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ORIGINAL ARTICLE

A Complex Code of Extrinsic Influences on Cortical Progenitor Cells of Higher Mammals

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Abstract

Development of the cerebral cortex depends critically on the regulation of progenitor cell proliferation and fate. Cortical progenitor cells are remarkably diverse with regard to their morphology as well as laminar and areal position. Extrinsic factors, such as thalamic axons, have been proposed to play key roles in progenitor cell regulation, but the diversity, extent and timing of interactions between extrinsic elements and each class of cortical progenitor cell in higher mammals remain undefined. Here we use the ferret to demonstrate the existence of a complex set of extrinsic elements that may interact, alone or in combination, with subpopulations of progenitor cells, defining a code of extrinsic influences. This code and its complexity vary significantly between developmental stages, layer of residence and morphology of progenitor cells. By analyzing the spatial-temporal overlap of progenitor cell subtypes with neuronal and axonal populations, we show that multiple sets of migrating neurons and axon tracts overlap extensively with subdivisions of the Subventricular Zones, in an exquisite lamina-specific pattern. Our findings provide a framework for understanding the feedback influence of both intra- and extra-cortical elements onto progenitor cells to modulate their dynamics and fate decisions in gyrencephalic brains.

Key words: DiI, neuronal migration, OSVZ, retrovirus, subplate

Introduction

The development of the cerebral cortex is a highly complex process involving a sequence of histogenetic events. This begins with progenitor cell amplification, followed by neurogenesis, neuronal migration, axon guidance, terminal differentiation and synaptogenesis. Regulation of progenitor cell proliferation and neurogenesis has been best studied at the level of cell-autonomous mechanisms, identifying a number of cell cycle proteins, transcription factors and other molecules that regulate the balance between cell proliferation and neurogenesis (Florio and Huttner 2014; Taverna et al. 2014; Fernandez et al. 2016). Cell non-autonomous mechanisms influencing these crucial early events are suspected to also be important but, in contrast, are much less well understood (Dehay and Kennedy 2007; Rakic 2009).

The assembly of the 6-layered mammalian cerebral cortex involves a complex orchestration of different processes that occur simultaneously, resulting in the temporal overlap of initial events (progenitor proliferation and neurogenesis) with final events (neuron migration, axon growth, synaptogenesis) (Issa et al. 1999; Rakic 2009). This scenario supports the possibility of cell non-autonomous regulation of these processes by means of mutual influence and extrinsic feedback mechanisms (Gong and Shipley 1995; Polleux et al. 1998; Dehay and Kennedy 2007; Seuntjens et al. 2009; Nelson et al. 2013). In vitro studies have shown that cortical progenitor cell proliferation is promoted by growing thalamic axons via bFGF signaling (Dehay et al. 2001), and inhibited by the neurotransmitters γ-aminobutyric acid (GABA) and glutamate when applied to brain slices (LoTurco et al. 1995; Haydar et al. 2000). Together, existing evidence points to a model where the timing of genesis of neurons for specific layers, and cortical area identity, are regulated by a dynamic interplay between cell-autonomous and cell non-autonomous effects onto cortical progenitor cells (Gillies and Price 1993; McConnell 1995; Polleux et al. 1998).

Current hypotheses propose that extrinsic factors influencing progenitor cell proliferation in the embryonic cerebral cortex in vivo may be secreted into the extracellular space by developing axons or tangentially migrating interneurons (Dehay and Kennedy 2007; Wang and Kriegstein 2009). The influence of extrinsic factors onto progenitor cells is believed to be mostly paracrine, relying on their physical proximity (Dehay et al. 2001; Dehay and Kennedy 2007). Although attractive, an essential undermining limitation of this model is that growing axons and migrating neurons in the embryonic cerebral cortex navigate far away from the ventricular zone (VZ), layer of residence for the cell body of Radial Glia Cells (RGCs), and their proximity with the cell body of the neurogenic Intermediate Progenitor Cells (IPCs, or basal progenitors) is also limited (Noctor et al. 2001, 2004). Here we present the Dynamic Extrinsic Code, a unifying framework on coordinated mechanisms of extrinsic influence onto cortical progenitor cells, which reconciles past and new evidence.

