Role of p35/Cdk5 in Preplate Splitting in the Developing Cerebral Cortex

The earliest generated cells of the mammalian cerebral cortex form the preplate layer (PPL). The subsequently born cortical plate (CP) cells split this layer into the superficial layer I (LI) and the deep subplate (SP). The cellular and molecular mechanisms that underlie this event are unclear. To investigate the role of the cyclin-dependent kinase 5 (Cdk5) and its activator p35 in preplate splitting, we used Nissl staining, carbocyanine dye tracing, cell birthdating, and immunohistochemistry for calretinin (CalR) in p35 and Cdk5 knockout mice. Our data demonstrated changes in early corticofugal and aberrant thalamic axon trajectories in these mice. Specifically, LI was thicker, and cell-dense and thalamic axons did not accumulate in the SP layer before invading the CP. Instead, they grew past the SP and more superficial cortical layers and coursed obliquely toward the pial surface. This behavior has been previously observed in reeler mice and suggests a defect in PPL splitting. CalR immunohistochemistry and bromo-deoxyuridine birthdating confirmed the abnormality in position of the earliest generated cortical cells of mutants. These observations suggest that the p35/Cdk5 pathway plays a role in preplate splitting in addition to regulating layer formation.

Keywords: axons, development, layer I, subplate, thalamus

Introduction

The preplate layer (PPL; Rickmann and others 1977), also known as primordial plexiform layer (Marin-Padilla 1971) or early marginal zone (Boulder Committee 1970), is the first layer of postmitotic cells to appear during mammalian corticogenesis. This layer is subsequently separated by emerging cortical plate (CP) neurons into the superficial layer I (LI) and the deep subplate (SP) layer (Luskin and Shatz 1985; Marin-Padilla 1998). Despite persistent efforts for more than 2 decades to unravel the PPL, the exact structure, origin, function, and fate of this layer and its derivatives remain unclear.

Preplate neurons are the earliest generated (embryonic day 10.5–12.5 in mouse) and precociously matured cortical neurons. Cajal-Retzius (C-R) cells are the most distinct neuronal type in the PPL located predominantly beneath the pial surface. They display diverse morphologies, and their axons remain within the PPL. The key biochemical features of these cells are the glycoprotein Reelin (D’Arcangelo and others 1995; Ogawa and others 1995) and the transformation-related protein p73 (Yang and others 2000). Further, they contain a wealth of other molecules, among them glutamate (del Rio and others 1995), calretinin (CalR) (del Rio and others 1995), Golgi proteins (Landry and others 1998; Tosic and others 2002), and a variety of receptors: glutamate (Lopez-Bendito, Shigemoto, Fairen, and Lujan 2002), γ-aminobutyric acid (GABA) (Lopez-Bendito, Shigemoto, Kuklik, and others 2002), ErbB4 (LI and others 2004), and Cxcr4 (Stumm and others 2003). They also express the pallial transcription factors Emx2 (Mallamaci and others 2000), Tbr1 (Hevner, Neogi, and others 2003), and Dbx1 (Bielle and others 2005). Apart from C-R cells, the PPL also comprises 1) pyramidal-shaped cells, representing future SP neurons, that express Golli (Landry and others 1998), Tbr1 (Hevner, Neogi, and others 2003), CalR (del Rio and others 2000), and low-affinity neurotrophin receptor p75 (reviewed by Allendoerfer and Shatz 1994), and 2) a number of small, migrating GABAergic interneurons, displaying various orientations that express calbindin (del Rio and others 2000) and Dlx gene products (Hevner, Neogi, and others 2003; Rakic and Zecvevic 2003). The PPL is also replete with monoamines (Marin-Padilla 1998) and extracellular matrix molecules including fibronectin (Pearlman and Sheppard 1996) and chondroitin sulfate proteoglycans (CSPGs; Sheppard and Pearlman 1997). Extracellular matrix proteins may have important roles in PPL splitting and cortical layer formation.

