

REVIEW ARTICLE

Molecular mechanisms of cortical differentiation

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Abstract

During development, several populations of progenitor cells in the dorsal telencephalon generate a large variety of neurons which acquire distinct morphologies and physiological properties and serve distinct functions in the mammalian cortex. This paper reviews recent work that has identified (i) key molecules involved in the specification and differentiation of cortical neurons, (ii) novel genes which distinguish distinct subsets of cortical progenitors and may be involved in the diversification of cortical neurons present in different cortical layers, and (iii) mechanisms involved in the generation of different projection neuronal subtypes in the well-studied model of layer 5 of the rodent cortex.

Introduction: neurogenesis and gliogenesis in cerebral cortex

All neurons, astrocytes and oligodendrocytes which form the adult nervous system originate ultimately from progenitor cells located in the ventricular zone of the embryonic neural tube. The cerebral cortex is composed of two main populations of neurons, projection (or pyramidal) neurons which are glutamatergic and excitatory, and interneurons which are GABAergic and inhibitory. Cortical projection neurons originate from progenitors located in the cortical ventricular zone. In contrast, most, if not all, cortical interneurons originate from progenitors located outside the cortex and primarily in the ventral telencephalon, at least in rodents (Marin & Rubenstein, 2001; Gorski *et al.*, 2002). Following their birth in the ventral telencephalon, interneurons use multiple and complex routes of tangential migration to reach their final position in the developing cortex (Marin & Rubenstein, 2001). Importantly, a significant fraction of GABAergic interneurons appear to be generated in the cortex in humans (Letinic *et al.*, 2002).

Cortical astrocytes, like projection neurons, originate from progenitors located in the cortex. Retrovirus-based lineage studies and *in vitro* culture experiments have shown that, early in cortical development, some of the progenitors located in the ventricular zone (VZ) are multipotent stem cells which generate both projection neurons and astrocytes (Davis & Temple, 1994; Williams & Price, 1995; Temple, 2001). These multipotent progenitors initially expand via symmetric divisions, but they soon generate more restricted progenitors, including neuronal- and astrocyte-restricted progenitors, which predominate during the later part of corticogenesis (Luskin *et al.*, 1988; Price *et al.*, 1995; Reid *et al.*, 1995; Williams & Price, 1995; Nieto *et al.*, 2001; Temple, 2001). Restricted neuronal and glial

progenitors have also been reported at very early stages of cortical development, raising the possibility that not all cortical neurons and glia originate from multipotent stem cells (Davis & Temple, 1994; McCarthy *et al.*, 2001).

As in the rest of the nervous system, neurons in the cortex are born and differentiate before astrocytes (Bayer & Altman, 1991). This is due to the sequential generation of neuronal and astrocyte precursors (Qian *et al.*, 2000) as well as to the delayed differentiation of astrocyte precursors, a process which involves multiple signalling, transcriptional and epigenetic mechanisms which are beginning to be elucidated (Morrow *et al.*, 2001; Sun *et al.*, 2001; Fan *et al.*, 2005; He *et al.*, 2005).

The origin of cortical oligodendrocytes is more complex than that of cortical astrocytes. During embryonic development, oligodendrocyte precursors in the telencephalon are thought to be generated outside the cerebral cortex, from stem cells located in the ventral telencephalon which also generate GABAergic interneurons (He *et al.*, 2001; Ross *et al.*, 2003). Like interneurons, oligodendrocyte precursors then migrate tangentially into the developing cortex (Thomas *et al.*, 2000; Tekki-Kessaris *et al.*, 2001). Fate-mapping of cortical progenitors in the mouse, however, indicates that many oligodendrocytes in the adult cortex originate from cortical progenitors (Gorski *et al.*, 2002). Indeed, a second postnatal wave of oligodendrocyte progenitors, which in contrast with earlier embryonic ones are glial-restricted, has been reported in the postnatal cortical subventricular zone (Ivanova *et al.*, 2003; Marshall *et al.*, 2003).

The rest of this review will focus on the generation of pyramidal neurons by cortical progenitors. The generation of new neurons is a complex process which involves a number of tightly coordinated steps, including the commitment of multipotent stem cells to a neuronal rather than a glial fate, the arrest of progenitor divisions, the acquisition of differentiated features by postmitotic precursors, including the growth of axons and dendrites, and the migration of

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nascent neurons outside the germinal zone towards a particular laminar position in the cortical plate. Important progress has been made in recent years in identifying some of the regulatory mechanisms involved. Proneural genes in particular have been shown to regulate many aspects of neurogenesis and, therefore, to play a central role in orchestrating the neurogenic process.

Regulation of the core programme of neurogenesis

Proneural genes

Proneural genes encode typical basic helix-loop-helix (bHLH) transcriptional activators, which heterodimerize with ubiquitously expressed bHLH partners called E proteins, and bind specific 6-bp motifs called E boxes. Binding of proneural E protein dimers to E boxes present in the promoter of target genes results in transcription activation. Although very few of their direct transcriptional targets have yet been reported, the biological activity of proneural proteins has been analysed in some detail in multiple model organisms, where they have been shown to play a critical role in neurogenesis (reviewed in Brunet & Ghysen, 1999; Bertrand *et al.*, 2002). In vertebrate embryos, a combination of gain-of-function and loss-of-function experiments have led to the idea that expression of proneural proteins in stem cells is both necessary and sufficient to promote the generation of postmitotic neurons and, therefore, that proneural proteins regulate the key steps of commitment of stem cells to the neuronal fate, cell cycle exit and initiation of a neuronal differentiation programme (Bertrand *et al.*, 2002).

Three proneural genes are expressed in the cerebral cortex of the mouse, *Neurogenin1* (*Ngn1*), *Neurogenin2* (*Ngn2*), and *Mash1* (Fode *et al.*, 2000; Nieto *et al.*, 2001), but *Ngn2* is clearly the most important of the three genes for corticogenesis. Only *Ngn2* mutants present a distinct corticogenesis defect (Fode *et al.*, 2000; Nieto *et al.*, 2001), and *Ngn2* is involved in regulating *Ngn1* (positively) and *Mash1* (negatively) in cortical progenitors, while the converse is not true (Fode *et al.*, 2000).

Neuronal commitment

The function of proneural genes in cortical development has been studied by analysis of mouse null mutants (Fode *et al.*, 2000; Nieto *et al.*, 2001; Schuurmans *et al.*, 2004), and by overexpression in dissociated cultures (Sun *et al.*, 2001).

