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A Molecular Neuroanatomical Study of the Developing Human Neocortex from 8 to 17 Postconceptional Weeks Revealing the Early Differentiation of the Subplate and Subventricular Zone

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We have employed immunohistochemistry for multiple markers to investigate the structure and possible function of the different compartments of human cerebral wall from the formation of cortical plate at 8 postconceptional weeks (PCW) to the arrival of thalamocortical afferents at 17 PCW. New observations include the subplate emerging as a discrete differentiated layer by 10 PCW, characterized by synaptophysin and vesicular gamma-aminobutyric acid transporter expression also seen in the marginal zone, suggesting that these compartments may maintain a spontaneously active synaptic network even before the arrival of thalamocortical afferents. The subplate expanded from 13 to 17 PCW, becoming the largest compartment and differentiated further, with NPY neurons located in the outer subplate and KCC2 neurons in the inner subplate. Glutamate decarboxylase and calretinin-positive inhibitory neurons migrated tangentially and radially from 11.5 PCW, appearing in larger numbers toward the rostral pole. The proliferative zones, marked by Ki67 expression, developed a complicated structure by 12.5 PCW reflected in transcription factor expression patterns, including TBR2 confined to the inner subventricular and outer ventricular zones and TBR1 weakly expressed in the subventricular zone (SVZ). PAX6 was extensively expressed in the proliferative zones such that the human outer SVZ contained a large reservoir of PAX6-positive potential progenitor cells.

Keywords: cell migration, cortical development, immunohistochemistry, synaptogenesis

Introduction

The patterning of gene expression by counter gradients of secretable, diffusible signaling molecules identifies the position and function of cells within the developing organism. Such mechanisms impose a protomap upon the cortical neuroepithelium prior to the emergence of the cortical plate (Rakic 1988; Mallamaci and Stoykova 2006). However, within the brain, an extra stimulus to development is provided by first spontaneous and then sensory evoked electrical activity transmitted between cells at synapses and gap junctions (Katz and Shatz 1996; Sur and Rubenstein 2005). In the cortex, this activity shapes the connectivity of neural circuitry from which consciousness emerges.

The gray matter of the mature neocortex arises out of the developing cortical plate, a dense, stratified mantle of cells that split the preexisting, more loosely packed preplate into an outer marginal zone and inner subplate from about 7 weeks postconceptional age onwards in the human (Meyer et al. 2000; Altman and Bayer 2002). The marginal zone and subplate are transient structures that have vital roles in development. Cajal–Retzius cells are located in the marginal zone. These neurons secrete reelin, a signaling molecule important in ordering the migration of cells into the cortical plate (Ogawa et al. 1995). The subplate provides a region through which cells migrate to the cortical plate and into which afferents grow and wait for a period before invading the cortex, becoming incorporated into transient local circuitry, (Rakic 1982; Chun and Shatz 1988; Meinecke and Rakic 1992). The first cortical synapses form in the marginal zone and subplate (Kostović and Rakic 1990).

The cells of the human marginal zone, cortical plate, and subplate are generated in the proliferative regions of the pallium or have migrated into the cortex from subpallial structures such as the ganglionic eminences (Letinic et al. 2002; Meyer et al. 2002a; Bystron et al. 2006). However, the primate neocortex has a highly developed subventricular zone (SVZ; Smart et al. 2002; Zecevic et al. 2005) that may contain many types of progenitor cell giving rise to cell types such as inhibitory interneurons (Letinic et al. 2002) or oligodendrocyte precursors (Ulfig et al. 2002; Rakic and Zecevic 2003a) that, in the rodent brain, almost exclusively migrate into the cortex from subpallial structures.

The present study aimed to characterize the molecular neuroanatomy, and clarify the boundaries, of the various compartments of the cerebral wall at the stage of neocortical development from the formation of the cortical plate up to the large scale innervation of the subplate by thalamic afferents. At this time, a large degree of cortical cell production and migration is taking place. Although this stage of development has been included as part of many wider ranging studies a more detailed study in terms of the numbers molecular markers examined and specimens available of this stage alone is reported here.

Expression patterns were studied by immunohistochemistry for the following proteins. Microtubule associate protein (MAP2) is found in the cell bodies and dendrites of postmitotic neurons (Bernhardt and Matus 1984). Glial fibrillary acidic protein (GFAP) is a cytoskeletal protein expressed by both radial glia and mature astrocytes in the primate brain (Levitt and Rakic 1980; Howard et al. 2006). Growth-associated protein 43 kDa (GAP43) is concentrated in growing axons and growth cones and shows formation of new axonal pathways (Benowitz and Routtenberg 1997). Synaptophysin is associated with synaptic vesicles in nerve terminals (Wiedenmann and Franke 1985; Leclerc et al. 1989). Immunolocalization of the vesicular transporter for the neurotransmitter gamma-aminobutyric acid (GABA) (vGAT) further clarifies the location and phenotype of nerve terminals (Takamori et al. 2000; Minelli et al. 2003).

Transcription factors show specific patterns of localization in animal studies. Pax6 expression is confined to the ventricular zone (VZ) and SVZ (Walther and Gruss 1991; Warren et al.

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1999). Tbr2 is also expressed in the VZ away from the ventricular surface but mostly in the SVZ, whereas Tbr1 is weakly expressed in the SVZ and intermediate zones but more strongly in the cortical plate, subplate, and marginal zones. Therefore, it has been proposed that Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons (Englund et al. 2005). Er81 is strongly expressed in subsets of layer V cortical plate neurons of both the rodent (Hevner et al. 2003) and macaque (Yoneshima et al. 2006). Ki67-immunoreactive labeling was employed to pinpoint the location of actively dividing cells (Brown and Gatter 1990; Meyer et al. 2002b).

Proteins characteristic of active GABAergic synapses were also studied as they generally form before glutamatergic synapses in the developing nervous system (Ben-Ari et al. 2004). Glutamate decarboxylase (GAD) is the synthesizing enzyme of GABA. Calretinin (CR) is a calcium-binding protein expressed by pioneer cells in the preplate of the developing human cortex (Meyer et al. 2000) but also by subtypes of GABAergic interneurons in the mature cortex (Markram et al. 2004), which are more numerous in human cortex than in other species (Gabbott et al. 1997). CR is expressed early in neocortical development, unlike other calcium-binding proteins which are predominantly expressed in the last trimester or postnatally (Cao et al. 1996; Honig et al. 1996; Yan et al. 1997). KCC2 is a potassium/chloride cotransporter and its expression during development marks a switch from the depolarizing to hyperpolarizing action of GABA on target neurons (Rivera et al. 2005; Vanhatalo et al. 2005). In the mature cortex, neuropeptide Y (NPY) expression is colocalized with GABA in nonpyramidal neurons (Hendry et al. 1984). Prominent expression of NPY by subplate neurons from 16 postconceptional weeks (PCW) in the human has been previously described (Delalle et al. 1997) and is considered a marker for functionally mature neurons in the subplate (Chun et al. 1987).

Materials and Methods

Brains were taken from 16 human fetuses ranging in age from 8 to 17 weeks postconception (PCW). Age was estimated from measurements of foot length and heel to knee length compared with a standard growth chart (Hern 1984), which is considered more reliable than reported weeks postmenstruation. Brains were dissected from fetal terminations of pregnancy obtained from the MRC-Wellcome Trust Human Developmental Biology Resource (HDBR collected following British national guidelines (Polkinghorne 1989), with appropriate maternal written consent and approval from the Newcastle and North Tyneside NHS Health Authority Joint Ethics Committee. Before sectioning, brains were fixed for at least 24 h at 4 °C in phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Before dissection, some brains were MRI (magnetic resonance imaging) scanned. Prior to scanning, brains were suspended in a 1% agarose gel to provide protection and stability. For MRI, a Bruker vertical 4.7-T scanner with a 60-cm-id bore equipped with an AC-44 actively shielded gradient set of the maximum strength 40 mT/m, and a 19.5-cm-id linear birdcage radiofrequency coil was used. High-resolution anatomical images were acquired by a T_1-weighted modified driven equilibrium Fourier transform (MDEFT) pulse sequence incorporating two adiabatic MDEFT preparation pulses. This sequence minimized B1 field inhomogeneities which, otherwise, would have occurred at high fields due to dielectric resonances (Lee et al. 1995).

Whole or half brains (divided sagitally) were transferred to 70% ethanol for storage at 4 °C prior to paraffin embedding. Eight-micrometer-thick paraffin sections were cut, mounted on slides, and used for immunohistochemistry. Brains for frozen sections were dissected into smaller blocks typically 3–5 per hemisphere) and stored in buffered fixative at 4 °C. Before sectioning, such blocks were immersed overnight in 30% phosphate-buffered sucrose at 4 °C, then 50-μm sections were cut with a freezing microtome and sections collected free floating in PBS.

