetic pathways at different stages of corticogenesis. Interestingly, in the spinal cord, differentiating neurons must also choose between a glutamatergic and GABAergic fate, and the homeodomain transcription factors *Tlx1* and *Tlx3* promote an excitatory neurotransmission phenotype, while inhibiting an alternative GABAergic fate (Cheng *et al*, 2004).

Coinciding with the shift from early *Ngn*-dependent to mid/late *Pax6*-dependent neuronal fate specification is a change in the competence of progenitor cells to respond to elevated levels of *Mash1*. That is, whereas early *Ngn* mutant cortical progenitors activate GABAergic differentiation pathways in response to an upregulation of *Mash1*, at E15.5, *Ngn2* mutant progenitors no longer activate a GABAergic pathway

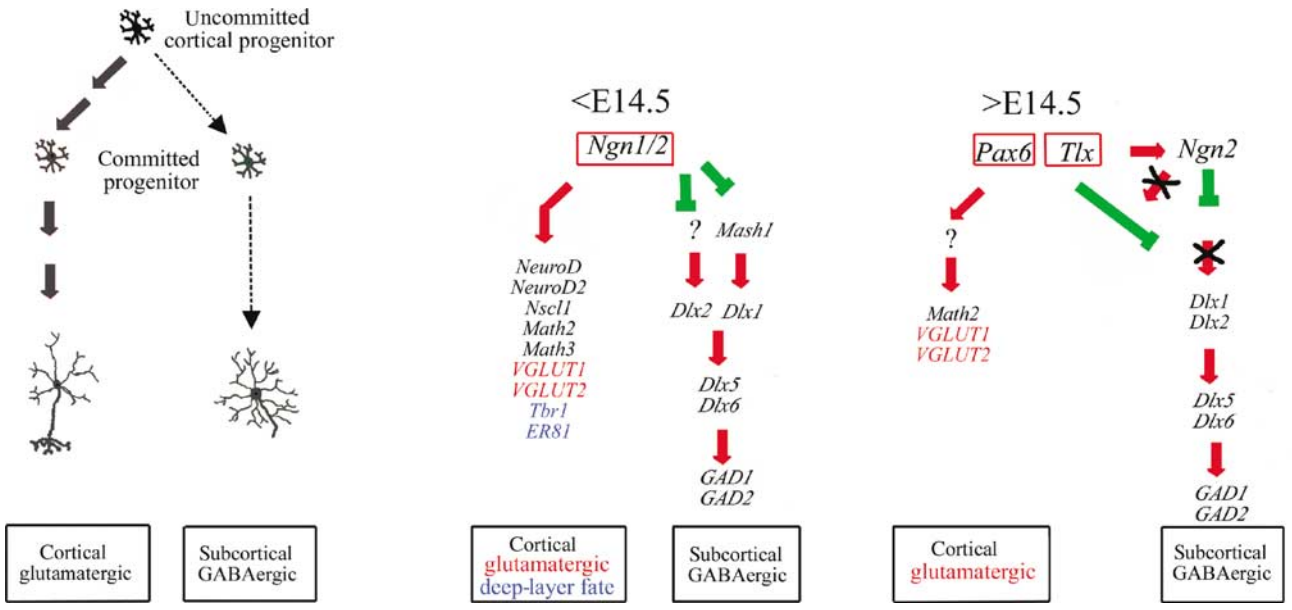

Figure 8 Two-phase model of neuronal fate specification in the neocortex. A schematic model of cellular and genetic cascades underlying early and late stages of neuronal phenotype specification in the neocortex. The early phase (<E14.5) is dependent on *Ngn1/2*, which induces expression of cortical, glutamatergic and lower-layer specification programs, while simultaneously repressing the differentiation of GABAergic neurons. The late phase of neuronal fate specification (>E14.5) occurs in the absence of *Ngn1/2* activity, and is dependent on synergistic interactions between *Tlx* and *Pax6*, which specify a cortical and glutamatergic identity while repressing a GABAergic phenotype.

in the same context. Presumably, this is not due to a loss of competence of late cortical progenitors to undergo GABAergic differentiation, since cortical progenitors are respecified in E15.5 *Pax6* mutants, but rather due to a shift in the genetic control of this binary decision, from *Ngn/Mash1* to *Pax6* dependent. Mechanistically, we showed that *Ngn1* and *Ngn2* inhibit a subcortical identity and GABAergic phenotype through the simultaneous repression of *Mash1*-dependent and *Mash1*-independent regulatory pathways (Figure 8). Components of a *Mash1*-dependent pathway have been previously identified, with *Mash1* capable of turning on *Dlx1*, which in turn induces *Dlx5/6* and *GAD* target genes (Fode *et al*, 2000; Letinic *et al*, 2002; Stuhmer *et al*, 2002), but the existence of a *Mash1*-independent pathway involved in specifying subcortical identities has only been inferred from the maintenance of a GABAergic phenotype in the ventral telencephalon of *Mash1* mutants (Casarosa *et al*, 1999; Horton *et al*, 1999).