A severe loss of proneural protein activity is observed in mice mutant for both *Ngn2* and *Mash1*, in which the cortex only retains residual expression of the third proneural gene *Ngn1*, which requires *Ngn2* for its normal expression (Fode *et al.*, 2000; Nieto *et al.*, 2001). In these mice, cortical neurogenesis is much reduced, as measured by expression of a neuronal precursor marker, *NeuroD*, and the size of the cortical plate (CP). Notch signalling, as measured by expression of *Hes5*, is also affected. Clonal culture experiments have shown that this neurogenesis defect is due to a loss of neurogenic progenitors in *Ngn2*/*Mash1* double-mutant cortex. Strikingly, this loss is associated with a compensatory increase in the number of astrocytic progenitors and progenitors which remain bipotent, thus demonstrating that proneural proteins are normally required to promote the choice of a neuronal fate, and inhibit the alternative astrocytic fate, in bipotent cortical stem cells (Nieto *et al.*, 2001).

Analysis of *Ngn1* activity in progenitor cultures has confirmed that this proneural protein has a dual function of promotion of neurogenesis and inhibition of astrogenesis, and has provided evidence that distinct mechanisms may underlie each of these

functions (Sun *et al.*, 2001). While the neurogenic activity of *Ngn1* requires an intact DNA binding domain and therefore involves the conventional transcriptional activation of target genes, such as the neuronal differentiation gene *NeuroD*, the antiastrogliogenic activity involves a novel mechanism which does not require DNA binding of *Ngn1* but instead requires interaction of *Ngn1* with intracellular components of several signalling pathways. Astrocyte differentiation, as measured by expression of the astrocytic marker GFAP, requires the synergistic activity of two signalling pathways, BMP-Smad and LIF-CNTF-JaK-STAT (Nakashima *et al.*, 1999). *Ngn1* has been shown to block the activity of Smad1 downstream of BMP, by sequestering a Smad1/P300 complex, and to interfere with the activation of STAT1 and STAT3 by LIF/CNTF (Sun *et al.*, 2001). A similar phenotype of reduced neurogenesis and premature or excessive astrogenesis has been observed in other brain regions in different proneural mutants (Tomita *et al.*, 2000; Parras *et al.*, 2004), suggesting that the neuronal commitment of bipotent neuronal-glial progenitors is a general and essential aspect of the function of proneural proteins.

Cell cycle exit

Cell cycle exit is an essential step in the progression of neuronal lineages, and is tightly coupled to specification of progenitors to a particular neuronal identity (which usually occurs during the last cycle; McConnell, 1995) and to the initiation of a differentiation programme (usually after cell cycle exit; Edlund & Jessell, 1999).

In a number of tissues, including muscles and endocrine cells, bHLH genes are part of the coupling mechanism, simultaneously promoting the differentiation of progenitors and their cell cycle exit, the later activity involving the regulation of cell cycle genes such as cyclin-dependent kinase inhibitors (Halevy *et al.*, 1995; Mutoh *et al.*, 1998). A similar role of proneural bHLH genes in promoting cell cycle exit has been reported in various parts of the nervous system, including peripheral ganglia (Lo *et al.*, 2002) and the spinal cord (Mizuguchi *et al.*, 2001), and this activity probably also involves the activation of cyclin-dependent kinase inhibitors, in particular p27/Kip1 (Farah *et al.*, 2000). Regulation of cell cycle progression and cell cycle length is a crucial process in corticogenesis and determines the total number of cortical neurons produced, as well as the relative ratios of neurons present in different cortical layers (Caviness *et al.*, 2003; Polleux *et al.*, 1997; Lukaszewicz *et al.*, 2005). The contribution of proneural genes to the regulation of cell cycle arrest by cortical progenitors has not been directly examined.

Two main populations of cortical progenitors contribute to the generation of cortical projection neurons, radial glial cells in the VZ, which possess a ventricular process and divide at the ventricular (apical) surface, and basal or nonsurface progenitors in the subventricular zone (SVZ), which originate from radial glial cells (or earlier from neuroepithelial cells), have lost their ventricular attachment and divide at a distance from the ventricle (Haubensak *et al.*, 2004; Miyata *et al.*, 2004; Noctor *et al.*, 2004). Indirect evidence from ectopic expression experiments by retrovirus infection of cortical slices suggests that *Ngn2* influences the mitotic behaviour of cortical progenitors, converting surface-dividing cells (radial glial cells) into non-surface-dividing cells (basal progenitors) rather than promoting outright cell cycle withdrawal (Miyata *et al.*, 2004). Further studies are required to elucidate the mechanism of coupling of cell cycle exit and differentiation of cortical progenitors, and the contribution of proneural genes to this mechanism.

Neuronal differentiation

Throughout the nervous system, proneural genes appear to be the main determinants of neuronal differentiation. Ectopic expression of a proneural gene is usually sufficient to initiate a full neuronal differentiation programme in undifferentiated neural progenitor cells (Ma *et al.*, 1996; Farah *et al.*, 2000; Mizuguchi *et al.*, 2001). Little is known about the individual genes that contribute to the generic programme of neuronal differentiation (i.e. the programme regulating the acquisition of features common to all neurons, such as the growth of axon and dendrites, as opposed to subtype-specific differentiation programmes, discussed in the next section). The best candidate 'neuronal differentiation genes' encode transcription factors which, like proneural proteins, belong to the bHLH class, in particular *NeuroD1* (Lee, 1997). These bHLH neuronal differentiation genes are sequentially expressed as neurons differentiate, and this regulatory cascade is activated by proneural genes in many regions of the nervous system (Bertrand *et al.*, 2002).

The contribution of proneural genes to the generic aspect of neuronal differentiation in the cortex has been difficult to assess from the analysis of proneural mouse mutants. This is due to the fact that loss of proneural activity in *Ngn2*/*Mash1* double-mutant mice results in a defect in neuronal commitment upstream of the neuronal differentiation programme (see above), while *Ngn2* single-mutants present a defect in specification of cortical neuron identity but no overt differentiation defect, due to compensation by up-regulation of *Mash1* (Fode *et al.*, 2000; Schuurmans *et al.*, 2004; see below). Nevertheless, mutant analysis has shown that *Ngn1* and -2 are required for normal expression in the cortex of many bHLH genes that are sequentially expressed in differentiating cortical neurons and are probably involved in their differentiation, including *NeuroD1*, *NeuroD2*, *Math2*, *Math3* and *Nsc1* (Schuurmans *et al.*, 2004). Moreover, other regulatory genes that are similarly sequentially expressed in cortical neurons and implicated in their differentiation, including the T-box genes *Tbr1* and *Tbr2* (Hevner *et al.*, 2001; Englund *et al.*, 2005) also depend on the function of *Ngn1* and -2 for their normal cortical expression (Schuurmans *et al.*, 2004). However, all the bHLH and *Tbr* differentiation genes regulated by *Ngn1* and -2 in the cortex are restricted to cortical progenitors and/or neurons in the embryonic telencephalon, and are not expressed by subcortical or basal ganglia progenitors and neurons (Schuurmans *et al.*, 2004). It is therefore unclear whether these genes are part of a generic neuronal differentiation programme or instead contribute to a regional differentiation programme involved in the acquisition of unique features of cortical projection neurons. Further analysis of the differentiation programmes of telencephalic neurons, and identification of their generic and subtype-specific components, is required to clarify the contribution of proneural genes to the differentiation of cortical neurons.