Paraffin sections were dewaxed and rehydrated according to standard procedures, treated with 3% hydrogen peroxidase (Sigma-Aldrich) for 10 min, and boiled in 10 mM citrate buffer before incubation with primary antibody in 0.3% PBS with 0.3% Triton X (Sigma-Aldrich, Poole, UK PBST) and 3% of the appropriate blocking serum (Vector Laboratories, Peterborough, UK). Sections were incubated on slide in a moist chamber at 4 °C overnight. Frozen sections were incubated free floating with gentle agitation overnight at 4 °C in PBST and 3% of the appropriate blocking serum (Vector Laboratories) and primary antibody. Table 1 provides details of all the primary antibodies used. Following washing in PBST,, sections were incubated for a further 2 h at room temperature with the appropriate biotinylated secondary antibodies (Vector Labs, diluted 1:200 in PBST) washed and then incubated for a further hour at room temperature with streptavidin–horse radish peroxidase (Vector Labs, diluted 1:200 in PBST). Sections were washed in PBS and then reacted with 0.05% diaminobenzidine (Sigma-Aldrich) and 0.003% hydrogen peroxide in PBS for up to 20 min. After further washing, frozen sections were mounted on gelatinized slides and air-dried. All sections were dehydrated, cleared, and mounted in Entellan (Merck, Nottingham, UK). Images were captured with a Zeiss Axiocam digital camera and figures prepared using Adobe Photoshop software. A conservative approach was adopted when attributing positive immunostaining to cells and weak staining was only accepted as immunopositive when areas of absolutely no staining were present in the section.

All images displayed in this study were taken from sections through the central part of the dorsolateral cortex except for Fig. 5*E,F*. Other areas were examined and any specific observations are given in the text. Because obvious differences were observed in CR expression between rostral and caudal poles at 12.5 PCW, a quantitative evaluation of CR-immunoreactive neurons was made. CR-immunopositive cells were counted in 500-μm-wide strips through the cerebral wall of the frontal, parietal, and occipital lobes from representative sections. To allow for differences in cortical thickness arising from differences in angle of cut and differential growth of the cortex, the cross-sectional area of each compartment within each strip sampled was calculated and the number of CR-positive neurons within each compartment expressed as the number of neurons/mm^2 cross-sectional area.

Results

Eight to 9 PCW; Establishment of the Cortical Plate

At 8–9 PCW, a cortical plate was recognizable in all regions of the neocortex although it appeared thickest in the lateral wall nearest to the internal capsule. Nissl preparations and anti-MAP2 and TBR1 immunostaining revealed the cortical plate to be several layers of cells thick and occupying about one eighth of the total thickness of the cerebral wall. MAP2- and TBR1-positive cells were also present in the marginal zone, sparsely distributed in the intermediate zone but more densely distributed in the SVZ, although TBR1 expression was weak in this location. Occasional MAP2-positive neurons were observed in the VZ (Figs 1–3). TBR2 was strongly expressed by some cells in the marginal layer and weakly expressed by a few cells in the cortical plate (Fig. 2). CR immunoreactivity marked a subpopulation of neurons, with individual CR neurons seen evenly spaced throughout the marginal zone and more densely in the cortical plate, particularly in the deeper parts, confirming the previous observations of Meyer et al. (2000). Intensely CR-positive neurons were rare in the intermediate or proliferative zones. GAP43-positive axons were plentiful in the marginal layer and in the intermediate zone, confirming a previous observation by Milosevic et al. (1995)

Table 1

A summary of all the primary antibodies used in this study, including their suppliers, examples of previously published use of this specific antibody in human fetal tissue, optimal dilution, species in which they were raised, and the species in which corresponding secondary antibodies were raised, and how clear the results were for different types of section

Primary antibody to	Supplier	Previous use in human prenatal post-mortem studies	Dilution	Species/blocking serum	Paraffin 8–13 PCW	Frozen 13–17 PCW
MAP2	Sigma-Aldrich	Rakic and Zecevic 2003b; Bystron et al. 2005; Howard et al. 2006	1:1000	Mouse monoclonal/horse	++	++
GFAP	Sigma-Aldrich	Clowry et al. 2005	1:400	Rabbit polyclonal/goat	++	++
GAP43	Sigma-Aldrich	Eyre et al. 2000; Koutcherov et al. 2003; Clowry et al. 2005	1:1000	Mouse monoclonal/horse	++	++
Synaptophysin	Sigma-Aldrich	Clowry et al. 2005	1:1000	Mouse monoclonal/horse	++	++
vGAT	Chemicon, Chandler's Ford, UK		1:5000	Guinea pig polyclonal/goat	+	−
GAD 65/67	Sigma-Aldrich		1:500	Rabbit polyclonal/goat	+	+
CR	Swant, Bellinzona, CHE	Koutcherov et al. 2003; Rakic and Zecevic 2003b; Howard et al. 2006	1:2000	Mouse monoclonal/horse	++	+
KCC2	Upstate Biotech, Lake Placid, NY		1:500	Rabbit polyclonal/goat	−	++
Neuropeptide Y	Sigma-Aldrich		1:8000	Rabbit polyclonal/goat	+	++
PAX6	Covance, Cambridge Bioscience, Cambridge, UK	Kerwin et al. 2004	1:300	Rabbit polyclonal/goat	++	+
TBR1	Abcam, Cambridge, UK		1:500	Rabbit polyclonal/goat	++	NA
TBR2	Abcam		1:200	Rabbit polyclonal/goat	+	NA
Er81	Dr T. Jessell	Clowry et al. 2005	1:20 000	Rabbit polyclonal/goat	−	++
Ki 67 (MIB-1)	Dako	Meyer et al. 2002b; Chan et al. 2006	1:200	Mouse monoclonal/horse	++	NA

Note: + indicates that immunostaining was of moderate intensity but still discernible, ++ indicates intense and specific immunostaining, − indicates no discernible specific staining, which could be because protein is not present or because either paraffin embedding or prolonged fixation (older specimens) destroys antigenicity for this protein. NA indicates that this particular immunostaining was not attempted at this age.

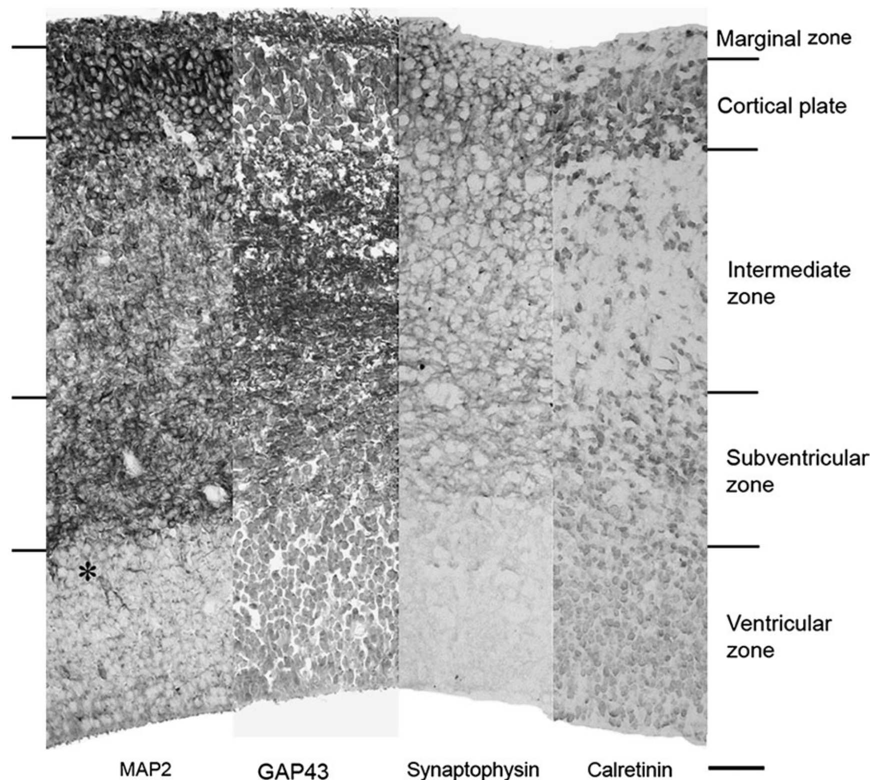

Figure 1. This figure shows cross-sections of the lateral wall of the cortex at 8 PCW near to its junction with the internal capsule and lateral ganglionic eminence. Adjacent paraffin sections have been immunostained for MAP2, GAP43, synaptophysin, and CR. The marginal zone expresses high levels of MAP2 and GAP43, but low levels of synaptophysin. Occasional CR-positive neurons are also seen. The cortical plate is clearly visible as a band of densely packed cells, which are strongly immunoreactive for MAP2. Some synaptophysin immunoreactivity is also observed and many CR-positive neurons are also present. GAP43 is most highly expressed in fibers in an intermediate zone, which contains few immunoreactive cell bodies. The SVZ is high in MAP2 expression and contains some GAP43-positive fibers. The VZ is characterized by a lack of synaptophysin immunoreactivity and little MAP2 immunostaining, although background staining reveals a high density of cell bodies. The occasional MAP2-positive neuron, can however, be seen in this region (asterisk) and may represent a tangentially migrating neuron. Scale bar = 50 μm.