The Dynamic Extrinsic Code links the variety of progenitor cell morphotypes recently discovered in the developing cerebral cortex of higher mammals (human, macaque and ferret) (Borrell and Gotz 2014) with the dynamic variations across time and space of cellular elements potentially providing extrinsic influence. We propose that cortical progenitors may sense extrinsic signals both through their cell body and also via their fine cellular processes, which are extended either at a short range in IPCs, or at a long range like the basal processes of RGCs. For example, signals such as calcium waves travel from the pia to the cell body of RGCs along their radial process (Weissman et al. 2004), and then calcium bursting in the cell body regulates progenitor cell proliferation and neurogenesis (Rash et al. 2016).

Focusing on the developing cortex of the gyrencephalic ferret, we combine axonal tracing, antibody stains and cell lineage labeling to discover that there are multiple potential sources of extrinsic influence (i.e., growing axon tracts and streams of migrating neurons). We demonstrate that these overlap extensively with progenitor cell bodies and processes, while also changing very dynamically between developmental stages. Hence, they represent a laminar arrangement of various signals (extrinsic code) that change dynamically in time and that may be read differently by each progenitor cell subtype depending on their particular spatial and temporal overlap. Finally, we provide functional evidence that developing thalamic axons exert significant and lamina-specific modulatory effects on cortical progenitor cells. Our findings further suggest that novel and very specific mechanisms of feedback interaction between differentiating neurons and cortical progenitor cell subtypes may have contributed to cortical expansion and an increase in functional complexity in higher mammals.

Materials and Methods

Animals and Tissue Collection

Pigmented sable ferrets (*Mustela putorius furo*) were obtained from Marshall Bioresources (North Rose, NY) and kept at the Animal Facilities of the Universidad Miguel Hernández or the Autonoma University of Madrid on a 16:08 h light:dark cycle. Ferrets were treated according to Spanish and European Union regulations, and experimental protocols were approved by the Institutional Animal Care and Use Committee of the respective Universities. All animals were perfused transcardially with 4% paraformaldehyde in phosphate buffer pH 7.4 (PFA), and brains were postfixed overnight at 4°C in the same fixative. For embryonic stages, pregnant dams were deeply anesthetized with ketamine/xylazine induction followed by Isoflurane, and embryos were extracted by cesarean section.

All analyses were performed on the caudal part of the developing ferret cerebral cortex, corresponding to the prospective visual cortex. In order to study the potential influence of extrinsic factors on the proliferative and neurogenic activity of cortical progenitor cells, we concentrate our analysis between embryonic day (E) 30 and postnatal day (P) 14, which includes the periods of birth and radial migration for neurons destined to almost all cortical layers (Jackson et al. 1989). This period of cortical development is equivalent to E12–E18 in mouse, E55–E94 in macaque, and 9–18 gw in human embryos (Rakic and Sidman 1968; Rakic 1974; Jackson et al. 1989; Bayer and Altman 2005). During this developmental period, we distinguished several germinal layers by their cytoarchitectonic organization as revealed by Nissl stains (Reillo and Borrell 2012): VZ and SVZ at E30–E34; VZ, ISVZ, and OSVZ between E36 and P14. Starting at P0 we distinguished 2 subdivisions within the OSVZ: an inner part (iOSVZ) with low cell density and striped alignment of cells, and an outer part (oOSVZ) with high cell density (Reillo and Borrell 2012).

Immunostaining

Fixed brains were cut parasagitally in 5 μm-thick paraffin sections, or 50 μm-thick cryosections. Sections were treated for antigen retrieval (when needed), blocked, and incubated overnight with the following primary antibodies: mouse anti-β-III-Tubulin (Covance, 1:1000); rabbit anti-Calbindin (Swant, 1:2000); rabbit anti-Calretinin (Swant, 1:2000); rabbit anti-Cx43 (Millipore, 1:100); rabbit anti-GABA (Sigma, 1:2000); rabbit anti-GFAP (DAKO, 1:1000); chicken anti-GFP (Aves, 1:1000); rabbit anti-Laminin (Millipore, 1:400); mouse anti-MAP2 (Chemicon, 1:1000); sheep anti-Neuropeptide Y (Chemicon, 1:2000); rabbit anti-Parvalbumin (Swant, 1:2000); mouse anti-Phosphovimentin (Abcam, 1:1000); rabbit anti-Pax6 (Millipore, 1:500); goat anti-Somatostatin (Santa Cruz, 1:250); mouse anti-Synaptophysin (Sigma, 1:1000); rabbit anti-Tbr2 (Abcam, 1:200); mouse anti-Vimentin (Chemicon, 1:400). Sections were then incubated with the appropriated fluorescently conjugated secondary antibodies and counterstained with DAPI (Sigma), or Nissl stain as required. Alternatively, sections were incubated with biotinylated secondary antibodies, ABC complex (Vector, 1:100), and developed with

nickel enhancement (Reillo and Borrell 2012). Bright field, fluorescence and confocal images were acquired using Leica, Olympus and Zeiss microscopy.