Preplate neurons have been thought to originate from the local cortical ventricular zone (Marin-Padilla 1998) and migrate radially toward the pial surface by “somal translocation” (Nadarajah and others 2001; Nadarajah and Parnavelas 2002). However, recent studies have shown that C-R cells originate from the discrete cortical proliferative zones of the cortical hem, ventral pallium, or septum and populate the PPL of complementary pallial regions by subpial migration (Bielle and others 2005; Yoshiida and others 2006). Other studies have suggested that C-R cells also come from the olfactory primordium (Zecvevic and Rakic 2001), retrobulbar area (Meyer and others 1999), and subpallium (medial ganglionic eminence; Lavdas and others 1999; Rakic and Zecvevic 2003). Although the vast majority of GABAergic interneurons in the PPL arise from the subpallium (Lavdas and others 1999), future SP projection neurons are most likely of pallial origin and are probably born within a day, around E11.5 or E12 in mouse. Precise data on the origin of SP neurons are not available as there are no SP-specific markers.

The underlying mechanism of PPL splitting is yet to be elucidated. At the time of splitting, most SP neurons seem to occupy the SP (de Carlos and O’Leary 1992), but the ratio of cells in LI and SP has not been established quantitatively. The SP contains a population of transient cells with long-range projections (McConnell and others 1989) and small GABAergic neurons (17% of its neurons; del Rio and others 2000). The former sends early fibers toward the internal capsule to guide early corticofugal and thalamocortical projections (McConnell and others 1989; Molnar and Blakemore 1995; reviewed by Lopez-Bendito and Molnar 2003). SP neurons receive functional synaptic input from the thalamus and relay this input to the CP well in advance of the invasion of thalamic axons into layer IV.
(for review, see Allenderfer and Shatz 1994). This early circuitry is believed to play a role in the maturation of the cortex and its emerging electrophysiological properties.

LI contains predominantly C-R cells (Frotscher 1997) as well as interneurons. C-R cells secrete the glycoprotein Reelin (D’Arcangelo and others 1995; Ogawa and others 1995), which is thought to regulate radial glia phenotype, proper splitting of the PPL, and “inside-out” layering of neocortical neurons (for review, see Tissir and Goffinet 2003). In the Reelin-deficient mouse, reeler, the partition of the PPL does not occur; hence, LI and SP neurons stay together in a layer called the “superplate” (Caviness 1982). The layers below the superplate are indistinct and are generated in an “outside-in” pattern (Caviness and Sidman 1973). Cyclin-dependent kinase 5 (Cdk5) is another molecule that significantly regulates neuronal migration in the developing cortex (Ohshima and others 1996; Chac and others 1997). It has been suggested that Reelin and Cdk5 pathways cooperate in regulating corticogenesis (Beffert and others 2004). In mice deficient in Cdk5 or its major activator p35, the PPL is split into LI and SP by the arrival of the early-born projection neurons (Gilmore and others 1998; Kwon and Tsai 1998). However, the late-born projection neurons fail to pass the SP and accumulate in a predominantly outside-in manner below it (Gilmore and others 1998; Kwon and Tsai 1998). Thalamocortical axons proved to be very sensitive indicators of cortical layering abnormalities (Molnar and others 2003). In normal mice, thalamic axons target SP neurons, whereas in reeler mice, they traverse the immature CP and extend in the direction of the superplate layer (Molnar and others 1998). In Cdk5 knockout (KO) mice, thalamic fibers have been described to target SP cells normally and were not thought to enter the compact CP prematurely or run toward the pia (Gilmore and others 1998). It has been proposed that the different arrangement of thalamocortical axons in reeler and p35/Cdk5 KOs is secondary to distinct positional defects of early-born cortical neurons. In the present study, we investigated further these questions by documenting the distribution of the earliest generated cells and the development of the early thalamocortical connectivity in p35 and Cdk5 KO mice.

Materials and Methods

Animals

All animal procedures were performed in accordance with institutional guidelines. Brains taken from p35 heterozygous and homozygous mice at different stages of development (E11.5-19.5) were fixed by immersion in 4% paraformaldehyde and used for Nissl staining, axon-tracing studies, and immunohistochemical experiments. p35 heterozygous mice did not show any abnormal phenotype and were used as controls. Cdk5 KOs and the corresponding controls at E18.5 were also used in this study.

Nissl Staining

Thionin and toluidine blue solutions were used to label cortical cytoarchitecture. Stained sections were also used to measure cortical thickness.

Axonal Tracing

Carbocyanine dyes were used to label axons in fixed brains of littersmates. For further technical details, see Molnar and others (1998). Briefly, single crystals of the fluorescent carbocyanide dye Dil (1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate) were placed in single locations in the dorsal thalamus under a dissecting microscope. After placement of dye crystals, the brains were stored in 4% paraformaldehyde at 37 °C for at least 3 days. Following incubation, the brains were embedded in 4% agarose and cut into 100-μm-thick coronal sections on a Vibratome. The cytoarchitecture of the brain was revealed by using bisbenzimide (BB).