with some immunoreactivity seen in the VZ. Synaptophysin immunoreactivity was completely absent from the VZ but seen at a low intensity everywhere else, including the cortical plate (Fig. 1). At this stage of development, no immunoreactivity for GFAP was observed. PAX6 expression was strong in the VZ and weaker in the SVZ, whereas TBR2 expression was mainly confined to the border between the VZ and SVZ in the proliferative zones (Fig. 2). Ki-67 immunoreactivity revealed

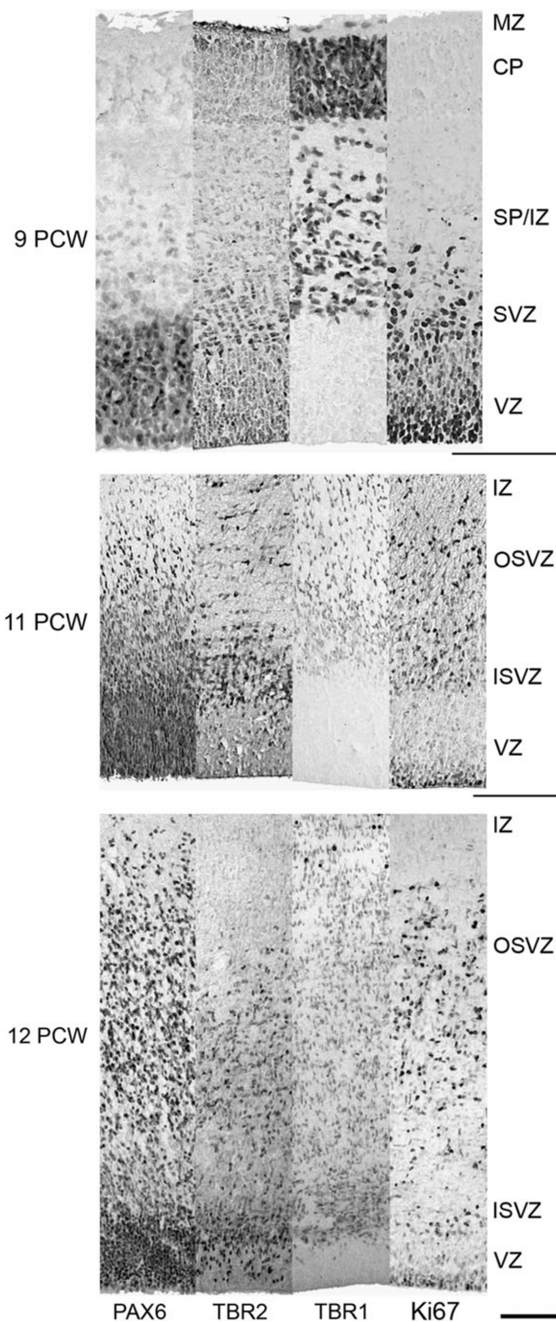

Figure 2. This figure compares nuclear expression patterns of PAX6, TBR2, TBR1, and Ki67 immunoreactivity in paraffin-embedded sections demonstrating the differentiation of the human SVZ occurring between 9 and 12.5 PCW. At 9–11 PCW, PAX6 immunoreactive cells are observed at very high density within the VZ, with weak expression in the SVZ at 9 PCW that increases by 11 PCW. TBR2 was expressed in a layer of cells traversing the VZ/SVZ border, whereas TBR1-expressing cells were present predominantly in the cortical plate (CP), although expression throughout the SVZ, subplate/intermediate zone (SP/IZ), and marginal zone (MZ) was also noted. Ki67-positive nuclei, a marker for dividing cells, were present throughout the VZ at 9 PCW, but exhibited a discrete banding pattern at the ventricular surface of the VZ at 11 GW. Strong but less dense Ki67 immunoreactivity was observed throughout the SVZ at 9–11 GW. By 12 GW, the SVZ has differentiated into three discernible layers, an ISVZ and an OSVZ with a cell sparse-layer in between (marked by arrow, see also Fig. 3). PAX6-positive cells were expressed throughout the proliferative zones with some also observed in the IZ. A number of strongly TBR2-expressing cells were present in the VZ, whereas a more dense expression pattern of this protein was observed in the ISVZ. TBR2-immunoreactive nuclei were also present in the OSVZ to a lesser extent. Outside of the CP and SP, TBR1 was found to be present in a few cells within the IZ, and throughout the SVZ but with weaker expression. Ki67 expression at the ventricular surface of the VZ was detected at 12 GW, with the majority of staining within the SVZ being located in the OSVZ. Scale bars = 200 μm.

numerous dividing cells at the ventricular surface and in the SVZ, but also some in the intermediate zone right up to the border with the cortical plate (Fig. 2, as previously described by Rakic and Sidman 1968, using autoradiography to detect tritiated thymidine incorporation).

10–13 PCW; Expansion of the Cortical Plate, Establishment of the Subplate and Differentiation of the SVZ

From 10 to 13 PCW, the thickness of the cortex increases approximately threefold (Figs 3, 8) largely due to an expansion of the cortical plate from about a fifth to a third of the total thickness of the cerebral wall, although the intermediate and SVZ also increased in size over this time. The VZ maintained its size in its absolute terms but declined as a proportion of the total thickness of the cortex (from about a quarter to a sixth, Fig. 8). Comparing different immunostains at these ages permitted identification of the different strata of the cortex. The marginal zone was characterized by intense MAP2, GAP43 (Fig. 3) synaptophysin and vGAT immunoreactivity (Fig. 4). Also present were GAD and CR-positive neurons (Fig. 5). Between 11 and 12.5 PCW, the marginal zone differentiated into two layers; cell bodies congregated to form the outermost layer (the previously described subpial granular layer, Meyer and Goffinet 1998; Rakic and Zecevic 2003b) and a cell poor, GAP43, MAP2, and synaptophysin rich layer formed between the granular layer and the cortical plate (Figs 3-5).

The cortical plate was easily identified in Nissl, MAP2, and TBR1 sections (Figs 2 and 3). TBR1 was expressed throughout the thickness of the cortical plate at this age (not shown). Its inner border was marked by a thin relatively cell free zone at 10–11.5 PCW. Just internal to this is a region of high GAP43 immunoreactivity (Figs 3 and 4) and this region also immunostained strongly for synaptophysin and vGAT (Fig. 4). Therefore, the intermediate zone seen at 8–10 PCW, by this stage of development appeared divided into an upper and lower layers. The upper layer containing a high density of markers for growing axons and synaptic terminals, and could thus be regarded as the subplate proper (see discussion). The lower layer, particularly at the occipital pole of the cortex, is further characterized by a band of radially orientated CR-positive nerve fibers (Fig. 5, similar to the trilaminar honeycomb matrix described by Altman and Bayer 2002, from Nissl-stained sections). The boundary between the intermediate zone and SVZ is relatively clear-cut in Nissl-stained sections, with the intermediate zone appearing cell poor compared with the SVZ.