Cloning and In Situ Hybridization

Ferret-specific ISH probes were cloned using the following primers:

Fgf10 fw: CTTGGTCAGGACATGGTGTAC;
Fgf10 rv: GAGCTCCTTTTCCATTCAATGCC;
Fgf2 fw: GGCCACTTCAAGGACCCCAAGCGG;
Fgf2 rv: CAGTTCGTTTCAGTGCCACATACCAAC;
Fgfr1 fw: ACTCTGCATGGTTGACCGTTCTGGAAGC;
Fgfr1 rv: CTTTGGTCACACGGTTGGGTTTGTCC;
Fgfr2 fw: AGAGATAAGCTGACGCTGGGCAAACC;
Fgfr2 rv: GAGGAAGGCAGGGTTCGTAAGGC;
Fgfr3 fw: ACACCACCGACAAGGAGCTAGAGGTTC;
Fgfr3 rv: CTCCCGCATGATCATGTACAGGTCGTG;
EphB3 fw: CAGCTCATGCTGGACTGTTGG;
EphB3 rv: GCATGTCCTGGATACTGCAGAG;
EphA4 fw: TTATTGGATTCCAGATCTGTTCAGGG;
EphA4 rv: GTACCAGCCATTCACCATCTGC;
EphA5 fw: TCTACTGGAACAGCAGCAACCC;
EphA5 rv: CTTCTTAGGATGAGCAGTTAGGTGG.

PCR was performed using Go Taq Flexi DNA polymerase (Promega), and the resulting amplicons were purified with Wizard SV Gel and PCR Clean-Up System (Promega) and cloned into pGEM-T Easy Vector System I. Ferret-specific sense and anti-sense cRNA probes were synthesized and labeled with digoxigenin (DIG; Roche Diagnostics) according to the manufacturer's instructions. In situ hybridization (ISH) was performed as described elsewhere (Reillo et al. 2011). Briefly, 50 µm-thick frozen brain sections were hybridized with DIG labeled cRNA probes overnight in hybridization solution [50% formamide (Ambion), 10% dextran sulfate, 0.2% tRNA (Invitrogen), 1× Denhardts solution (from a 50× stock; SIGMA), 1× salt solution (containing 0.2 M NaCl, 0.01 M Tris, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 5 mM EDTA, pH 7.5)]. After sections were washed, alkaline phosphatase-coupled anti-digoxigenin Fab fragments were applied. For visualization of the labeled cRNAs, sections were incubated in nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution [3.4 µl/ml from NBT stock and 3.5 µl/ml from BCIP stock in reaction buffer (100 mg/ml NBT stock in 70% dimethylformamide; 50 mg/ml BCIP stock in 100% dimethylformamide; Roche)].

Virus Injections and Plasmid Electroporation

High titer MMLV-based VSVG-pseudotyped retrovirus (5×10^7–5×10^8 pfu/ml) encoding *Gfp* under the CAG promoter were prepared, concentrated and viral titer estimated as described (Tashiro et al. 2006). Ferret kits were deeply anesthetized and maintained with 1.5% Isoflurane during surgery. Virus injections were aimed at the medial ganglionic eminence (MGE) or the lateral telencephalic ventricle by means of stereotaxic coordinates. Viral solutions were injected using pulled glass micropipettes. Postnatal electroporation of ferret kits was performed as described (Borrell 2010) using DNA plasmids encoding for *Gfp* under the CAG promoter. After the appropriate survival period, kits were overdosed with sodium pentobarbital (80 mg/kg, ip.), perfused transcardially with PFA, the brain collected and postfixed, and further processed for immunostaining as described above.

Binocular Enucleation

Enucleation was always performed bilaterally and on ferret kits aged P1. Kits were deeply anesthetized and maintained with 1.5% Isoflurane during surgery. Eyelids were cut open, the connective tissue and muscle surrounding the eye ball was cut all around, the optic nerve severed and the eye ball removed.

Dye Tracing

Ferret kits aged between E30–P14 were perfused with PFA, and their brains were stored in the same fixative at room temperature. Small crystals of DiI and DiA (Molecular Probes) were delivered to the parietal cortex, lateral geniculate nucleus (LGN) or corpus callosum (CC). Dyes were allowed to diffuse at room temperature for 2–3 months, and then the brains were vibratome-sectioned at 80 µm and counterstained with DAPI (Sigma).