Specification of cortical neuron identity

The mechanisms underlying the specification of neuronal identity have been explored in a number of invertebrate and vertebrate systems. In mammals, specification of both spinal motor neurons and retinal neurons, among the best-studied models, involves a cooperation between neural bHLH genes and homeodomain genes (Lee & Pfaff, 2003; Hatakeyama & Kageyama, 2004). Both proneural bHLH genes (Bertrand *et al.*, 2002) and homeodomain genes (Shirasaki & Pfaff, 2002) have also been separately implicated in the specification of a large variety of neuronal types. Cortical neurons are no exception, and the proneural genes *Ngn1* and -2 and the homeodomain gene *Pax6* have been shown to have an important role in

specification of several aspects of the phenotype of cortical projection neurons.

Role of *Ngn1* and -2

Ngn1 and -2 and *Mash1* have largely complementary expressions in the telencephalon, in cortical progenitors and basal ganglia progenitors, respectively, raising the possibility that these genes contribute to the specification of the distinct neuronal fates of these two progenitor populations (Fode *et al.*, 2000). A combination of loss-of-function and gain-of-function studies has confirmed this hypothesis, demonstrating that *Ngn1* and -2 act as selector genes in cortical progenitors to regulate a binary choice between two distinct and alternative regional differentiation programmes. In wild-type mice, expression of *Ngn1* and -2 in dorsal telencephalic progenitors activates a programme that provides neurons with a glutamatergic neurotransmission phenotype (marked by expression of vesicular glutamatergic transporters vGlut1 and -2) and a cortical regional character (marked by expression of genes such as *NeuroD1* and -2, *Math2* and *Tbr1* and -2; see above). In *Ngn1*-*Ngn2*-mutant mice, a large fraction of dorsal telencephalic progenitors activate instead a programme conferring on neurons a GABAergic phenotype (marked by expression of the biosynthetic genes *GAD1* and *GAD2* and of GABA transporters) and a basal ganglia regional character (marked by expression of the *Dlx* family of homeodomain genes; Schuurmans *et al.*, 2004). This role of *Ngn1* and -2 in selecting the regional and neurotransmission identity of cortical neurons appears to involve several parallel activities. *Mash1* is de-repressed in *Ngn1*- and *Ngn2*-mutant cortex, suggesting that at least part of the function of *Ngn1* and -2 in specification of cortical neurons may involve repression of *Mash1* and the *Mash1*-induced basal ganglia programme. Indeed, many of the basal ganglia- and GABAergic-specific genes ectopically activated in *Ngn1*- and *Ngn2*-mutant cortex remain low in *Ngn2*/*Mash1* double-mutant mice, indicating that *Ngn1* and -2 repress the basal ganglia differentiation programme in part via repression of *Mash1*. In contrast, cortical and glutamatergic-specific genes fail to be activated in both *Ngn2* single- and *Ngn2*/*Mash1* double-mutant cortices, indicating that *Ngn2* also directly activates the cortical glutamatergic programme, independently of *Mash1* regulation (Schuurmans *et al.*, 2004; see also Fig. 1).

Ngn2 specifies other properties of cortical projection neurons including their characteristic unipolar dendritic morphology, which gives them their 'pyramidal' shape (Hand *et al.*, 2005). Interestingly, this activity requires phosphorylation of a tyrosine residue in the C-terminal part of *Ngn2*, which is only present, among Neurogenin proteins, in mammalian *Ngn2*, suggesting that it is part of an evolutionarily recent mechanism regulating cortical neuron specification. The signalling cascade involved in *Ngn2* phosphorylation in the cortex is presently unknown.

Importantly, *Ngn1* and -2 do not appear to be involved in specification of all cortical neurons. In *Ngn1* and -2 mutants, neurons born during the first part of corticogenesis [embryonic day (E)11.5–E14.5] are misspecified, but neurons born later (E14.5–E17.5) normally express cortical and glutamatergic markers. In keeping with this finding, *Ngn2* single-mutants, analysed after birth when cortical layers can be distinguished, present defects in expression of laminar-specific markers in early born neurons in layers 6 and 5, while late-born neurons in layers 4 and 2/3 appear normal (Schuurmans *et al.*, 2004). This result suggests that distinct determinants regulate the specification of early-born and late-born neurons, including for the acquisition of pan-cortical properties such as glutamatergic neurotransmission. Among the candidate regulators of late-born cortical neuron identity is the paired-homeodomain factor *Pax6*. *Pax6* plays

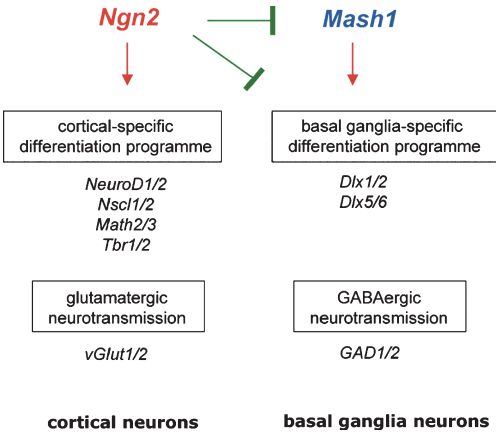


FIG. 1. Model of the genetic programmes controlling neuronal specification in the mouse telencephalon. The proneural genes *Ngn1*, *Ngn2* and *Mash1* are involved in the specification of cortical neurons and basal ganglia neurons, respectively, by coordinately regulating several aspects of their identity, including their regional identity and neurotransmission phenotype. Some of the genes involved in region-specific differentiation and neurotransmission in cortical and basal ganglia neurons, which are regulated by *Ngn1* and -2 and by *Mash1*, respectively, are listed. See text and Schuurmans *et al.* (2004) for further details.

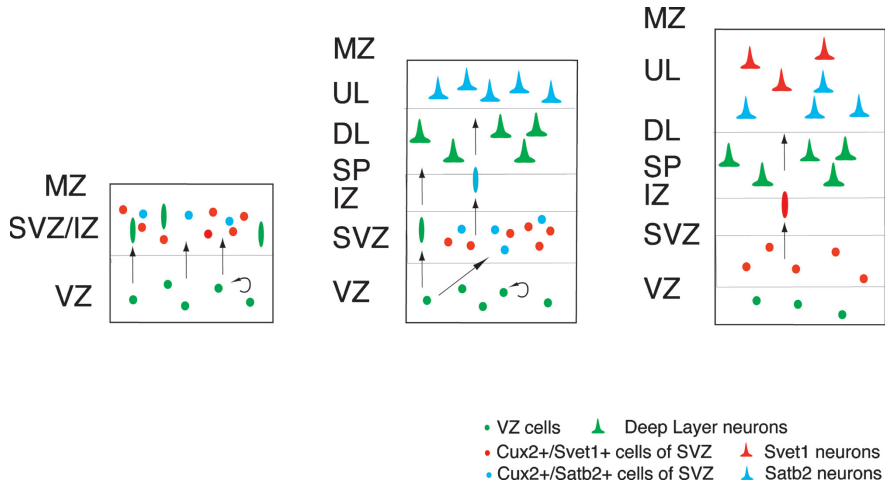


FIG. 2.