Immunohistochemistry

Immunohistochemistry was performed on 100-μm-thick Vibratome sections or 15-μm sections cut on a Cryostat. CalR antigen was visualized using rabbit anti-CalR antibody and immunoperoxidase/3,3′-diaminobenzidine method. CalR is expressed in PPL neurons and in the emerging SP and LI. It is also found in prenatal corticopetal fiber systems, including thalamic axons (Fonseca and others 1995). A mouse anti-Nogo-A antibody was also used as a marker of developing axons (Hunt and others 2003).

BrdU Injections and Staining

BrdU was injected intraperitoneally into timed-pregnant mice at E11.5 (for PPL splitting analysis) or E13.5, E15.5, and E17.5 (for cortical layering analysis), and the distribution of BrdU-labeled cells was studied at E15.5/E18.5 (in the former) and P12 (in the latter; P-postnatal day). BrdU staining, single or double (with anti-CalR antibody), was performed using standard immunofluorescence (PPL splitting) or immunoperoxidase (cortical layering) techniques.

Quantitative Measurements

Coronal sections of p35+/− and p35−/− forebrains, 100-μm thick, were immunolabeled using antibodies to BrdU and CalR and counterstained with BB to label layers. Immunolabeled sections were analyzed using a confocal microscope. Multiple z-plane images (z-stacks) were acquired throughout each section and analyzed using Metamorph software. BrdU− cells were counted in LI, CP, and SP on each z-plane confocal image that had clearly visible BrdU/CalR/BB staining.

Results

Thick, Multicellular, and Disorganized LI in p35 and Cdk5 KOs

We first investigated whether there were changes in prenatal neocortical layers in p35−/− mice compared with controls by examining Nissl-stained sections. At E14.5, in both p35+/− and p35−/− genotypes, we noted splitting of the PPL that resulted in the formation of LI and SP. At this stage, p35 KO and control cortices showed no significant differences in the thickness of LI (22.5 ± 3.7 vs. 22.5 ± 4.7 μm) and CP (35.5 ± 2.8 vs. 38.5 ± 1.3 μm) (Fig. 1AA,EF). However, a clear difference in neocortical layering between the 2 genotypes appeared at E16.5. The compact CP, located between LI and the SP, was significantly thinner in p35−/− mice (67.5 ± 7.8 vs. 124.2 ± 1.5 μm) (Fig. 1BB,F), and there was a visible accumulation of cells below the CP in these animals (Fig. 1F,1F′, vertical line). These cells, situated within the intermediate zone (IZ), represented “ectopic” CP cells; we refer to this cellular part of the IZ as ectopic CP. Nogo-A immunohistochemistry at this stage revealed that, although axons in the IZ/SP of p35+/− mice ran as an uninterrupted bundle (Fig. 1C), the region of ectopic CP in p35−/− animals was devoid of labeling (Fig. 1C′, vertical line). It has been hypothesized that fasciculation of early cortical axon systems is impaired in p35−/− mice (Kwon and others 1999). This phenomenon of axonal “defasciculation” could be explained as nonautonomous and secondary to accumulation of projection neurons in the IZ. Our present study confirms this and shows that fibers in p35 KOs are widely spread in the IZ as they have to navigate through numerous cells that could be acting as a physical barrier for their outgrowth. Further, there was no...
significant difference in LI thickness (38.8 ± 5.5 vs. 33.3 ± 3.8 μm) between p35 KOs and control mice (Fig. 1E). By E19.5, and just before birth, the difference between p35 hetero- and homozygous cortices became more striking. LI in p35 KO brains was significantly thicker (96.7 ± 4.2 vs. 63.3 ± 7.2 μm) compared with controls, whereas the compact CP was significantly thinner (81.7 ± 3.1 vs. 283.3 ± 6.7 μm) (Fig. 1D). Interestingly, the accumulation of cells at 3 sites (LI, compact CP, and ectopic CP) in p35 KO mice gave an impression of a “triple cortex” (Fig. 1D). Similar to p35 KO mice, the existence of a triple cortex was confirmed in Cdk5−/− mice (Fig. 1D). Toluidine blue-stained semithin (1 μm) sections showed in greater detail clear differences between p35 +/− and p35 −/− cortices at E18.5 (Fig. 2). In p35 −/− mice, the telencephalic wall was thicker, and the compact CP was thinner (Fig. 2B) compared with control mice (Fig. 2A). In addition, cortical axons in KOs were intermingled among ectopic CP neurons located in the IZ, giving an impression of a fan (Fig. 2B). These axons even seemed to penetrate the compact CP (Fig. 2B, arrows). These preparations also confirmed the thickening of LI in p35 −/− brains and the “cellular” appearance of this layer showing no clear border with the compact CP (Fig. 2D) compared with LI in control brains (Fig. 2C).