Neonatal CTB Injections for Anterograde Axonal Tracing

Ferret kits aged P2 ($n = 3$) and P4 ($n = 2$) were deeply anesthetized and immobilized on a Kopf small animal stereotaxic apparatus. A borosilicate micropipette (20 µm outer Ø) containing 2% CTB in PB pH 6.0 (List Biological Labs, Campbell, CA) was lowered into the LGN (1.2 mm posterior to Bregma; 1.1–1.2 mm lateral to midline; 4.8–5.5 mm under the pial surface). CTB was injected by iontophoresis applying positive current pulses 2 µAmp, 7 on/off for 10 min. Because the head size of newborn ferrets may vary significantly between animals and between litters, in some cases 2 such deposits were made within the same LGN to guarantee a successful labeling. After surgery the kits were allowed to fully recover before placed back to the dam. After 2–4 days of survival kits were overdosed with sodium pentobarbital and perfused transcardially with PFA. Brains were postfixed in the same fixative, cryoprotected and sectioned coronally at 50 µm. CTB was detected following a standard protocol (Angelucci et al. 1996), and then sections were mounted, dehydrated and coverslipped.

Results

Diversity of Progenitor Cell Morphologies

The embryonic cerebral cortex contains 2 principal types of progenitor cells: apical and basal progenitors. Apical progenitors include apical Radial Glia Cells (aRGCs) and Short Neural Precursors (SNPs) (Fig. 1A) (Miyata et al. 2001; Noctor et al. 2001; Gal et al. 2006; Taverna et al. 2014). Basal progenitors undergo mitosis mostly in the SVZ and include Subapical Progenitors, Intermediate Progenitor Cells (IPCs) and basal Radial Glia Cells (bRGCs) (Fig. 1A–C,F) (Fietz et al. 2010; Hansen et al. 2010; Reillo et al. 2011; Wang et al. 2011; Garcia-Moreno et al. 2012; Kelava et al. 2012; Pilz et al. 2013; Borrell and Gotz 2014; Martinez-Martinez et al. 2016). In primates and carnivores, the SVZ is subdivided in inner and outer SVZ (ISVZ and OSVZ, respectively) (Smart et al. 2002; Reillo et al. 2011), which are abundantly populated by bRGCs and IPCs, the cell bodies of which spread over the entire apical-basal extent of these layers (Fietz et al. 2010; Reillo et al. 2011; Betizeau et al. 2013; Martinez-Martinez et al. 2016). Hence, whereas all aRGCs coalesce within