PAX6-immunoreactive cells are almost entirely contained within the VZ and SVZ, although a few highly immunoreactive cell nuclei might be present in the deeper reaches of the intermediate zone (Figs 2 and 4). PAX6 expression was uniform in all regions of the cortex at these ages. In general, PAX6 immunoreactivity was of moderate intensity but present in all cells in the VZ. In the SVZ, the inner part (ISVZ) was characterized by a similar moderate intensity of labeling but in the outer part (OSVZ) fewer cells expressed PAX6, although

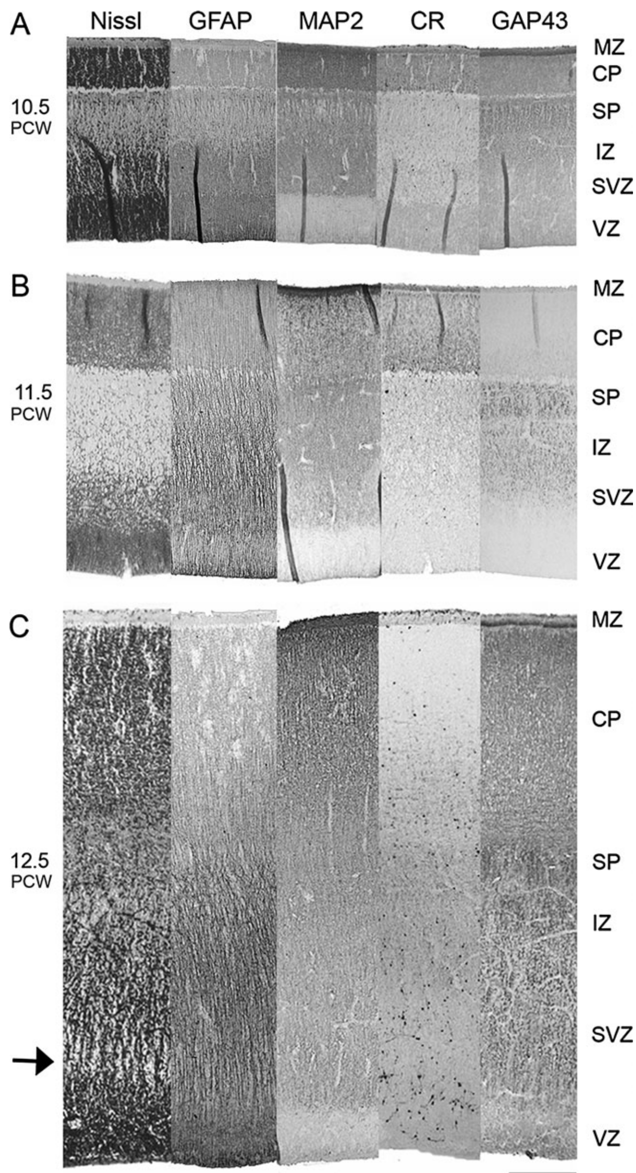

Figure 3. These montages are made from paraffin sections taken from the central/dorsolateral region of the cerebrum and illustrate the rapid growth in thickness of the cortex between 10 and 13 PCW, and in particular, the expansion of the cortical plate (CP) which is most clearly seen with Nissl staining. CR-positive neurons are found in the cortical plate around the border with the subplate (SP). The marginal zone (MZ) grows slowly in thickness. By 12.5 PCW (C) CR neurons form the very outermost layer of the marginal zone (the subpial granular layer) with strong GAP43 and MAP2 immunoreactivity sandwiched between this layer and the cortical plate. The SP and intermediate zone (IZ) appears as a homogenous cell poor zone in the Nissl-stained sections, although GAP43 immunostaining reveals a band particularly rich in growing axons in the outer part of this zone. GFAP immunoreactivity appears in these two compartments from 11.5 PCW (B). The SVZ appears as a homogenous band up to 11.5 PCW (B) but by 12.5 PCW (C) cell rich regions are divided by a cell poor band (arrow, see Fig. 2). The cell poor band and inner parts of the SVZ also contain numerous CR-positive neurons. The VZ is characterized by dense Nissl staining and an absence of MAP2 or GAP43 immunoreactivity. GFAP immunoreactivity is present at the ventricular surface only at 10.5 PCW (A) and but labels fibers throughout the VZ and SVZ at later stages of development. Scale bar = 200 μm.

immunoreactivity was more intense in those that did (Fig. 2). TBR2 expression was largely confined to the ISVZ, with some positive cells also found in the VZ but away from the ventricular surface. TBR1 was weakly expressed in the SVZ and IZ but not in the VZ. Ki67 immunoreactivity was seen in nuclei of the VZ, mostly at the ventricular surface, but also in the SVZ and even in the intermediate zone.

GFAP showed an interesting expression pattern. At 10.5 PCW, it was confined to the VZ at the ventricular surface (Fig. 3*A*). By 11.5 PCW, GFAP was expressed strongly throughout the VZ, SVZ, intermediate zone, and subplate, but was weaker in the cortical plate. This continued up to 13 PCW (Fig. 3*B*,*C*).

Localization of CR expression changed between 10 and 13 PCW. Early on, immunoreactive cells were restricted to the marginal zone and either side of the thin cell free zone that marks the border between cortical plate and subplate. At older ages, many more CR-immunoreactive cells were found in all parts of the cerebral wall (Figs 3, 5, and 8). Many had the appearance of migrating neurons; bipolar with trailing and leading processes. Approximately half were aligned roughly parallel to the surface of the cortex (tangential migration), whereas half exhibited radial migration (at right angles to the cortical surface). Migratory cells could be found in the cortical plate, VZ, and the layers in between, although the largest numbers were present in the SVZ (Fig. 5B–D). The density of CR cells was greater toward the rostral pole of the cortex, as was confirmed by counts of CR neurons at this age (Fig. 5E–G). In general, the difference between frontal and parietal lobes was not large but there was a consistently higher density of CR neurons in the frontal lobe. The exceptions were the cortical plate of the frontal lobe which had a much larger density of CR neurons, and the VZ, where only a slightly higher density of CR neurons were found in the parietal lobe. The difference between frontal and occipital lobe was much larger, the frontal lobe containing several fold more CR neurons/mm^2 in all compartments except for the marginal zone where the difference was less than 2-fold (Fig. 5*G*).

A very small number of NPY neurons were observed scattered through the subplate by 12.5 PCW, although they were relatively plentiful in the hypothalamus and internal capsule (not shown). No KCC2 immunostaining was observed in paraffin sections from 10 to 12.5 PCW specimens.

13–17 PCW; Growth and Differentiation of the Subplate

Between 13 and 17 PCW, the cortex continued to dramatically increase in thickness but it was predominately the subplate region that underwent expansion. Other layers remained the same or are slightly expanded. A clear distinction between the subplate and intermediate zone emerged, with the intermediate zone being dominated by fibers running in a tangential direction (Fig. 6). This was particularly marked in the occipital cortex. By 14 PCW, the cortical plate showed up as a distinct layer on MRI scans of the fixed brain prior to sectioning, and by 16–17 PCW a boundary between the subplate and the intermediate zone was discernible (Figs 5 and 6). The VZ was also clear, but a distinction between the SVZ and the intermediate zone was not apparent in MRI scans (Fig. 6).The boundary between these two layers was apparent in PAX6-immunostained sections, a PAX6 was confined to VZ and SVZ (Fig. 6*I*). Er81 immunoreactive nuclei were also present in these locations (Fig. 6*H*). Many GFAP-positive cell bodies could be seen in the VZ and ISVZ, but not in the OSVZ where GFAP immunoreactivity was mainly confined to radial fibrous processes (Fig. 6*J*).

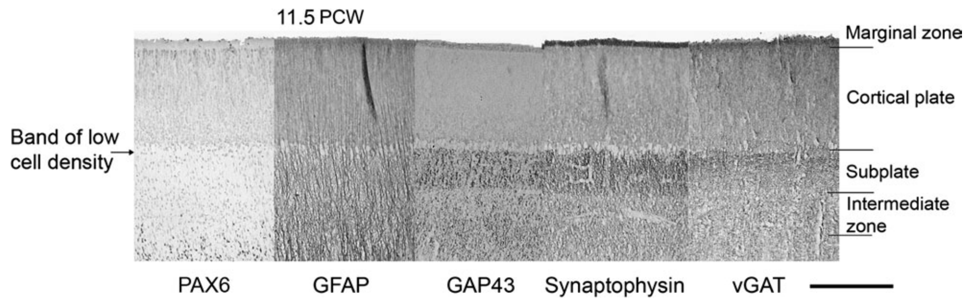

Figure 4. These montages are made from paraffin sections taken from the parietal region, adjacent to those used in Fig. 3. A thin band of very low cellular density (arrow) can be clearly seen at the proximal border of the cortical plate, and this has been interpreted as representing the nascent subplate in a previous study (Kostović and Rakic 1990). However, GAP43, synaptophysin, and vGABA immunostains reveal a wider band displaying intense immunoreactivity for all three, similar to the marginal zone. We interpret this wider band as marking the full extent of the subplate (see text for further discussion). GFAP immunostaining also shows a subtle transition between the intermediate zone, where glial fibers are relatively disordered, and our definition of the subplate, where glial fibers adopt a parallel orientation, which is also seen in the cortical plate. The boundary between the intermediate zone and the SVZ is most clearly seen in the PAX6 immunostained section where immunoreactivity is almost completely confined to VZ and SVZ. Scale bar = 200 μm.