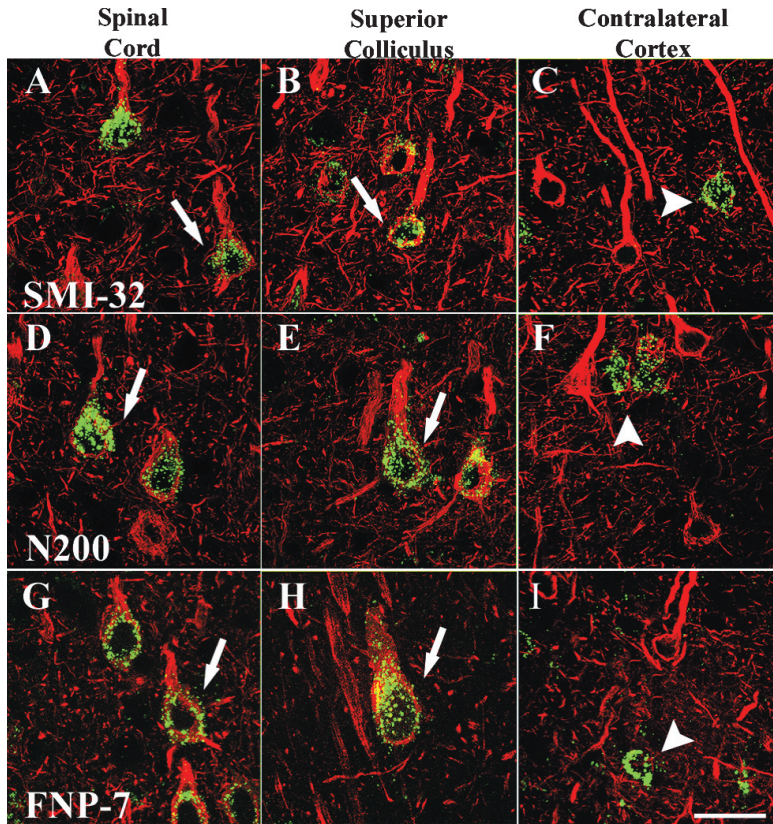


FIG. 3.

multiple roles in development of the cerebral cortex (see below). Some of these functions are probably mediated by *Ngn2*, particularly during early phases of cortical development, as *Pax6* is a direct transcriptional regulator of *Ngn2* in cortical progenitors (Scardigli *et al.*, 2003).

Transcription factor Pax6 in cortical specification

Pax6 is a member of the evolutionarily conserved *Pax* family of transcriptional regulators with a restricted expression in the developing CNS and the eye (Walther & Gruss, 1991; reviewed by Simpson & Price, 2002). *Pax6* contains two DNA binding domains, the paired domain and the paired-type homeodomain (Bopp *et al.*, 1986; Treisman *et al.*, 1991). In vertebrates, alternative splicing generates two isoforms: the canonical *Pax6* and the *Pax6-5a* isoform, where an insertion of 14 amino acids into the paired domain changes the binding properties of the *Pax6-5a* isoform (Czerny *et al.*, 1993; Epstein *et al.*, 1994).

Pax6 in the regional patterning of the telencephalon

Pax6 is a pattern-forming gene involved in the dorsoventral specification of telencephalic progenitors. As early as E9.0, *Pax6* shows a regionalized expression confined to the neuroepithelium of the dorsal part of the telencephalic primordium or pallium, the anlage of the future cortex (Walther & Gruss, 1991; Stoykova & Gruss, 1994; Stoykova *et al.*, 1997). Only one active allele of the homeobox-containing genes *Pax6* or *Emx2* is sufficient to promote the morphogenesis of the cerebral cortex, indicating that these two genes act in parallel to induce corticogenesis (Muzio *et al.*, 2002b). In the absence of *Pax6* and *Emx2* function, pallial neuroblasts are converted into subpallial neuroblasts which accumulate in an aberrant structure resembling basal ganglia (Muzio *et al.*, 2002b). In the cortical primordium, the early expression of *Pax6* and the homeobox gene *Gsh2* outline the anlagen of the pallium and subpallium, respectively, and cross-repressive interactions between these two genes establish the pallial–subpallial border (PSPB) (Toresson *et al.*, 2000; Yun *et al.*, 2001). Disruption of the PSPB in the *Pax6*–*Small eye* mutant, where *Pax6* is not functional (Hill *et al.*, 1991), permits more cells to migrate from the subpallium into the cortex (Chapouton *et al.*, 1999). A number of studies have demonstrated striking specification defects in the telencephalic neuroepithelium of homozygous *Small eye* (*Sey/Sey*) embryos, with a dorsal shift of subpallial markers, initially limited to the ventral and the lateral pallium, but later spreading progressively into the VZ of the entire pallium (Stoykova *et al.*, 1996, 2000; Toresson *et al.*, 2000; Yun *et al.*, 2001; Muzio *et al.*, 2002a; Kroll & O'Leary, 2005). Because *Ngn2* is a direct downstream target of *Pax6* (Scardigli *et al.*, 2003), part of the dorsoventral patterning defect of the telencephalon in the *Sey/Sey* embryos could be due to the abrogation of the *Ngn2*-dependent suppression of subpallial fate as discussed above. A recent genetic lineage tracing study has shown that the

molecular ventralization of pallial progenitors in *Pax6* mutants results in the respecification of the neuronal progeny of a subset of cortical glutamatergic progenitors into a subpallial GABAergic interneuron phenotype (Kroll & O'Leary, 2005). Thus, *Pax6*, like *Ngn2*, is required for the establishment of a correct neurotransmitter phenotype in pallial neurons (Schuurmans *et al.*, 2004; Kroll & O'Leary, 2005).

The graded expression of *Pax6* in cortical progenitors suggests that it may provide positional information for region-specific differentiation of the developing cortex. During corticogenesis, *Pax6* is expressed at its highest level in progenitors of the ventral pallium, which appear to be defective in *Pax6* mutants based on the absence of the markers *sFrp2* and *Dbx1*, specific for this region (Kim *et al.*, 2001; Yun *et al.*, 2001; Assimacopoulos *et al.*, 2003). At least one allele of either *Pax6* or the nuclear orphan receptor *Tlx* is required for the expression of *sFrp2* in the neuroepithelium of the ventral pallium, suggesting a genetic interaction between these two genes in patterning of the lateral telencephalon (Stenman *et al.*, 2003). The strong expression of *Pax6* in ventral pallial progenitors is essential for the specification of the claustrum, the endopiriform nucleus and the piriform cortex (Stoykova *et al.*, 2000) as well as the amygdalar lateral, basolateral and basomedial nuclei and the nucleus of the lateral olfactory tract, which all fail to form in the *Small eye* mutant (Tole *et al.*, 2005).