Prenatal “Overshooting” of Thalamocortical Axons in p35 KOs

To study in more detail the appearance of oblique fibers in the compact CP of p35 KO mice, we placed DiI crystals in the dorsal thalamus at E16.5 and E18.5. In p35 +/− and p35 −/− animals, labeled thalamic axons were present in the IZ and accumulated within the SP (Fig. 3A–D). In the former group of animals, these axons appeared to run tangentially within the upper IZ as a thick, compact band and without dispersion (Fig. 3A,C). A slow radial growth of these axons toward the CP was observed at E16.5 (Fig. 3A), and this growth was more substantial 2 days later (Fig. 3C). In the latter group of mice, growth of thalamocortical axons in the cortex was considerably different compared with their heterozygous littermates. Specifically, these fibers were rather scattered within the IZ (Fig. 3B,D), and many appeared to leave the SP, course obliquely through the CP, and stream toward the pial surface (Fig. 3B,D). This overshooting of thalamic axons was first observed at E16.5, became more evident at E18.5, and disappeared shortly after birth (data not shown). In agreement with other studies (Molnar and others 1998), we rarely observed such behavior.

Figure 1. Loss of Cdk5 activity alters the morphology of the developing neocortex. Coronal sections of the developing cortex stained with Nissl (thionin). (A, A’) No difference in the overall appearance of the cortex was observed between p35 +/− (A) and p35 −/− (A’) mice at E14.5. (B, B’) The cCP was thinner in the p35 −/− mice at E16.5. (C, C’) Nogo-A+ fibers in the cortex of p35 +/− and p35 −/− mice at E15.5. Note a space below the SP of the mutant cortex devoid of labeled axons. Vertical lines in (B’) and (C’) show the position of eCP neurons below the SP. (D, D’, D”) The cCP was thinner, whereas LI and the IZ were thicker in the p35 +/− and Cdk5−/− KOs compared with controls (D). The position of the triple cortex is indicated by 3 (*) in (D’) and (D”). Note obliquely running axons within the cCP in p35 +/− and Cdk5−/− KOs indicated with the arrows. The thickness of LI (D) was significantly increased in p35 KOs compared with controls at E19.5, whereas the thickness of the cCP (D’) was significantly decreased in p35 KOs compared with controls from E16.5. The thickness of cortical layers was measured, and the results were expressed as mean ± standard error of the mean (SEM). Error bars represent SEM. Three brains were used for each genotype for each of the 3 ages (E14.5, E16.5, and E19.5) analyzed. ***, p < 0.005, Student’s t-test. cCP, compact cortical plate; eCP, ectopic cortical plate; SVZ, subventricular zone. Scale bars, 200 μm (A, A’, B, B’, C, C’); 250 μm (D, D’, D”).
of thalamic axons in normal mice. Corticofugal and early thalamic projections outside the cortex were not affected in p35 KOs (data not shown).

We used CalR immunohistochemistry to confirm that thalamic axons and neurons that derived from PPL cells were misplaced in p35 +/- mice. CalR-labeled thalamic axons were present in the IZ/SP in both p35 +/- and p35 +/- mice at E17.5 (Fig. 3E-M). However, they were more dispersed in the p35 KOs, and many entered the CP prematurely streaming in the J regions (Fig. 3I). In conclusion, in p35 +/- mice, a portion of the developing thalamocortical axons enter the CP prematurely and stream obliquely toward LI (E16.5–E18.5) without stopping at the SP. Two hypotheses may be suggested from this observation: 1) PPL does not split properly in p35 KOs (incomplete reeler phenotype), resulting in mispositioned SP cells and, thus, misguided thalamic axons, or/and 2) thalamic axons do not respond adequately to molecular cues, repulsive or attractive, present in the embryonic SP and CP due to lack of active Cdk5.