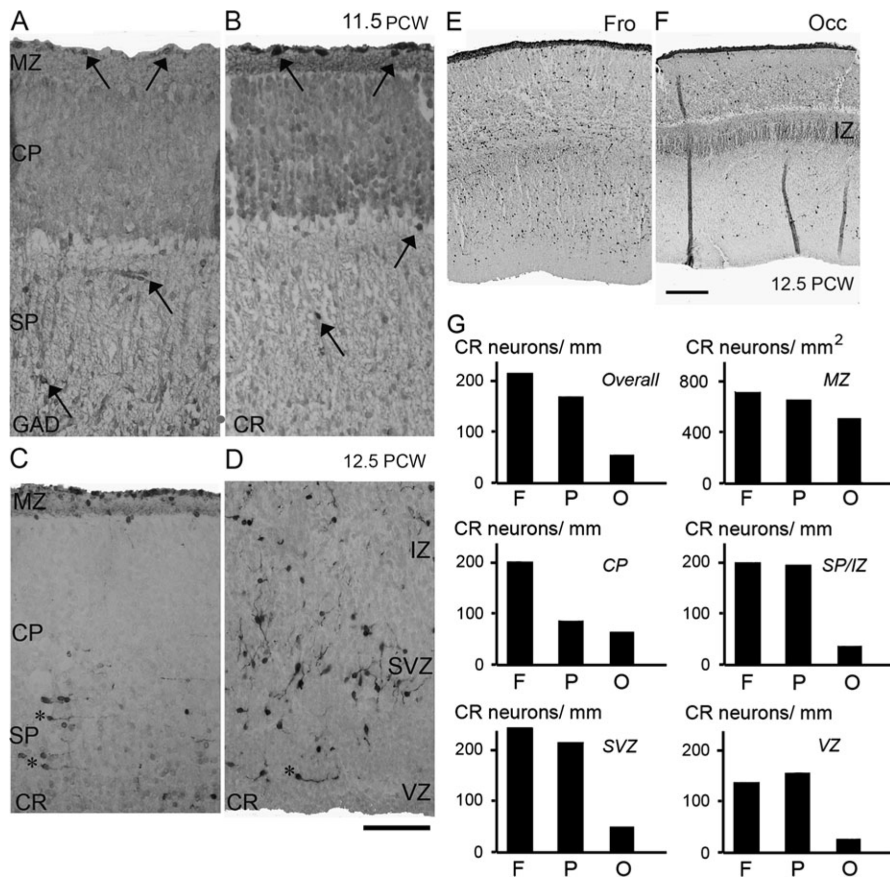

Figure 5. (*A–D*) Paraffin sections taken from the parietal region at 11.5 and 12.5 PCW, immunostained for GAD (11.5 PCW only) and CR. At 11.5 PCW, GAD- and CR-positive cells are found in similar locations in the marginal zone (MZ) and subplate (SP, arrows) however, a higher density of CR neurons is seen in the marginal zone and on the border between the cortical plate (CP) and subplate. By 12.5 PCW many more CR-positive cells appear, particularly in the SVZ. Many have the bipolar morphology characteristic of migrating neurons. Many are aligned for radial migration, but in addition tangentially migrating neurons can be observed in all zones including the SP and VZ (asterisks). IZ is the intermediate zone. At 12.5 PCW, CR expression was markedly different in the frontal (*E*, Fro) and occipital (*F*, Occ) lobes of the cortex with many more CR-positive neurons visible in the frontal section in all compartments except for the marginal zone. In addition, CR-positive fibers banded together in parallel bundles in the intermediate zone (IZ) of the occipital lobe only. (*G*) A series of bar charts summarizing counts of CR neurons (see text for details) in the different compartments of the cerebral wall of the frontal (F), parietal (P), and occipital (O) lobes at 12.5 PCW. It confirms there is a higher density of CR neurons is found in the frontal lobe compared with the occipital lobe in particular at this stage. Scale bar = 100 μm.

The cortical plate appeared as a homogenous band in Nissl-stained material, but Er81 immunostaining revealed a distinct layer in the cortex (Fig. 6*E*,*F*) which is likely to be layer V (Hevner et al. 2003). It was present throughout the rostro-caudal extent of the cortex and also in the temporal cortex. Er81-negative bands were present in the cortical plate both inside and outside the Er81-positive band. The subplate was rich in synaptophysin (Fig. 7*A*,*B*) GAP43 (previously