Pax6 and the specification of the cortical progenitors

Cortical progenitors are heterogeneous. At the onset of the neurogenesis, *Pax6* is expressed by the majority of radial glial cells that divide at the apical surface of the cortical VZ (Malatesta *et al.*, 2000; Hartfuss *et al.*, 2001; reviewed by Götz *et al.*, 2002). The basal progenitors, located and dividing at some distance from the apical surface of the VZ (Haubensak *et al.*, 2004), do not seem to express glial markers GLAST, hGFAP-Cre transgene and *Pax6* (Malatesta *et al.*, 2003), but the majority of them express instead *Tbr2* and *Ngn2* (Miyata *et al.*, 2004; Englund *et al.*, 2005).

Pax6 is an intrinsic molecular determinant of radial glial progenitors and, in the absence of *Pax6*, these cells present defects in their mitotic cycle, molecular phenotype and morphology (Götz *et al.*, 1998). In the *Small eye* mutants the proliferation of cortical progenitors increases at early stages (Götz *et al.*, 1998; Warren *et al.*, 1999; Estivill-Torres *et al.*, 2002), suggesting that corticogenesis in *Pax6* loss-of-function results from the residual neurogenic activity of basal progenitors only (Heins *et al.*, 2002; Haubst *et al.*, 2004). A number of approaches, including cell lineage analysis in dissociated and slice cultures (Malatesta *et al.*, 2000; Miyata *et al.*, 2001; Noctor *et al.*, 2004) and fate-mapping studies *in vivo* (Malatesta *et al.*, 2003; Anthony *et al.*, 2004), have now demonstrated that radial glial cells act as pluripotent progenitors, generating both neuronal and glial cells. Interestingly, both loss- and gain-of-function experiments have shown that *Pax6* endows radial glial progenitors with their neurogenic activity

FIG. 2. Model of cortical lamination. A pool of multipotent VZ progenitors could generate deep layer (DL) neurons, a process depending on the function of *Ngn* genes (Schuurmans *et al.*, 2004). VZ progenitors also produce a secondary proliferative zone, the subventricular zone (SVZ). Evidence available so far indicates that the SVZ contains at least two subpopulations of cells: *Satb2*⁺ cells and *Svet1*⁺ cells. Both cell subtypes can be detected in the SVZ at the stage E13.5. *Satb2*⁺ cells start migrating out of the SVZ shortly after their initial detection. *Svet1*⁺ cells, in contrast, need to spend several days in the SVZ and start to migrate only after stage E17.5. *Pax6* seems to be required for the differentiation of the *Svet1*⁺ cells, but not for the *Satb2*⁺ cells. Both cell subpopulations appear to express *Cux1* and *Cux2*.

FIG. 3. Confocal microscopic images of layer 5 pyramidal neurons showing that the three neurofilament antibodies (red) specifically stained neurons which project subcortically, but they did not stain cells with projections to the contralateral cortex (all populations contain green microspheres). (A and B) SMI-32, (D and E) N200 and (G and H) FNP-7 were specifically located in (A, D and G) the spinal cord and (B, E and H) superior colliculus projecting layer 5 neurons (arrows indicate examples of double labelling). (C, F and I) Contralateral cortex-projecting neurons (green microspheres indicated by arrowheads) did not express (C) SMI-32, (F) N200 or (I) FNP-7. The open arrowheads indicate neurons expressing the neurofilaments, but do not contain green microspheres and thus do not project to the contralateral cortex. Scale bar, 20 µm. (Reproduced with permission from Voelker *et al.*, 2004).

(Heins *et al.*, 2002; Haubst *et al.*, 2004). Analysis of the cortical phenotype of *Pax6* mutants with defects restricted to either the paired- or the homeodomain indicated, furthermore, that the control of progenitor proliferation, neurogenesis and dorsoventral patterning is mediated mostly by the canonical *Pax6* isoform which only depends on the function of the paired domain (Haubst *et al.*, 2004).

One of the several enhancers of *Ngn2* (*E1-Ngn2*) is only active in the part of the *Pax6* expression domain (mostly in the ventral and lateral pallium all along the PSPB) that expresses *Pax6* at its highest levels. The activity of this enhancer is directly regulated by *Pax6* in a dosage-dependent manner (Scardigli *et al.*, 2003). *Ngn2* expression in this region is lost in a *Pax6*-null mutant background (Stoykova *et al.*, 2000; Schuurmans *et al.*, 2004) while it becomes expanded when the dose of *Pax6* is increased (Scardigli *et al.*, 2003). Thus, the concentration level of patterning protein *Pax6* in cortical progenitors determines the expression pattern of proneural gene *Ngn2*, providing one of the first pieces of evidence in vertebrates of a direct regulatory link between neural patterning and neurogenesis (Scardigli *et al.*, 2003).

Pax6 in cortical layer formation

The cortex is organized radially into six layers (each of which contains neurons with distinct morphologies, projection patterns and neurotransmitter phenotype) and tangentially into numerous functional domains (Mallamaci & Stoykova, 2006). Postmitotic neurons of different layers are born in the proliferating progenitors cells in the VZ and SVZ in an inside-first, outside-last pattern (reviewed by McConnell, 1988). The time of birth of the neurons produced in the cortical germinal zones at sequential developmental stages is tightly linked with their laminar identity: early progenitors (in mouse between embryonic stages E10 and E14) generate the neurons of the lower layer 6 and layer 5, while progenitors after stage E14.5 produce exclusively neurons for the upper supragranular cortical layers 4, 3 and 2 (McConnell, 1988). Data from transplantation experiments indicated that during development the fate potential of the neuronal progenitors in the VZ is progressively restricted and by the end of the corticogenesis the progenitors are lineally committed to producing upper layer neurons (Frantz & McConnell, 1996; Desai & McConnell, 2000).

As discussed in the preceding paragraphs, elimination of *Ngn1* and -2 function appears to affect mostly the specification of neurons born early in corticogenesis which will migrate into the lower cortical layers (Schuurmans *et al.*, 2004). In contrast, accumulating recent evidence indicate that the neurogenic factor *Pax6* is required predominantly for the specification of neurons born in the later part of corticogenesis, which migrate into the upper cortical layers. Previous data from morphological (Schmahl *et al.*, 1993) and bromodeoxyuridine-labelling studies in embryos of the mouse (Caric *et al.*, 1997) and the rat *Small eye* mutants (Fukuda *et al.*, 2000) suggested that the supragranular cortical neurons are specifically affected in the mutant CP, possibly due to non-cell-autonomous migration defects of late-born neurons (Caric *et al.*, 1997) or defective radial glial processes, hampering the normal migration of neurons to their target locations in the CP (Götz *et al.*, 1998). Expression analyses using molecular markers for distinct layers have also suggested that in the *Sey/Sey* cortex the upper cortical layers are malformed while the lower layers are generally not affected (Nieto *et al.*, 2004; Tarabykin *et al.*, 2001; Schuurmans *et al.*, 2004; Zimmer *et al.*, 2004). Thus, the generation of neurons of the lower and upper cortical layers appear to be a specific output of *Ngn1-Ngn2*- and *Pax6*-controlled genetic pathways, respectively (Schuurmans *et al.*, 2004).