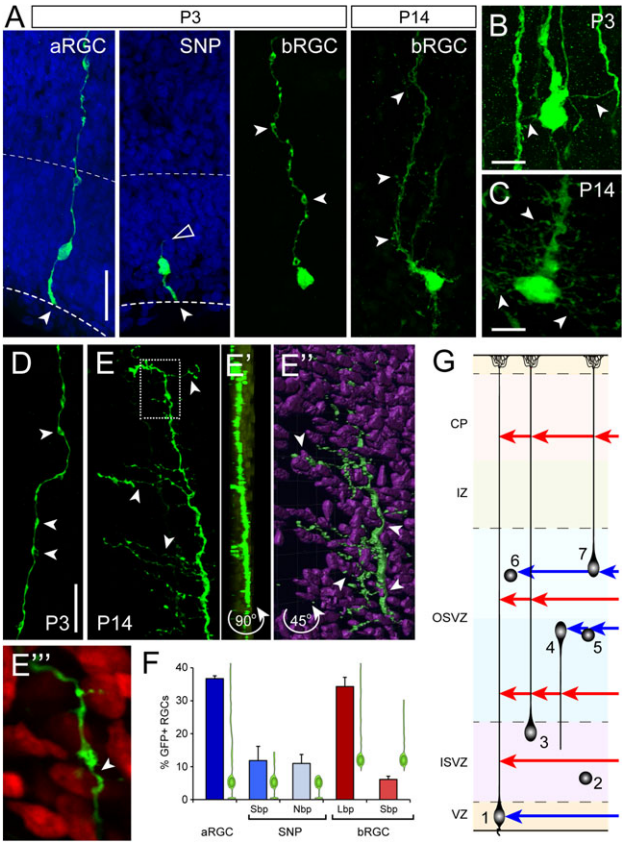

Figure 1. RGC morphotypes in ferret. (*A*) Morphotypes of aRGC and SNP in VZ, and bRGCs in ISVZ, at the indicated stages after rv::*Gfp* delivery in the ventricle at P1. Solid arrowheads in aRGC and SNP indicate the anchoring to the ventricular surface. Open arrowhead in SNP points to the short basal process. Solid arrowheads in bRGCs indicate the basal process. (*B*,*C*) High magnification of the soma of bRGCs in ISVZ. Solid arrowheads indicate lamellate expansions extending from the cell soma. (*D*–*E*‴) Details of the basal fiber in ISVZ at the indicated ages, with solid arrowheads indicating varicosities (*D*) and lamellate expansions (*E*). (*E*′,*E*″) Z-stacks of confocal images of the basal fiber shown in (*E*), where the same entire field of view is rotated 90° (*E*′) and 45° (*E*″) to demonstrate its 3-dimensional structure and intermingling with adjacent cells (DAPI stain in purple). (*E*‴) Enlarged view of the area boxed in (*E*) showing the close intermingle between basal fiber (green) and DAPI-stained nuclei of neighboring cells (red). (*F*) Relative abundance of GFP + RGC morphotypes in ferret cortex at P6 after rv::Gfp delivery at P1. Lbp, cell with long basal process; Nbp, cell with no basal process; Sbp, cell with a short basal process. (*G*) Summary schema of the different progenitor cell morphotypes and their laminar location. Numbers indicate: 1- aRGC; 2- IPC in ISVZ; 3 bRGC-basal-P in ISVZ; 4, bRGC-apical-P in OSVZ; 5- IPC in lower OSVZ; 6- IPC in upper OSVZ; 7- bRGC-basal-P in upper OSVZ. Blue arrows represent extrinsic influences onto the cell bodies, red arrows represent extrinsic influences onto different levels of RGC radial fibers. Notice that depending on the direction and length of the radial fiber, the number and nature of potential extrinsic interactions may vary significantly. IPCs essentially receive influence via their cell soma. Scale bar: 25 μm (*A*,*D*,*E*), 10 μm (*B*,*C*).

the relatively thin VZ, the location of the cell body of bRGCs and IPCs is significantly heterogeneous (Fig. 1*G*).

In addition to the diversity of locations of cortical progenitor cell bodies, their morphology is also quite diverse. Most progenitor cells are highly polarized: SNPs and aRGCs have an apical process extended to the ventricular surface, which exposes a primary cilium to the cerebro-spinal fluid, and then a radially oriented basal process which either extends outside the VZ (aRGCs), or is short and remains within the VZ (SNPs) (Fig. 1*A*,*F*) (Borrell and Gotz 2014). In the young postnatal ferret (P6; equivalent to mouse E16.5, macaque E85 and human 16 gestational weeks, gw), when neurons destined to layer 2/3 are being born and migrating radially, we found that SNPs displayed either a short basal process (Fig. 1*A*) or no basal process at all, with both morphologies being at a similarly low abundance (Fig. 1*F*). Most bRGCs lack an apical process contacting the ventricle but display a long basal process (Fietz et al. 2010; Hansen et al. 2010; Reillo et al. 2011). Studies in primates show that the detailed morphology of bRGCs is really diverse and includes 4 main morphotypes: cells bearing only a basal process (bRG-basal-P), cells bearing only an apical process (bRG-apical-P), cells bearing both apical and basal processes (bRG-both-P), and cells alternating between 2 transient states: having an apical and/or a basal process, or no process (tbRG) (Betizeau et al. 2013). In ISVZ and OSVZ of P6 ferrets we found that the virtual majority of bRGCs were bRG-basal-P, and that most of these had a long process extending more than 200 μm (Lbp), versus a small minority extending a shorter process (less than 200 μm; Sbp) (Fig. 1*A*,*F*). The cell bodies of bRGC subtypes were found similarly distributed across the thickness of OSVZ and ISVZ, with no specific laminar pattern.

In the embryonic cortex of human, macaque and ferret, the basal process of RGCs is decorated with fine lamellate expansions that protrude laterally to their immediate vicinity for up to 40 μm, and are especially abundant and elaborate at late stages of development (Fig. 1*A*–*E*″) (Rakic 1972; Sidman and Rakic 1973). The function of these protrusions is unknown, but they could mediate cell-cell interactions or be used for sensing extracellular signals, like the growth cone of developing axons, the spines of neuronal dendrites or the fine processes of astrocytes (Perea et al. 2009; Tan et al. 2016). Consistent with this idea, we observed that these lamellate expansions were much more conspicuous and abundant at late (P14) than early (P3) stages of postnatal development (Fig. 1*D*,*E*), and they intermingled closely with cells resident in the germinal layers (Fig. 1*E*″,*E*‴). This correlates with the developmental increase in complexity of their cellular environment, further supporting the notion that these fine elaborations may serve as part of a sensing system.