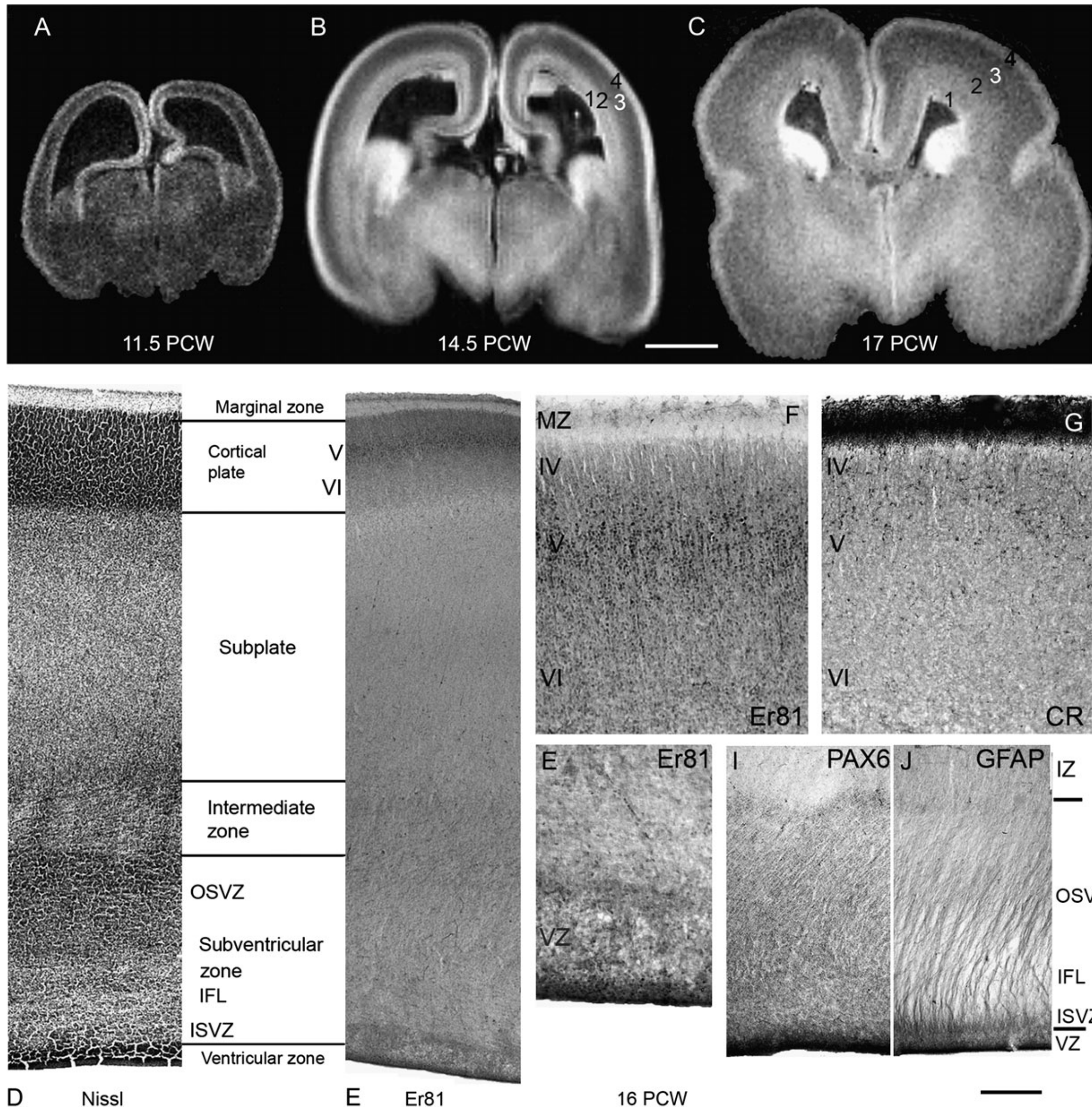

Figure 6. (*A*) (0.15 × 0.15 × 0.5 mm/voxel), (*B*) (0.25 × 0.25 × 1.0 mm/voxel), and (*C*) (0.25 × 0.30 × 1.0 mm/voxel) are from MRI scans of fixed brains at different developmental stages showing the parietal lobe in a coronal section. Higher signal intensities represent greater density of cellular material. They illustrate both the rapid growth and differentiation of the cortex. At 11.5 PCW (*A*) three layers can be identified in the cerebral wall; the proliferative layer (inner, white) and cortical plate (outer, white) separated by the intermediate/subplate zone. At 14.5 (*B*) and 17 PCW (*C*) four layers can be seen. Layer1 is very thin and not discernible everywhere and is probably the VZ. Layer 2 corresponds to the subventricular/intermediate zones and layer 3 to the subplate (as observed at 18 PCW by Radoš et al. 2006) and 4 is the cortical plate. (*D* and *E*) The entire cross-section of the cortex at 16 PCW. Nissl staining shows the different zones in the cerebral wall. The cell rich and cell poor layers in the SVZ first seen at 12.5 PCW are more pronounced by 16 PCW. Er81 immunostaining is found in cell nuclei the VZ and SVZ (*H*), and at high density in a layer in the middle of the cortical plate likely to be layer V (see text). This is shown in close-up in (*F*). CR immunoreactivity has a different location, being intense in the marginal zone (cell bodies and neurites) with CR-positive cell bodies in the cortical plate located mainly in a putative layer IV (*G*). In addition, lower power images (*I*, *J*) show dense PAX6 immunoreactivity in cell nuclei throughout the VZ and SVZ at this stage of development which coincides with GFAP immunoreactivity visible in cell bodies in the VZ and ISVZ but mostly localized to radial fibers in the rest of the SVZ. IZ intermediate zone; IFL, intermediate fiber layer. Scale bar = 5 mm in *A–C*, 500 μm in *D*, *E*, *I*, *J* and 200 μm in *F*, *G*, *H*.

demonstrated by Honig et al. 1996) and MAP2 immunoreactivity, suggesting the presence of dendrites, axons, and terminals. Examination of synaptophysin immunoreactivity at high magnification revealed a punctuate staining suggestive of synaptic terminals (Fig. 7*B*). Unfortunately, immunostaining for vGAT in frozen sections was poor and so this observation was hard to confirm at later stages.

Although the subplate appeared as a relatively cell poor zone, there were neurons present that had undergone a degree of phenotypic specialization. By 16 PCW, many neurons expressing NPY were found in the outer subplate which extended processes into the cortical plate itself (Fig. 7*C*). The numbers of neurons in the subplate expressing NPY increased between 13.5 and 16 PCW. The highest density of NPY neurons was observed in the temporal cortex, the lowest in the occipital cortex, with intermediate densities observed in the frontal and parietal regions (qualitative observations only). At younger ages, most NPY expressing neurons appeared bipolar and could be orientated either radially

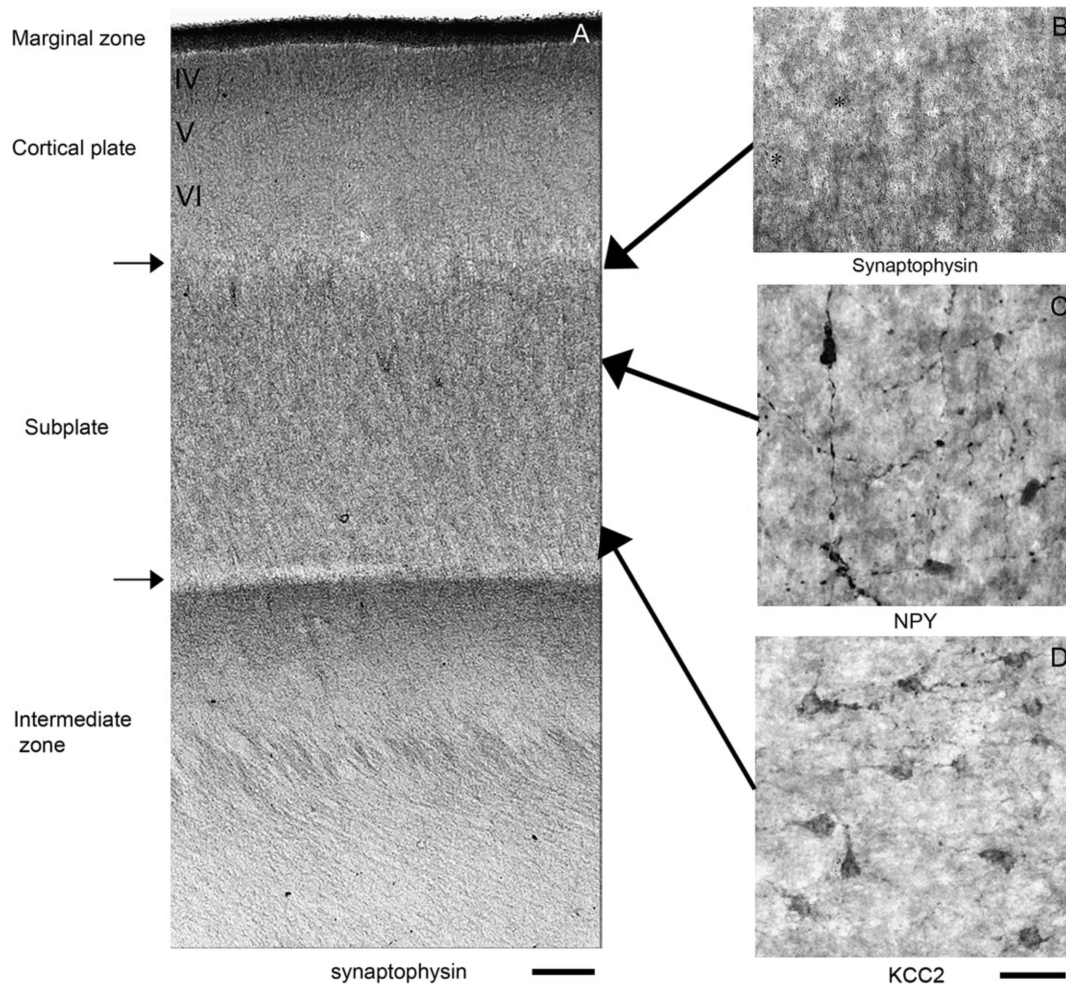

Figure 7. This figure is taken from frozen sections from parietal cortex at 16 PCW. Synaptophysin immunoreactivity (*A*) shows clearly different regions in the cerebral wall. The marginal zone is intensely immunoreactive. The cortical plate is less so but immunoreactivity appears densest in layer IV. The subplate is found between the two small arrows and shows moderately dense immunoreactivity. In the intermediate zone, immunoreactivity appears to be expressed by fibers which are radial in the deeper parts but tangential near to the border with the subplate. (*B*) Synaptohysin immunoreactivity at a higher magnification at a border between the subplate and the cortical plate. Distinct punctuate staining which may correspond to synaptic terminals is seen in the neuropil sometimes appearing to surround a putative cell body (asterisk). (*C* and *D*) NPY and KCC2 immunoreactive neurons in the subplate, the large arrows show where in the subplate they are found. NPY neurons can be bipolar, orientated radially or tangentially, or multipolar, with varicose processes. KCC2 neurons are often pyramidal in shape. Scale bars = 100 μm in *A*, and 20 μm in *B*, *C*, *D*.

or tangentially. At older ages, more NPY neurons displayed a multipolar morphology. In all cases, dendrites had a beaded appearance. Smaller numbers of NPY neurons could also be found in the marginal zone, intermediate zone, SVZ, and VZ (data not shown).

Nearer to the intermediate zone, neurons expressing KCC2 immunoreactivity could be observed (Fig. 7*D*). These cells were quite large, often pyramidal in shape with several dendritic processes. Unlike at earlier ages, CR-immunoreactive neurons were few in the subplate but there was intense immunoreactivity in the marginal zone, present in both cell bodies and processes. CR-positive cell bodies were also seen in the outer part of the cortical plate (Fig. 6*G*).

Discussion

At present, molecular neuroanatomical studies of post-mortem tissue are one of the few available approaches for studying specifically human brain development that will provide detailed information about cell type, location, and state of differentiation that can be related to functional properties. It is very important to test the validity of applying schemes of brain development derived from the study of animal models to humans by making observations in human tissue. This study provides a detailed description of the molecular neuroanatomy of the developing human cortex during the time period 8–17 PCW. In general, features common to all regions of the cortex have been described, although some areal differences have been noted.