Results from several studies suggest that the SVZ may specifically contribute to the generation of the upper cortical layers in primates (Sidman & Rakic, 1973; Smart *et al.*, 2002) as well as in mice (Smart & McSherry, 1982; Smart & Smart, 1982; Tarabykin *et al.*, 2001). By using cell cycle-specific markers in birthdating experiments, Lukasiewicz *et al.* (2005) recently convincingly demonstrated that indeed the progenitors of the SVZ in primates produce the supragranular layer neurons. The role of molecular cues in the SVZ for changing the temporal identity of cortical progenitors from early progenitors (producing lower cortical layers) to late progenitors (producing upper cortical layers) has only recently begun to be understood. Genes with a transient or specific expression in the cortical VZ and SVZ and in distinct cortical layers would be appropriate candidates for exerting control functions for specification of the temporal identity of cortical progenitors. Several recent studies support the idea that *Pax6* might have an essential role in this process. The recently identified cDNA sequence *subventricular tag* (*Svet1*) shows an intriguing expression pattern confined to both SVZ progenitors and upper cortical layers at a perinatal stage (Tarabykin *et al.*, 2001). In the homozygous *Sey/Sey* embryos the expression of the POU homeodomain gene *Otx1*, which is normally restricted to the VZ and the deep cortical layers (Frantz *et al.*, 1994), is not altered (Stoykova *et al.*, 2000; Tarabykin *et al.*, 2001). However, the expression of *Svet1* in the abnormal SVZ and CP of *Sey/Sey* cortex is abolished, suggesting that *Pax6* is involved in the determination of the upper cortical layer identity in the SVZ (Tarabykin *et al.*, 2001; see Fig. 2). In the developing cortex, *Cux1* and *Cux2*, two homologues of *Drosophila Cut*, are specifically expressed in the VZ and in mitotically active cells of the SVZ, respectively, whereas later on their expression is confined to distinct subpopulations of the upper cortical layers (Nieto *et al.*, 2004; Zimmer *et al.*, 2004). Interestingly, the expression of *Cux2* in the SVZ and the expression of both *Cux2* and *Cux1* in the supragranular cortical layers is severely diminished in the *Pax6*-mutant cortex, suggesting that in addition to *Svet1* these two genes might also act as determinants for the specification of the upper cortical layer neurons in a *Pax6*-dependent pathway (Nieto *et al.*, 2004; Zimmer *et al.*, 2004).

It is unclear whether the expression of *Svet1* and *Cux2* is an intrinsic property of distinct cell subpopulations of the SVZ or whether their expression is induced by environmental cues which are available within the SVZ at late developmental stages. Interestingly, GABAergic interneurons which normally migrate from the subpallium into the SVZ of the pallium might play a role in providing such environmental signals. As recently shown, GABA can dramatically increase the proliferation of VZ progenitors whereas the proliferation of the SVZ is substantially inhibited (Haydar *et al.*, 2000).

In addition to *Pax6*, the nuclear orphan receptor *Tlx* also appears to be involved in the generation of upper cortical layers (Land & Monaghan, 2003a,b). Loss of function of both *Pax6* and *Tlx1* genes results in increased severity of the defects in the dorsoventral telencephalic patterning (Stenman *et al.*, 2003) and in specification generation of the upper cortical layers in the rostrolateral cortex, implying a genetic cooperation between these two factors (Schuurmans *et al.*, 2004).

Molecular determinants of cortical layer formation

The great cellular heterogeneity of the cerebral cortex renders difficult the classification of cortical neuron subtypes solely on the basis of their developmental birthday and morphological and physiological characteristics. A number of specific molecular markers for neurons of distinct cortical layers have been recently identified (see Table 1) and

these have a profound impact on the elaboration of a more comprehensive classification of the cortical neurons.

Many of these genes are not expressed uniformly within a given layer. Their expression borders often demarcate boundaries between different cortical areas within a certain layer or have rostrocaudal or mediolateral gradients of expression. For instance, *Cadherin-6* (Suzuki *et al.*, 1997) and *ephrin-A5* (Mackarehtschian *et al.*, 1999) are predominantly expressed in the parietal cortex, while the expressions of *Lmo4* and *Clim1a* (Bulchand *et al.*, 2003) are predominantly confined to the upper layers of the frontal and occipital cortex, respectively. Some genes with layer-restricted expression have been mutated and shown to play an essential role in normal corticogenesis. Mutation of the T-box transcription factor *Tbr1* causes a specific defect of the generation of the layer 6 neurons as well as Cajal–Retzius cells of the marginal zone and subplate neurons, while the production of upper cortical layers is not affected (Hevner *et al.*, 2001). Consistent with its restricted expression in layer 6 and layer 5, the homeobox transcription factor *Otx1* is required for the normal connectivity of layer 5 neurons (Weimann *et al.*, 1999). Two other genes with restricted expression in the supragranular cortical layers which encode the POU-domain transcription factors *Brn1* and *Brn2* were found to have essential roles for normal production and migration of layers 2–4 neurons (McEvelly *et al.*, 2002; Sugitani *et al.*, 2002).

Given the importance of molecular markers in defining layer-specific neuronal subtypes (Hevner *et al.*, 2003), efforts have recently been made to identify novel genes with laminar-restricted expression in the cortex. In one set of experiments, cDNA subtraction between mRNA pools isolated from different stages of cortical development

was performed and resulted in isolation of several novel genes with expression restricted to distinct cortical layers (Tarabykin *et al.*, 2001; Britanova *et al.*, 2005). One of these genes, *Svet1*, is a marker of proliferating cells in the SVZ and some cells in the upper cortical layers, as mentioned above. Two other genes, *Satb1* and *Satb2*, members of a novel family of transcription factors, are expressed in a mutually exclusive manner in the developing cortex. *Satb1* expression is detected in cells of the marginal zone (layer 1) and in the deep layers, overlapping with *Tbr1* expression, while most *Satb2*-positive cells are located within layers 2–4. Although a great number of supragranular neurons express *Satb2*, it seems that *Satb2* is not a universal marker of the upper cortical layer neurons. Thus, most of the cells that express *Tbr1* at a high level do not coexpress *Satb2*, and *Satb2*-positive upper cortical neurons do not coexpress *Svet1* (Britanova *et al.*, 2005). Neurons born in the SVZ stay several days in this region ('sojourn phase') before they migrate out towards their final location in the CP (Bayer & Altman, 1991). In contrast to *Svet1*-positive cells which stay several days in the SVZ and appear to require *Pax6* function for their differentiation, *Satb2*-positive upper cortical layer neurons do not seem to stay in the SVZ, or to be affected in the *Small eye* mutant (O. Britanova, P. Gruss and V. Tarabykin, unpublished observations). It is still not clear whether *Satb2*⁺ neurons are derived from *Cux2*-expressing cells in the SVZ or whether these are two independent cell subpopulations. Altogether, current evidence suggests that two subtypes of upper cortical layer neurons might coexist whose generation might be, respectively, dependent and independent of *Pax6* function.