**Defective Splitting of the Preplate**

To test the first hypothesis that the overshooting of thalamic axons is due to defective PPL splitting, neurons of this layer were labeled with BrdU at the time of their birth (E10.5–12.5 in mice), and their distribution in LI, CP, and SP was examined later in corticogenesis (E15.5 and E18.5) (Fig. 4).

BrdU+ cells were located mainly above and below the CP in both genotypes, indicating splitting of the PPL. However, the organization of the PPL derivatives and the accuracy of its splitting were different between the 2 groups of mice. BrdU+ cells in p35 +/- mice at E15.5 were aligned in 2 well-separated layers, LI and SP (Fig. 4A). In contrast, in p35 +/- mice, BrdU+ cells had a more random distribution and appeared disorganized (Fig. 4B). Triple immunolabeling, using antibodies to BrdU and CalR (markers of prenatal PPL cells) and BB (to label layers) was performed at E18.5 in order to examine the positions of cells relative to specific layers. In p35 +/- mice, BrdU+ cells were mostly located in LI and SP layer, with only a few scattered in the CP (Fig. 4C). However, in p35 +/- mice, many more BrdU+ cells were observed in LI and CP and fewer in the SP (Fig. 4D).

We next examined the relative distribution of BrdU+ cells in LI, CP layer, and SP layer of both genotypes by counting labeled cells in each layer in rostral (septum level, Fig. 4E), medial (anterior commissure level, Fig. 4F), and caudal (hippocampus level, Fig. 4G) regions of the cortex (n = 6 brains for each genotype). The relative distribution of BrdU+ cells in each layer (LI, CP, or SP) was calculated as a percentage of the total number of BrdU+ cells in all 3 layers, revealing a significant shift in the distribution of BrdU+ cells in p35 +/- compared with p35 +/- mice. Regardless of the region examined, there was a significant increase of BrdU+ cells present in LI and CP and a significant decrease of BrdU+ cells in the SP (Fig. 4F-G). The difference was more prominent in rostral regions, and this was in accord with the distribution of CalR+ fibers (Fig. 4J) and somata (data not shown).

Surprisingly, we observed CalR+ thalamic axons (Fig. 5A, arrowhead) growing over E11.5-born BrdU+ or BrdU+/CalR+ cells, the latter being SP neurons (Fig. 5A, B, C, arrows), in p35 +/- mice at E18.5 (Fig. 5D). These observations were originally made on images containing maximal projections of numerous z-stack confocal planes (each 2 μm apart). To check for the possibility that thalamic axons were not in the same plane as the cells they were apparently growing over, we carefully examined each z-plane and confirmed colocalization of single CalR+ axons coursing over BrdU+ or BrdU+/CalR+ cells in the SP (Fig. 5E–J).

**Misplaced Mature Cortical Layers in p35 KOs**

The position of layers (II–VI) in mature neocortex depends on the time of their origin (Angervin and Sidman 1961). Previous studies suggested an outside–in production of the layers in p35 KO mice (Chae and others 1997). The BrdU birthdating...
of p35 and extend toward the altered LI (overshooting). These events can be explained by improper splitting of the PPL, adding Cdk5 to the list of molecules that are involved in its correct partition.

In p35 KO mice, LI became thicker compared with controls as early as E15.5, but this phenotype was clearly visible from E17.5. More cells were found in p35-/- LI from E14.5 (data not shown), and in addition, the border of LI with the compact CP, which was relatively sharp in control mice, was uneven and hardly visible in p35 KOs. It has been reported that the Doublecortin (Dcx) mutation gives rise to a neuronal migration disorder in females known as subcortical band heterotopia or "double cortex" (Gleeson and Walsh 2000). Interestingly, Tanaka and others (2004) described a similar phenotype in Cdk5 KO mice. In the present study we observed that in either p35 or Cdk5 KO mice, apart from the accumulation of cells in the compact CP and the ectopic CP located within the IZ, there was an additional thick layer of cells within LI, contributing to the formation of a triple cortex. In normal mice, thalamic axons are mainly associated with the SP during early development (reviewed by Allendoerfer and Shatz 1994). If these axons access the CP, they do so by sending short radial branches to the lower part of the CP. In contrast, in p35 KOs, numerous thalamic axons enter the CP from early on, run obliquely in larger fascicules, and enter LI. By labeling thalamocortical axons in 2 different ways, either with carbocyanine dyes or antibody against CalR, we observed that overshooting of axons was common in p35-/- mice as well as Cdk5 mutants (data not shown). This was not fully recognized in previous studies on the development of thalamic axons in p35-/- (Kwon and others 1999) or Cdk5-/- (Gilmore and others 1998) mice, although Gilmore and others (1998) observed L1-positive thalamic fibers crossing over the entire CP but omitted to include this finding in their final model of neuronal cytoarchitecture in Cdk5-/- KOs.