This period of human development is comparable with the period E50–E90 in the macaque monkey. This should be considered a critical period in cortical development because, as this study confirms, the first synapses form (Kostović and Rakic 1990) and thus spontaneous network activity in the marginal layer and subplate could influence development of the cortical plate. The thickness of the cerebral wall increased dramatically during the period studied, however, different compartments undergo major growth at different times. To begin with the cortical plate and the SVZ were the fastest growing regions. Later on the subplate became the fastest expanding region

(summarized in Fig. 8). Three significant and novel observations have been made. First, the subplate is recognizable as a distinct structure by 10.5 PCW that expresses synaptophysin and vGAT and thus is capable of forming a spontaneously active synaptic network. It is also distinguishable in MRI scans by 14.5 PCW. Second, by 16 PCW there is further differentiation of the subplate with NPY neurons located principally at the border with the cortical plate and KCC2 expressing neurons found at the border with the intermediate zone. Third, PAX6 is not only expressed in the VZ but by many cells in a large and complex SVZ.

Differentiation of the Cortical Plate

During the period of the study, MAP2-positive cells that will form layers IV–VI migrate to and collect in the cortical plate, but the cells remain densely packed suggesting little growth either of dendritic processes, despite high MAP2 expression, or invasion by axonal processes, demonstrated by low GAP43 expression. From 9 to 12 PCW, the entire width of the CP is filled with TBR1 expression, a marker for layer VI in rodent (Hevner et al. 2001). Our observations strongly suggest that by 16 PCW the cortical plate contains both layers V and VI, with layers IV and above in the process of forming. This is assuming that Er81 is a marker for Layer V, as has been demonstrated in both the rodent and macaque (Hevner et al. 2003; Yoneshima et al. 2006). Clearly, the outer SVZ would be established in time to contribute to formation of the layers II–IV in the human (see below). By 16 PCW, CR neurons are predominantly found in the newly forming layer IV (for further discussion, see below) a region which, by this stage, also shows some evidence of synaptophysin expression occurring at higher levels than in the deeper layers.

Delineation of the Subplate

The subplate shows marked expansion in the later part of our study beginning after 12.5 PCW and continuing to 17 PCW, and beyond, as has previously been shown (15–26 PCW, Kostović et al. 2002). In the macaque, the subplate begins rapid expansion at E72 and it continues until E100 (Kostović and Rakic 1990; Smart et al. 2002). The previous study of Kostović and Rakic (1990) described a presubplate layer being present between formation of the cortical plate at 7–8 PCW and the rapid expansion of the subplate proper after 13 PCW or so. This presubplate comprised cell types belonging to the preplate that exists before cortical plate formation such as the CR-positive pioneer neurons described by Meyer et al. (2000) that project

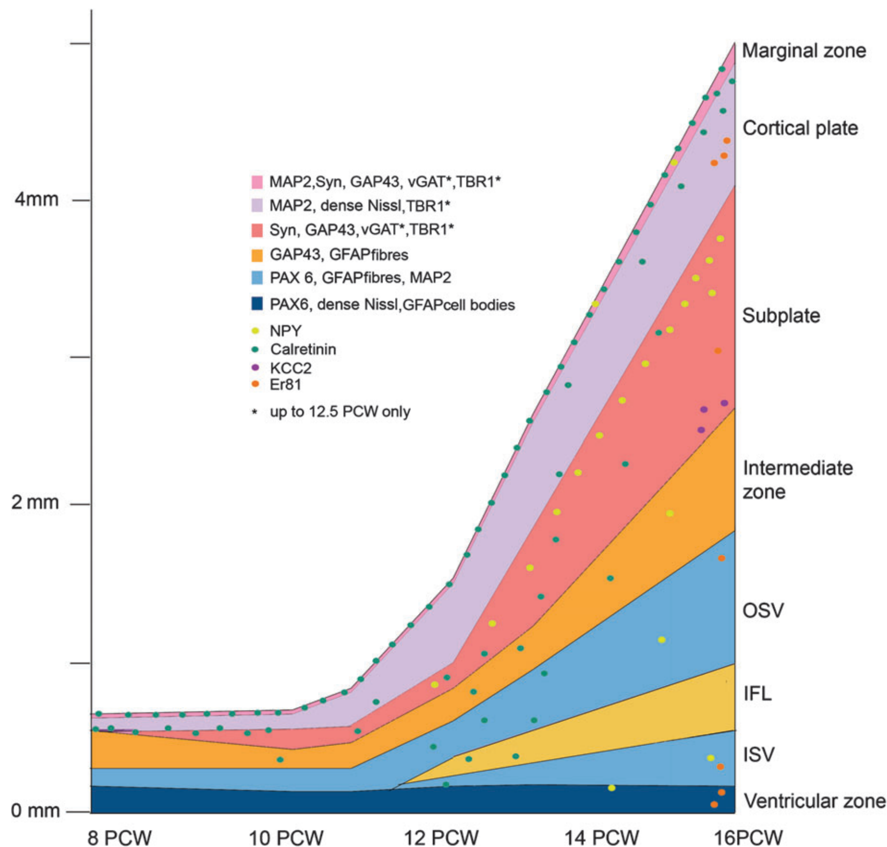

Figure 8. A qualitative summary diagram that shows the ontogenetic expression of several proteins found in all cortical regions and the average growth of the cerebral wall also across regions. Generalized immunostaining of many cells and/or their processes in a given layer is represented by blocks of color, whereas immunoreactivity observable in individual cells is represented circles of different colors. It can be seen that, initially, the cortical plate grows rapidly in thickness, but during the second half of the period studied it is the subplate and SVZ that undergo the greatest degree of expansion and differentiation. The SVZ divides into OSV and ISV cell rich regions that express PAX6 indicating that both compartments contain neural progenitor cells. This figure shows that CR-positive neurons are amongst the first differentiated neuronal cell types observed seen particularly in the marginal zone, but that a second cohort appears around 11–13 weeks, followed by migration to a final resting place in the cortical plate. NPY and KCC2 neurons appear at different locations in the subplate. Er81 positive neurons are born in the VZ but settle in layer V of the cortex. The scale bar should be considered as only giving approximate values for thickness of the cerebral wall and each layer, as this diagram is derived from sections that have undergone different methods of tissue processing and have different angles of cut.

axons into the intermediate zone and toward the internal capsule. Studies in rodent also predict that this early subplate should contain TBR1-positive neurons (Hevner et al. 2001) which this study confirms. However, the internal boundary of this presubplate layer was not clear. In our sections, an inner boundary with the intermediate zone is evident from 10.5 PCW (Fig. 3 and 4) showing that a distinct subplate forms a substantial compartment rich in synaptophysin and vGAT immunoreactivity and comprising about 12–15% of the total thickness of the cerebral wall in the neocortex between 10 and 13 PCW (Fig. 8). In this respect, we concur with Smart et al. (2002) who concluded from their studies of the macaque that a proper subplate forms at E65 prior to the invasion by subcortical afferents (Kostović and Goldman-Rakic 1983; Kostović 1986) that results in a rapid expansion of the subplate from E72 onwards, or 13 PCW in human. This thickness of subplate observed between 10 and 13 PCW could contain within it the branched neurites that are extended by immature pyramidal neurons at this age, as observed in Golgi preparations (Mrzljak et al. 1988). Such neurites could provide sites for synaptogenesis. Potential presynaptic elements could include small numbers of catecholaminergic fibers that invade the region from 7 to 11 PCW (Zecevic and Verney 1995) and early arriving basal forebrain afferents (Kostović 1986) as well as locally derived axons.

MRI reveals a distinct subplate from 14.5 PCW. In the adult brain, a T_1-weighted MR image provides sharp contrast between white and gray matter, with white matter giving higher signal intensity. However, the T_1 difference is negligible for neonatal brain due to lack of myelin (Koenig et al. 1990). It is expected that the signal intensity should be predominantly proportional to proton density in spite of a T_1-weighted pulse sequence used in this study. MRI reveals distinctly reduced intensity corresponding to a subplate (Fig. 5*B*,*C*) from 14.5 PCW, further demonstrating that it is the decreased cellular density accompanied by increased amounts of extracellular matrix that makes the subplate discernible in such scans (Kostović et al. 2002; Gupta et al. 2005; Radoš et al. 2006).Our studies provide the earliest published time point for identification of the subplate by MRI.