Koral is another newly identified gene which encodes a protein of 378 amino acids (NM_177869) of still unknown function (V. Tarabykin and P. Gruss, unpublished observations). In the mouse developing cortex, *Koral* expression is detected from E15.5 onwards in a subpopulation of layer 5 neurons within the upper part of the *Otx1* expression domain, while its expression is downregulated after birth and in adult brain. The expression of the gene *Clsn2* is first detected in migrating neurons of the intermediate zone of the cortex and later on in young neurons of layers 2–4 (O. Britanova, P. Gruss and V. Tarabykin, unpublished observation).

By using a similar cDNA subtraction approach, Zhong *et al.* (2004) have identified *Unc5h4* as a gene expressed mostly in layer 4 neurons. Interestingly, *Svet1* transcript, which does not seem to encode any protein, is transcribed from an intron of the *Unc5h4* gene, and both genes have a highly similar expression patterns (V. Beilinson and V. Tarabykin, unpublished observation), suggesting that the same enhancer might control their expression. Three other genes, *deltex-like* gene, *stem cell factor* (*SCF*) and *myocyte-specific enhancer factor-2C* (*MEF-2C*) have also been identified in this study as novel markers with a restricted expression in layers 2–4.

Recently, a genome-scale expression analysis of genes encoding all putative transcription factors revealed layer-restricted expression pattern for some of them (Gray *et al.*, 2004). The transcription factors *FoxO1*, *NR4A2* (*Nurr1*), *bHLHb5* and *Lmo4* are expressed in cells of the upper layers, while the expression of *Sox5*, *Klf6*, *Zfp312* and *NR4A3* is confined to cells of the deep layers (see Table 1). Apart from their layer-specific expression, some of the genes are expressed in a region-specific manner or form gradients. For instance, *NR4A3* is expressed with a prominent medial (high) to lateral (low) gradient, while *Mef2C* is more abundantly expressed in the lateral than in the medial cortex (Gray *et al.*, 2004).

Finally, a recent microarray analysis of the transcription profile of callosal and corticospinal cortical projection neurons has revealed novel marker genes for these neuronal subpopulations (Arlotta *et al.*, 2005; see below). Fourteen genes identified in this study showed

TABLE 1. Summary of some layer-specific genetic markers

Gene	Layer					
	1	2/3	4	5	6	6b
<i>bHLHb5</i> ^d		+	+			
<i>Brn2</i> ^c		+				
<i>Clim1a</i> ^b				+		
<i>CTIP2</i> ^h				+		
<i>Cux2</i> ^{d,e}		+	+			
<i>Deltex-like</i> ^g		+	+			
<i>Er81</i> ^c				+		
<i>FoxO1</i> ^d		+				
<i>Klf6</i> ^d			+			
<i>Koral</i>				+		
<i>Latexin</i> ⁱ					+	
<i>Lmo3</i> ^b		+	+		+	
<i>Lmo4</i> ^{b,d}		+	+		+	
<i>MEF-2C</i> ^{d,g}		+	+			
<i>NR4A3</i> ^d				+	+	
<i>Otx1</i> ^c				+	+	
<i>Reelin</i> ^c	+					
<i>ROR-b</i> ^c			+			
<i>Satb1</i> ^a	+			+	+	+
<i>Satb2</i> ^a		+	+			
<i>SCF</i> ^g		+	+			
<i>SCIP</i> ^c		+			+	
<i>Sox5</i> ^d				+	+	
<i>Svet1</i> ^f		+	+			
<i>Tbr1</i> ^c	+				+	+
<i>Unc5h4</i> ^g			+			
<i>Zfp312</i> ^g				+	+	

^aBritanova *et al.* (2005); ^bBulchand *et al.* (2003); ^cHevner *et al.* (2003); ^dGray *et al.* (2004); ^eNieto *et al.* (2004) and Zimmer *et al.* (2004); ^fTarabykin *et al.* (2001); ^gZhong *et al.* (2004); ^hArlotta *et al.* (2005); ⁱArimatsu *et al.* (1999).

expression confined to morphologically distinct subpopulations of corticospinal neurons, which might potentially be instructive concerning the generation and/or differentiation of corticospinal motor neuron subtypes.

Specification of the layer 5 pyramidal neuronal subtypes

The cortical projection neurons are further classified by their morphology, electrophysiology, hodology and neurochemical characteristics in relation to the laminar position of the cell body (Peters & Jones, 1985). In this part of the review we shall describe recent studies on subtypes identification of layer 5 pyramidal neurons in the rodent cerebral cortex, as they provide an exceptional model system to study cortical neuronal specification within the same cortical lamina.

Two major classes of layer 5 pyramidal cells; an accessible model for the study of target selection and dendritic and physiological differentiation

Pyramidal cells of layer 5 of the adult rodent cortex fall into two major classes which can be distinguished on the basis of their projection site, morphology and physiological properties in the adult (Klein *et al.*, 1986; Hallman *et al.*, 1990; Larkman & Mason, 1990). Pyramidal neurons of layer 5 in the rat are born between E15 and E18, migrate to layer 5 by E19–20 (Miller, 1998) and appear indistinguishable when they first migrate into their final position in the CP, all having stout apical dendrites reaching layer 1 with terminal tufts, and none of them fire bursts after the injection of depolarizing currents. However, there seems to be a sequential expression of characteristics (see Table 2). Shortly after they have migrated into the CP, the pyramidal neurons grow correctly at E19 to postnatal day (P)0 towards the appropriate target: type I neurons reach superior colliculus, basal pons or spinal cord, while type II neurons reach the contralateral hemisphere. However, both type of pyramidal neurons do not invade the target regions until the early postnatal period (P0–3), and morphologies of the two types do not begin to diverge until \approx P5 (Koester & O'Leary, 1992, 1993). Type I cells with axons to the superior colliculus maintain their thick tufted apical dendrites, and begin to fire bursts

after the second postnatal week (Kasper *et al.*, 1994). Type 2 cells with axons to the contralateral hemisphere transform to cells with slender apical dendrites with fewer oblique branches that end without terminal tufts, usually in the upper part of layers 2/3, and never fire bursts (Kasper *et al.*, 1994). Larkman & Mason (1990) also showed that, in adult rat visual cortex, these two groups differ in other intrinsic physiological properties, such as input resistance and time constant. Interestingly, recent studies have revealed that the electrophysiological differences emerge earlier than previously believed (Christophe *et al.*, 2005). Deisz (1988) proposed that a calcium-activated potassium current controls the generation of a depolarizing $\text{Ca}^{2+}/\text{Na}^{+}$ envelope underlying the bursts in neurons, but the factors controlling its development are not known (Schwindt & Crill, 1997). While most experiments were performed in rat visual and somatosensory cortices, the existence of two similar types of layer 5 neurons in other cortical regions (Games & Winter, 1988; Chagnac-Amitai *et al.*, 1990) suggests that it is a general feature of rodent neocortex.