This study and others have identified several genes, including *Gli3*, *Pax6*, *Emx2*, *Tlx* and *Ngn1/Ngn2*, which are all required to repress GABAergic differentiation in cortical progenitors (Theil *et al*, 1999; Fode *et al*, 2000; Stoykova *et al*, 2000; Muzio *et al*, 2002). Given the multitude and complexity of these genetic mechanisms, it is not surprising that the vast majority of GABAergic cortical interneurons are derived from the ventral subpallium in mice (Anderson *et al*, 2002). However, we have found that *Mash1* is expressed in a significant number of cortical progenitors (C Schuurmans and F Guillemot, unpublished), suggesting that *Mash1* may indeed play a role in the rodent cortex, but that this function may be distinct from its capacity to activate a GABAergic differentiation program. Interestingly, in humans, the majority of cortical interneurons appear to be dorsally derived, and cortical progenitors express several genes that are normally at least partially repressed in mouse, including *Mash1*, suggestive of a role for *Mash1* in specifying GABAergic interneurons in the human cortex (Letinic *et al*, 2002). In future, it will be interesting to investigate whether the production of GABAergic neurons in the human neocortex reflects changes during evolution in the expression or function of *Ngn*s, *Pax6* and/or other genes that repress subcortical GABAergic fates in rodents.

Ngns participate in specifying the laminar identity of lower-layer cortical neurons

Here, we showed that *Ngn1/2* are essential for the specification of laminar identity in a subset of deep-layer neurons. Similarly, we hypothesize that *Pax6* and *Tlx* act synergistically to specify the laminar identity of upper-layer neurons, but, because there was a complete loss of layer II–IV neurons in the rostral cortex of *Pax6;Tlx* double mutants, we cannot ascertain whether *Pax6* and *Tlx* are directly required to activate an upper-layer-specific differentiation program, or if the loss of upper-layer markers is secondary to the transformation of these neurons to a ventral-like phenotype. Despite this, from our studies, we come to the unexpected conclusion that early and late phases of neuronal specification in the neocortex are regulated by independent genetic mechanisms, and that the specification of laminar identity, an aspect of neuronal phenotype that changes during cortical development, is directly coupled to the acquisition of common regional and neurotransmission properties, at least in lower-layer neurons. A possible consequence of the existence of independent programs of neuronal specification for different phases of corticogenesis is that evolutionary changes in deep and upper layers could be uncoupled through alterations in the genetic program controlling only one of the two populations. A clear example of such uncoupling is the expansion of supragranular layers II/III in primates (Kornack and Rakic, 1998), possibly resulting from a specific

modification of upper-layer genetic programs (*Tlx/Pax6*) without changing lower-layer programs (*Ngn*) in the primate cortex, an exciting prediction that deserves further study.

Materials and methods

Maintenance and genotyping of mice

Ngn2^{lacZ}, *Ngn1*, *Mash1*, *Ngn2*^{KIM1}, *Pax6 (Sey)* and *Tlx* mutant lines were maintained as single and double heterozygotes, with mutants generated and genotyped as previously described (Ma *et al*, 1998; Parras *et al*, 2002; Stenman *et al*, 2003).

Microarray hybridization

Cortices were dissected from E13.5 wild-type, *Ngn2*, *Ngn1*, *Ngn2;Mash1* and *Ngn1;Ngn2* mutants. RNA was extracted with Trizol reagent, total RNA from three cortices was pooled to average genetic variation and 10 μg RNA was used to generate probes, which were hybridized to U74A and U74B chips following standard Affymetrix guidelines. The experiment was performed twice for single mutants and once for double mutants. Gene accession numbers are listed in Supplementary data. Normalization of data and comparative analysis were performed with GeneChip software.

RNA in situ hybridization

Brains were dissected from staged embryos, fixed overnight in 4% paraformaldehyde (PFA), impregnated in 20% sucrose and embedded in OCT (Tissue-Tek). Sections (10 μm) were cut using a cryostat. Nonradioactive and radioactive RNA *in situ* hybridization and double *in situ*/immunohistochemistry were previously described (Gradwohl *et al*, 1996; Fode *et al*, 2000). Probe sources are described in Supplementary data.

Immunohistochemistry

Brains were fixed for 2 h in 4% PFA and processed for frozen sectioning as above. Sections were blocked for 1 h in 10% normal goat serum/PBS/0.1% Triton X-100 (PBT), and then incubated overnight at 4°C in primary antibody diluted in blocking solution. Sections were washed with PBT, exposed for 1 h to secondary antibodies and washed again. Primary antibodies included mouse monoclonal anti-Mash1 (1:1), rabbit polyclonal pan-Dll antibody (1/500, Grace Panganiban), rabbit polyclonal VGLUT1/2 (1/500; SySy) and rabbit polyclonal anti-Cux1 (1/250, H Tang and JM Cunningham).

Histology and BrdU birthdating

Brains were fixed in Bouin's fixative, transferred to 70% ethanol and processed for paraffin sectioning as described (Rhinn *et al*, 1998). For histology, brains were stained with cresyl violet. For birthdating, pregnant females were injected intraperitoneally with 100 μg/g BrdU on E12, E14 and E16, killed at P0, processed for wax sectioning and immunostained with anti-BrdU as described (Gradwohl *et al*, 1996). Darkly stained BrdU+ nuclei were considered born at the time of injection, and lightly stained nuclei derived from progenitors that had undergone additional cell divisions. For quantification, the cortex was divided into bins corresponding to CP layers I (MZ), II–IV, V, VI, IZ and GZ. Two brains/genotype and six sections/brain were counted and averaged.

Axonal tracing and zinc staining

Dissected brains were fixed overnight in 4% PFA. Corticofugal projections were labeled by placing a DiI crystal in the dorsal cortex as described (Seibt *et al*, 2003). Brains were incubated in 4% PFA for 6 weeks, embedded in 3% agar, and 100 μm thick vibratome sections were cut and analyzed. To chelate and stain synaptic zinc, mice were administered sodium selenite (5 mg/ml intraperitoneal) 60 min prior to dissection, and cortical hemispheres were sectioned and processed as described (see Supplementary data for details; Danscher, 1982).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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