Differentiation of a GABAergic Network

The expression of GAD, CR, and vGAT in the human cortex from early stages suggests that GABAergic neural networks can form early in development. Studies in the neonatal rat show that there is intense expression of vGAT in the marginal zone and weaker expression in deeper layers of the cortical plate and the subplate by birth (Minelli et al. 2003). This coincides with the detection of $GABA_A$-mediated synaptic currents in both Cajal–Retzius cells and subplate neurons at this time (Hanganu et al. 2001; Kilb and Luhman 2001). As we have made similar observations of vGAT immunoreactivity in the human at 11.5 PCW, this suggests that there may be GABA mediated synaptic activity at this stage of development. Our concomitant observations of widespread synaptophysin immunoreactivity in the marginal zone and subplate, coupled with previous electron microscopic identification of synapses in small samples of these zones at this stage (Kostović and Rakic 1990), further strengthen this conclusion. The spontaneous network activity first generated by GABAergic synapses is believed to be crucial to nervous system development (Penn and Shatz 1999; Ben-Ari et al. 2004). It appears to be present from a much earlier stage in humans than is generally studied in animal models.

At the early stages of development of GABAergic circuitry in the rodent cortex, $GABA_A$ receptor activation has a depolarizing effect upon target neurons which switches to the hyperpolarizing action seen in mature neurons. This is due to the expression of the KCC2 chloride transporter around postnatal day 7 which changes the equilibrium potential for chloride ions (Rivera et al. 2005). We have observed expression of KCC2 in a subset of subplate neurons by 16 PCW. Certain trophic factors such as brain derived neurotrophic factor appear to promote both synaptogenesis and KCC2 expression at early developmental stages (Aguado et al. 2003; Rivera et al. 2005) providing further evidence for development of GABAergic synaptic networks at this stage of human subplate development. However, other subplate neurons and their potential targets in the cortical plate do not express KCC2 at this time, suggesting that most neurons would still be under the depolarizing influence of GABA. Indeed the subplate and cortical plate continue to generate spontaneous network activity dependent on the depolarizing action of GABA until shortly after birth in the human (Vanhatalo et al. 2005).

NPY expression has also been previously considered a marker for mature, active neurons in the developing subplate (Chun et al. 1987). In common with previous studies, we see NPY-positive neurons in the human subplate which could form constituents of early GABAergic circuitry (Delalle et al. 1997; Uylings and Delalle 1997; Wai et al. 2004). In the adult cortex, NPY is exclusively expressed by a subset of GABAergic interneurons (Hendry et al. 1984) and NPY acts on G-protein coupled receptors to suppress calcium entry into neurons. Presynaptically this acts to suppress transmitter release (Bacci et al. 2002). Postsynaptic actions are less well understood. As well as being located in the subplate, NPY neurons were seen in other locations, including the SVZ. NPY has been demonstrated to regulate cell proliferation via activation of the ERK sub group of MAP kinases (Hansel et al. 2001). Therefore, in the same way that thalamocortical afferents are believed to communicate with proliferative zones by release of transmitters or growth factors from their growth cones as they grow past on the way to the subplate (Edgar and Price 2001) migrating neurons may also release trophic peptides into the SVZ.

Migration of GABAergic Neurons

A major difference between the human and rodent brain lies in the origin of inhibitory cortical interneurons. It appears that in the rodent 95% of inhibitory interneurons are born in the medial (MGE) and caudal (CGE) ganglionic eminences of the subpallium and migrate tangentially into the cortex (Márin and Rubenstein 2001). In humans, it has been estimated that only 35% come from this source, the rest being born on the cortex itself (Letinic et al. 2002). Furthermore, in the rodent, parvalbumin- and somatostatin-positive inhibitory neurons arise from the MGE and mostly populate the rostral two thirds of the cortex, whereas CR-positive inhibitory neurons arise from the CGE and mostly populate the caudal pole (Xu et al. 2004), although they do find their way to all parts of the cortex (Polleux et al. 2002). In the present study, CR neurons were present at the earliest stages studied and were probably original preplate neurons and not GABAergic (Meyer et al. 2000). However, we found that the numbers of CR-positive neurons increased from about 12 PCW, confirming the previous

observations of Meyer et al. (2002b). Furthermore, at this stage of development, the rostral cortex was far more densely populated with CR neurons than the caudal pole, suggesting that they did not necessarily arise from the CGE. Perhaps many more CR interneurons are born locally in the cortex in the human. CR interneurons are more prevalent in the adult primate than the rodent (Conde et al. 1994; Gabbott et al. 1997; Wonders and Anderson 2005) and it has been previously suggested that these extra numbers of inhibitory neurons might be generated in the cortex itself (Wonders and Anderson 2005).

CR-positive neurons were observed with the appearance of radially migrating neurons in this study. Therefore, these might have been born in the cortical neuroepithelium or be undergoing radial migration having first reached the appropriate location in the cortex by tangential migration (Nadarajah et al. 2002). Many CR neurons also appeared to be migrating tangentially and these were generally most numerous in the SVZ and intermediate zone, although they were observed in all compartments including the cortical plate as previously described (Meyer et al. 2002b). In rodents, tangentially migrating neurons are largely confined to regions containing fiber tracts such as the intermediate zone, subplate, and inner fiber layer of the SVZ (Nadarajah et al. 2002; López-Bendito et al. 2004).

Thus, the present study suggests that if CR-positive interneurons in humans are generated in the subpallium, this should include more rostral regions such as the medial and lateral ganglionic eminences. Alternatively, CR neurons are generated directly in the rostral pallium and migrate to other areas of the pallium from there; in vitro preparations of human cortical progenitor cells from the VZ/SVZ of the frontally cut hemispheres can give rise to CR-positive neurons under culture conditions (Mo et al. 2007). However the possibility that CR interneurons are generated in the rostral cortex early in development and at the caudal pole later in development than was studied here cannot be ruled out. Although at 12–13 PCW CR neurons are seen throughout the developing neocortex, by 16–17 PCW they are predominantly located in the newly forming layer IV of the cortex, which suggests that they arrive relatively early in development but are destined to reside in the later formed layers of the cortex.

Differentiation of the SVZ

The growth of a large SVZ, which divides into two recognizable layers separated by a cell poor/fiber rich region, appears to be characteristic of primate development as it has already been described in the macaque monkey (Smart et al. 2002) and humans (Zecevic et al. 2005). In the monkey, inner and outer layers of the SVZ emerge between E65 and E72, and this is recognizable in our human material from differences in PAX6, GFAP, TBR2, and Ki 67 immunoreactivity that emerge between 10.5 and 11.5 PCW. Observations of both PAX6 and Ki67 immunoreactivity external to the cell poor/fiber rich region confirm that this outer layer is indeed part of the SVZ as both are markers for dividing cells (Brown and Gatter 1990; Warren et al. 1999; Chan et al. 2006) and disagrees with Altman's and Bayer's (2002) assertion that the SVZ is restricted to the inner layer. However, the ISVZ is distinct from the OSVZ. The ISVZ shows higher expression of TBR2 and Ki67 and may represent intermediate or basal progenitor cell layer described in rodents (Miyata et al. 2004; Noctor et al. 2004). TBR2 can be expressed both by mitotic intermediate progenitor cells and postmitotic neurons (Englund et al. 2005) but these postmitotic neurons presumably downregulate TBR2 as they migrate toward the cortical plate, losing expression of TBR2 even before they reach boundary of the OSVZ with the intermediate zone. On the other hand, PAX6 expressing cells are numerous up to this boundary. PAX6 is believed to suppress the exit of progenitor cells from the cell cycle and the expression of postmitotic markers such as TBR2 (Quinn et al. 2007). Therefore, it appears that the human OSVZ retains a large reservoir of PAX6-positive potential progenitor cells. These cells may return to the VZ or ISVZ to undergo cell division, but Ki67 expression clearly demonstrates that cell division also occurs throughout the OSVZ.

The cell poor/fiber rich layer intervenes by 12.5 PCW in the human and E78 in the macaque. At this time in the macaque, the VZ decreases dramatically in thickness and it has been proposed that the outer layer of the SVZ becomes the mitotic layer that provides neurons for layers II–IV of the cortical plate (Smart et al. 2002; Lukaszewicz et al. 2005; Molnár et al. 2006). By this stage, in our specimens, the most intense PAX6 expression appears in cells in the SVZ, as has been described in mouse at embryonic day 14.5 (Warren et al. 1999). However, in humans, the VZ is still prominent and PAX6 immunoreactive at 16PCW and also contains cells that express Er81 and GFAP. It is possible that radial glia are still giving rise to layer V projection neurons at this stage, suggesting that it retains a role in cortical cell generation for longer than in mice.

In conclusion, this study forms part of continued efforts to link modern molecular neuroanatomical data with traditional Nissl-stained sections and the appearance of the developing human brain in MRI scans. In this way, it should be possible to match developmental lesions and malformations to specific events in cortical development, such as establishment of the subplate, synaptogenesis, and generation and migration of certain types of cortical neuron.

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