The emergence of the differences between these two distinct cell classes provides an excellent model system for studying the contribution of genetic and epigenetic factors during cortical development and provide numerous technical advantages. Layer 5 pyramidal cells are among the largest cells in the cerebral cortex; this is of considerable advantage for recording and single-cell RT-PCR studies (Christophe *et al.*, 2005). The subclasses reach their specific targets before any distinctions can be observed in their morphology or physiology so it is relatively easy to label them selectively in living or fixed tissue through their specific projections and before their full differentiation with fluorescent latex beads or carbocyanine dyes (Koester & O'Leary, 1992; Kasper *et al.*, 1994). This permits the selective identification of the different cells in combination with immunohistochemistry, *in situ* hybridization or single-cell recording (Hevner *et al.*, 2003; Voelker *et al.*, 2004; Arlotta *et al.*, 2005; Christophe *et al.*, 2005). In spite of these advantages of this system, it seems that our current knowledge of interneuron populations is much better (Cauli *et al.*, 1997; Markram, 2004) than that of pyramidal cells. The paucity of specific markers for classes and subclasses of pyramidal neurons not only holds back our efforts to understand development but also hinders our studies of functional cortical circuits.

TABLE 2. Time frames of the sequential differentiation of the two major classes of pyramidal neurons in the rat cortex

Feature	Age	Type I	Type II
Cell birth	E15–16		
Innervation of target	E21–P0	Spinal cord	Contralateral cortex Striatum
		Superior colliculus Basal pons Tufted	Non-tufted
Somatodendritic morphology	P5–7		
Firing properties	P15–21	Bursting	Non-bursting

Both types of pyramidal neurons are born in rat at E15–16. By E19–P0 both set reach their appropriate target: type I neurons reach superior colliculus, basal pons or spinal cord while type II neurons reach the contralateral hemisphere and, shortly afterwards (P0–P3), invade their target regions. By P5 the morphologies of the two types start to diverge. Type I cells with axons to the superior colliculus maintain their thick tufted apical dendrites while type II cells with axons to the contralateral hemisphere transform to cells with slender apical dendrites without terminal tufts. Type I neurons start to fire bursts of action potentials from the end of the second postnatal week, whereas Type II cells never fire bursts. The data for the Table have been collected from Koester & O'Leary (1992), Kasper *et al.* (1994) and Larkman & Mason (1990).

Molecular characterization of the layer 5 pyramidal cells

Previous and recent studies have isolated molecules which serve as molecular markers for layer 5 neurons: Otx1, a transcription factor expressed in layers 5/6, which is specific to type I neurons (Frantz *et al.*, 1994; Weimann *et al.*, 1999); SMI-32, N200 and FNP-7, which are neurofilaments only expressed in type I neurons (Hornung & Riederer, 1999; Voelker *et al.*, 2004; see Fig. 3); ER81, an ETS transcription factor expressed in both neuronal cell types (Hevner *et al.*, 2003; Yoneshima *et al.*, 2006); Lmo4, a LIM domain-containing protein known to be expressed in layers 2/3 and 5 (Bulchand *et al.*, 2003), which is a specific marker for type II neurons (Arlotta *et al.*, 2005); and *CTIP2*, *Fez6* genes specifically expressed in layer 5 type I neurons (Arlotta *et al.*, 2005; Molyneaux *et al.*, 2005).

Co-localization studies with Otx1 and ER81 suggested that the two markers are not expressed within the same postnatal layer 5 neurons; moreover, ER81 is also expressed in some type II layer 5 pyramidal neurons (Hevner *et al.*, 2003; Yoneshima *et al.*, 2006). Although ER81 and N200 have been shown to label type I projection neurons in layer 5, retrograde labelling and immunohistochemistry for the two markers revealed that they are never coexpressed in the same projection neurons, suggesting that there are at least two distinct neurochemical subpop-

ulations within type I layer 5 pyramidal cells (Rolph *et al.*, 2005; A. Cheung, C. Voelker, R. Rolph and Z. Molnar, unpublished observations; see also Fig. 3). An important aim of these studies is to be able to unify molecular classification with other aspects of layer 5 neuronal classification in the adult and during development (Molnár & Cheung, 2006). As pointed out above, potential molecular markers for layer 5 neurons are continually being found and the correlation of these markers with other aspects of neuronal phenotype will result not only in a more comprehensive classification of layer 5 projection neurons but also in the better understanding of the genetic and epigenetic programs of differentiation.

Layer 5 neurons have, furthermore, been shown to be divided into five major classes based upon multidimensional cluster analysis of a series of dendritic morphological variables, revealed by biocytin staining, in mouse primary visual cortex (Tsiola *et al.*, 2003). Using electrophysiological recording of prelabelled neurons from their specific projection sites, combined with morphological reconstruction and immunocytochemistry and with single-cell gene expression profiles for various markers (Audinat *et al.*, 1996; Christophe *et al.*, 2005; Sugino *et al.*, 2006), will further advance our understanding into the differences (or similarities) between the two neurochemically distinct populations. By performing these studies at specific periods of differentiation (Arlotta *et al.*, 2005; Christophe *et al.*, 2005), we hope to get an insight into the mechanisms controlling the developmental decision making.

Conclusions

This is an exciting time for researchers involved in the study of cortical development, as rapid progress is made in identifying molecules playing key roles in the specification and differentiation of cortical neurons, and long-standing issues, such as the degree of heterogeneity of cortical progenitors and the lineal relationships of different progenitor subpopulations, are finally being addressed. This review has focused exclusively on intrinsic regulators of cortical neurogenesis, reflecting the interests and biases of the authors. However, throughout neurogenesis, cortical progenitors and neurons are influenced by extrinsic signals. An important challenge ahead is to acquire a more comprehensive understanding of the regulation of cortical development, including both intrinsic and extrinsic determinants, and to identify the mechanisms by which the signalling pathways and transcription factors involved interact.

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Abbreviations

bHLH, basic helix-loop-helix; CP, cortical plate; E, embryonic day; *Ngn1*, *Neurogenin1*; *Ngn2*, *Neurogenin2*; P, postnatal day; *Sey/Sey*, homozygous *Small eye*; *Svet1*, *subventricular tag1*; SVZ, subventricular zone; VZ, ventricular zone.

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