Discussion
The formation of the PPL and its splitting by radially migrating CP neurons into LI and SP are key events in early mammalian corticogenesis. This is followed by the ascending creation of the CP layers and the arrival of the thalamic fibers that initially elaborate in the region of the SP (Rakic 1981; Luskin and Shatz 1985). The present study reveals that in mice lacking Cdk5 or its major embryonic activator p35, 1) LI exhibits a complex hypercellular appearance without a clear border with the compact CP and 2) prenatal thalamic axons appear to ignore the SP and extend toward the altered LI (overshooting). These events can be explained by improper splitting of the PPL, adding Cdk5 to the list of molecules that are involved in its correct partition.
We hypothesize that the incomplete PPL splitting, which results in misplaced SP neurons, underlies the overshooting phenomenon in p35−/− mice. This hypothesis was confirmed by our present BrdU birthdating study and CalR staining, which indicated a shift in the distribution of cells derived from the PPL toward superficial positions. However, the idea that the PPL might not be properly split in Cdk5-like KOs is not a completely novel suggestion. Studies by Tsai and coworkers showed 1) that LI and SP, identified histologically and by CalR staining, are markedly less distinct in p35−/−/p39−/− double KOs (Cdk5-like) when compared with normal cortices (Ko and others 2001) and 2) that migration of early-born CP neurons, destined to split the PPL, was defective in p35−/− animals as observed by time lapse (Gupta and others 2003). Thus, Cdk5 emerges as an important participant in the early compartmentalization of the cerebral cortex. Interestingly, in reeler mice that express Reelin in proliferative zones under the nestin promoter, a Cdk5-like phenotype was observed (Magdaleno and others 2002). These mice had a thin CP and ectopic SP with most of the projection neurons beneath it. Although ectopically expressed Reelin induced splitting of the PPL in these transgenic reeler mice, and the splitting appeared qualitatively normal, the figures (Fig. 5) suggest that there may be a subtle abnormality in PPL splitting. Thus, the correct amount and probably the position of Reelin are important for proper PPL splitting (see below).

How Does the Preplate Split?
The partition of the PPL in the mammalian cortex is an extremely accurate process and probably requires a programmed interaction between the host environment (PPL cells, fibers, and extracellular matrix) and guests (emerging radial glia fibers and migrating projection neurons). It is not known whether segregation of LI and SP cells occurs before the arrival of CP neurons or whether it is a consequence of their appearance. The molecular mechanisms that control PPL formation and its splitting are yet to be fully defined.

From the phylogenetic point of view, PPL splitting is an evolutionary adaptation and, although the PPL emerges in amphibians, it is not split before the reptile evolutionary level (from review of Marin-Padilla 1998). The multilayered cortex, formed in ascending order, appeared with the development of mammals (from review of Marin-Padilla 1998). In zebra fish, the dorsal pallium comprises nuclei rather than layers, and yet, Reelin is found in the vast majority of embryonic pallial cells (Costagli and others 2002). Naturally, this begs the question whether Reelin truly mediates layer formation. Furthermore, early Reelin expression in reptiles and mammals is mostly confined to the pallial PPL (see review of Bar and others 2000), and this localized Reelin expression coincides with the evolutionary appearance of C-R cells and PPL partition. However, this restricted Reelin appearance, which eventually expands into the IZ/subventricular zone and even lower CP (Alcantara and others 1998; Bar and others 2000; Rodriguez and

Figure 4. p35 deficiency affects proper splitting of the preplate. Pregnant dams were injected with BrdU at E11.5 and sacrificed at E15.5 and E18.5. BrdU-labeled preplate derivatives at E15.5 (A, B) and at E18.5 (C, D) in p35 +/− (A, C) and p35 −/− (B, D) mice. (E-G) Relative distribution of BrdU-labeled cells at E18.5 in LI, CP layer, and SP layer, at different brain levels: (E) rostral, (F) middle, and (G) caudal. Data represent mean percentage of BrdU+ cells per layer ± standard error of the mean (SEM) from 6 brains for each genotype. Error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.005, Student's t-test. Scale bar, 50 μm.
Is the Reelin Pathway Necessary for PPL Splitting?