The basal process of aRGCs and bRGCs spans radially through multiple layers of the developing cortex, from the cell body to the pial surface or, occasionally, to a blood vessel (Ramon y Cajal 1891; Rakic 1972; Sidman and Rakic 1973; Takahashi et al. 1990; Misson et al. 1991; Miyata et al. 2001; Noctor et al. 2001; Reillo et al. 2011; Tan et al. 2016). As a result, aRGCs and bRGCs are prone to be influenced by a combination of extrinsic signals onto their radial process, which will differ depending on the exact location of their cell body across the germinal layers (Fig. 1*G*). In contrast, IPCs only extend short processes from their cell body, and thus may sense extrinsic signals only from their immediate vicinity (Noctor et al. 2004; Stubbs et al. 2009; Nelson et al. 2013). In summary, the plethora of progenitor cell morphologies, and locations of their cell body, offers a wide palette of possibilities for the extrinsic regulation of progenitor cell types in the different germinal layers, with a high degree of selectivity (Fig. 1*G*).

Germinal Layers are Populated by Different Combinations of Callosal and Thalamic Afferents

It has been proposed that one of the main sources of extrinsic influence onto progenitor cells of the developing cortex are axonal afferents, both extra-cortical and cortico-cortical (Gong and Shipley 1995; Dehay et al. 2001; Callaway and Borrell 2011;

Reillo et al. 2011). Focused on the ferret, we began by identifying the types of afferents that overlap with progenitor cells in the developing cortex, by means of tracing with lipophilic dyes (Fig. 2A). Analyses were performed at different developmental stages starting at embryonic day (E) 30, the onset of cortical neurogenesis and prior to the appearance of the SVZ (Reillo and Borrell 2012). At E30 (equivalent to mouse E12, macaque E55 and human 9 gw), growing thalamo-cortical axons had just reached the parietal part of the cortex, with only some axons beginning to navigate into the somatosensory cortex (Fig. 2B,B'). At this early stage, cortico-fugal axons were not observed away from the injection sites. At E32, thalamo-cortical and cortico-cortical axons were just beginning to reach the dorsal-most aspect of the cortex, but still not growing into the medial part (Fig. 2C–C'''). At this stage, cortico-thalamic axons were just reaching the internal capsule (IC) (Fig. 2C'). Two days later (E34), thalamo-cortical axons and cortico-cortical axons were found in more advanced positions towards the midline (Fig. 2D,D'). At E36, when layer 5 neurons are being born (equivalent to mouse E13.5, macaque E65 and human 10 gw), thalamo-cortical axons and cortico-cortical axons already covered the entire latero-medial extent of the cortex, and they remained near the emerging CC (Fig. 2E). Between E36 and postnatal day P2 (equivalent to mouse E15.5, macaque E78, human 15 gw), thalamo-cortical axons extended all the way to the occipital (visual) cortex, and cortico-cortical axons crossed the midline forming the incipient

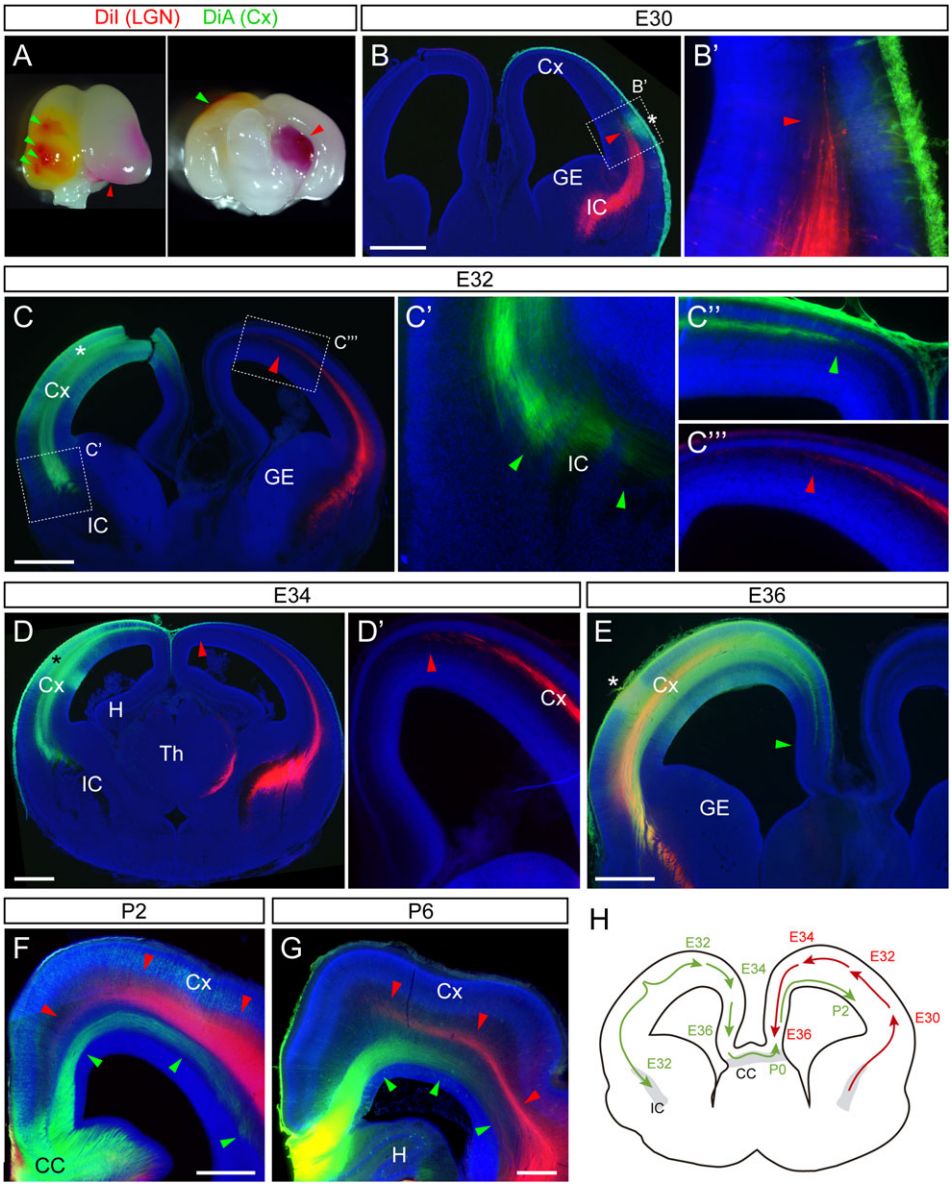

Figure 2. Development of major axon fiber systems in the cerebral cortex. (A) External views of E34 (left) and P0 (right) ferret brains injected with DiI in the thalamic lateral genicular nucleus (LGN; red arrowheads) and DiA in the cortex (Cx; green arrowheads). (B–G) Coronal sections through brains injected as in (A) at the indicated developmental ages, showing thalamo-cortical (red) and cortico-fugal (green) axons. Panels (F,G) show detailed images from the dorsal cortex at the indicated postnatal ages. In postnatal animals, DiA crystals (green) were injected in the corpus callosum (CC). Asterisks indicate the location of DiA injections. Colored arrowheads indicate the front of advance of the axonal systems (B'–D') or their laminar location (F,G). Dashed boxes indicate the source of the high-magnification images, as indicated. Image in (C'') was obtained from a different section. (H) Diagram showing a summary analysis of the developmental progression of axonal tracts as seen in coronal sections. GE, ganglionic eminence; H, Hippocampus; Th, Thalamus. Scale bar: 1 mm (A–E), 200 μm (F,G).

CC. From P2 onwards, callosal axons populated the contralateral cortical hemisphere (Fig 2F–H).

At P6, when neurogenesis is near completion and layer 2/3 neurons are migrating radially (equivalent to mouse E16.5), the main axonal tracts displayed a precise laminar distribution (Fig. 3A). We have previously shown that the ferret OSVZ is subdivided into inner and outer domains (iOSVZ and oOSVZ, respectively). Focused on the visual cortex, we found that most axons labeled upon DiI injection in the thalamus run in 2 parallel tiers: one in the top half of the iOSVZ, and the other in the top part of the oOSVZ and the intermediate zone (IZ; Fig. 3A). DiI-traced axons were absent from the rest of the germinal layers. Callosal axons, originated in the contralateral cortex, populated densely and exclusively the iOSVZ, specifically avoiding the oOSVZ, ISVZ and VZ (Fig. 3A). These callosal axons overlapped partially with the lower tier of thalamic axons, in the central part of OSVZ (Fig. 3A,B). Taken together, these tracing analyses revealed that callosal and thalamo-cortical afferents developed with a very precise timing, and run through different and very stereotyped paths (Fig. 2H).