Studies that utilized animal models with altered amounts of Reelin have contributed to understanding the splitting of the PPL during cortical formation. These models include animals that "lack" Reelin, contain "decreased" levels of Reelin, have "increased" amounts of Reelin, have been presented with "external" sources of Reelin, or show "absence" of Reelin "lack" Reelin, contain "decreased" levels of Reelin, have PPL during cortical formation. These models include animals that "lack" Reelin, contain "decreased" levels of Reelin, have "increased" amounts of Reelin, have been presented with "external" sources of Reelin, or show "absence" of Reelin 

pathway molecules (for summary, see Table 2). Taken together, these studies have pointed to the need of "normal" levels of Reelin for proper PPL splitting. 

Our study has demonstrated that the p35/Cdk5 complex belongs to the list of molecules that play a role in PPL splitting. Interestingly, p35/Cdk5 regulates N-cadherin-mediated adhesion, and lack of Cdk5 activity increases cell adhesion (Kwon and others 2000). Thus, Cdk5 might be involved in the proper segregation of L1 and SP neurons prior to the arrival of CP neurons. Brm1 and Brm2 are transcription factors that control the expression of Cdk5 regulatory subunit p35. Although in Brm1/Brm2 double KOs (McEvilly and others 2002; Sugitani and others 2002) PPL splitting has been reported, reevaluation of the finding is necessary as separation of L1 and SP might be inaccurate. In conclusion, the p35/Cdk5 complex might play a redundant role in PPL splitting, completely independent of the Reelin pathway, which is absolutely necessary for this process. Alternatively, it may be that the p35/Cdk5-deficient PPL and/or emerging pyramidal cells do not respond normally to correct amount of Reelin present in p35/Cdk5 KOs. 

Are C-R Cells Necessary for PPL Splitting and Cortical Lamination?

If the number of C-R cells is reduced by local application of domoic acid in newborn mice, the migration of late-born neurons is affected (Super and others 2000), suggesting an important role for C-R cells in corticogenesis. However, in the Wnt3a KO, the cortical hem does not develop, and the vast majority of C-R cells are missing (Yoshida and others 2006). Despite this, a low level of Reelin expression is maintained, and strikingly, this KO does not show a defect in PPL splitting or a disruption in cortical lamination. Similarly, in the p73 KO, p73-expressing C-R cells are lost from early on (E12.5), although Reelin is faintly expressed in LI; the partition of PPL and early CP formation are not disturbed (Meyer and others 2004). Likewise, in Dbx1 KOs, there is a substantial loss of C-R cells and Reelin expression in the cortex, but defects in PPL splitting and cortical lamination have not been fully characterized (Bielle and others 2000). Reelin-expressing Dbx1+ cells could rescue the appearance of the reeler-like phenotype in mutants lacking the cortical hem–derived Reelin+ cells (Wnt3a and p73 KOs) and vice versa. Interestingly, other sources of Reelin in the developing cortex, apart from C-R cells, such as its expression in interneurons (Alcantara and others 1998; Rodriguez and others 2000; see Table 2), have been suggested as important for proper late cortical layering (Alcantara and others 2005). It is yet to be determined whether this source of Reelin is exhausted in C-R cells associated Reelin KOs. In conclusion, it is still not certain that C-R cells are essential for proper PPL partition and cortical lamination.
Is PPL Splitting Important for Proper Layering of the Cortex?

If PPL splitting does not occur or is defective, cortical layers are not properly formed. This has been confirmed in all mouse models that show defective PPL splitting (see Table 2). However, aberrant layering of the cortex can also occur independent of PPL splitting as has been reported in presenilin1 (Wines-Samuelsen and others 2005), p35 (Meyer and others 2004), and MAP1B (Gonzalez-Billault and others 2005) KOs. This suggests a level of independence from the PPL in proper cortical (II–VI) layering, and that could be explained in some cases by lack of Reelin (p73 and presenilin1 KOs) at later stages.

More than a Defect in PPL Splitting in the p35 KOs

The aberrant thalamocortical axon ingrowth that initially targets the superplate has been described in reeler mice (Caviness and others 1988). The current study in p35 or Cdk5 KOs revealed that the thalamic axons have similar premature targeting of the region below the pial surface. The mechanism by which thalamic axons approach the subpial region, and ignore the embryonic CP in reeler or in p35 or Cdk5 KOs, is not clear. It was demonstrated that embryonic CP is not permissive for thalamocortical ingrowth (Gotz and others 1992; Molnar and Blakemore 1995; Tuttle and others 1995). Nevertheless, thalamic projections traverse the immature CP to reach the subpial region in reeler and in p35 and Cdk5 KOs (Caviness and others 1988 and present study). It has been suggested that thalamic axons have special association with SP projections and later with SP cells as they grow through the internal capsule and subsequently accumulate below the CP (Allendoerfer and Shatz 1994; Molnar and Blakemore 1995), and this association could mediate the ingrowth through the CP in the reeler mouse (Molnar and others 1998) and p35/Cdk5 KOs (this study). Because thalamic axons express L1 and SP cells express a brain-specific CSPG (neurocan), it has been suggested that L1/neurocan heterophilic interaction might mediate the selective fasciculation (Emerling and Lander 1996; Fukuda and others 1997). Interestingly, our results also revealed that thalamic axons do not seem to stop or accumulate around the presumptive SP neurons in p35 /–KO mice (Fig. 6) as they proceed toward the pial surface. There might be several explanations for this behavior. Perhaps some of the BrDU/+/–/+/CalR cells observed within the SP/CP of the p35 KO are not truly SP cells. They can conceivably represent a cell population of C-R cells that appeared in the SP/CP layer instead of L1 after improper PPL splitting. The distinctions between the L1 and SP cell populations at the time of their split and the different classes of cells contributing to these layers are not understood. Therefore, the overshooting phenomenon in p35 /–KO mice could be explained as axon nonautonomous (secondary response to a primary, p35-dependent neuron migration defect, such as improper PPL splitting) or axon autonomous (primary
However, the observation described in Figure 6 prompted us to further consider the phenomenon showing that thalamic axons ignore presumptive SP neurons (BrdU(E11.5)/CalR*) in p35 mutants. Perhaps these early-generated BrdU(E11.5)/CalR* cells with long-range projections toward the internal capsule are SP cells stranded in the CP, but SP neurons themselves do not give instructions to thalamic axons to stop and accumulate within them; they simply provide a permissive environment for their growth and accumulation. It is also conceivable that they have to be present in large numbers within the same waiting compartment (SP or superplate) to make a considerable impact on the thalamic projections.

In conclusion, the p35 and Cdk5 mutants provide excellent model systems to further study the splitting of the PPL, cortical layer formation, and establishment of thalamocortical and corticofugal pathways. The alterations of early cell positioning cause a distinct sequence of developmental changes, which elicit further alterations providing excellent paradigms to test our current ideas about cortical ontogeny.

**Notes**

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Address correspondence to John G. Parnavelas, Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK. Email: j.parnavelas@ucl.ac.uk.

**References**


**Figure 6.** Indistinct and misplaced postnatal cortical layers in p35 KO mice. Schematic illustrations of neuronal birthdating experiments depicting a defect in neuronal migration in p35 KO mice. Neuronal progenitors were labeled with BrdU at E13.5 (red), E15.5 (yellow), and E17.5 (green). The positions of BrdU+ cells in the neocortex were analyzed at P12. Each dot corresponds to a single BrdU+ cell. Dotted panels represent the overall relative distribution of labeled cells throughout the cortex but not the real number of BrdU+ cells. Roman numerals designate mature neocortical layers. Dotted panels, representing cells born at E13.5 (red), E15.5 (yellow), and E17.5 (green), were superimposed in order to illustrate the relation between the layers born at different times. In p35 +/- (A), the neocortical layers were well separated and formed in an inside-out fashion, whereas in p35 +/- (B), these layers, born at different times, were mixed. Scale bar, 100 μm.

defect in axons due to a lack of p35). Thus, we cannot rule out the possibility that the position of thalamic axons in p35 +/- mice might be due to their defective responsiveness to chemotropic or contact guidance cues, such as semaphorins and slits present in the SP/CP region (Skalar and others 1998; Marillat and others 2002; Tamamaki and others 2003). The fact that the proximal part of the thalamic pathway looks identical in the mutant and normal brains does not support this possibility. These ideas could be tested further in coculture experiments with thalamic explants from the mutants and with wild-type cortices.