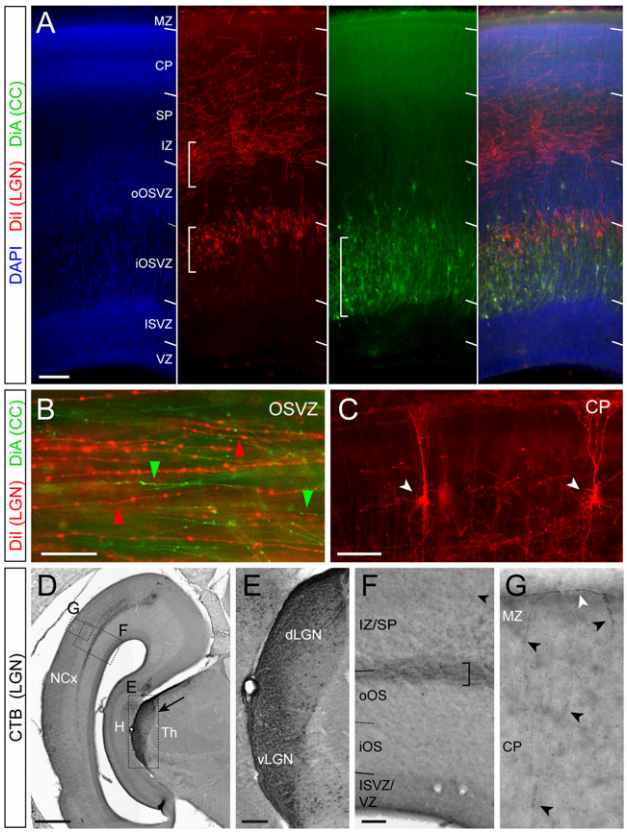

Figure 3. Laminar segregation of axon fiber bundles across germinal layers. (*A*) Dual tracing of axonal projections at P6 demonstrates the laminar segregation of axons of the thalamo-cortical loop (DiI, red) and callosal axons (DiA, green). Axons labeled upon injection in the lateral geniculate nucleus (LGN) run in 2 tiers through the developing cortex, along the upper part of iOSVZ (iO), and the upper part of oOSVZ (oO), intermediate zone (IZ). Axons in iOSVZ were cut perpendicular to the sagittal plane of section, whereas in oOSVZ these were largely parallel. Callosal projections run exclusively through iOSVZ (iO), also perpendicular to the sagittal plane. (*B*) Few DiI- and DiA-traced axons run parallel and overlap in the upper part of iOSVZ, as seen in coronal sections. (*C*) Cortical pyramidal neurons in the cortical plate (CP; arrowheads) retrogradely labeled from the LGN. (*D–G*) Anterograde tracing of thalamo-cortical projections with CTB (black reaction product) at P6. Boxed areas in (*D*) are presented at higher magnification in (*E–G*), showing the CTB injection site in LGN (*E*; arrow in *D*) and a dense band of thalamo-cortical axons running through the upper part of oOSVZ and IZ (oOS; bracket and black arrowheads), but no labeling in iOSVZ (iOS). dLGN, vLGN, dorsal and ventral Lateral Geniculate Nucleus; NCx, neocortex; Th, thalamus. Scale bars: 200 μm (*A,F*), 100 μm (*B,E*), 25 μm (*C,G*), 1 mm (*D*).

An important caveat of lipophilic dye tracing is its bidirectionality, labeling both anterogradely and retrogradely from the injection site, and thus not defining the place of origin for the axons traced. Indeed, our above injections in visual thalamus (lateral geniculate nucleus, LGN) always labeled the optic tract, containing axons projected by retinal ganglion cells (not shown), and frequently labeled pyramidal neurons in cortical layer 5 projecting to LGN (Fig. 3C) (Clasca et al. 1995). To define unequivocally the site of origin of the 2 bands of DiI-labeled axons running along the OSVZ, we injected CTB in the LGN of newborn ferrets, a very efficient and unidirectional anterograde tracer (Fig. 3D–G) (Angelucci et al. 1996). These injections labeled very conspicuously, but selectively, a compact band of axons running along the upper half of oOSVZ and lower IZ, precisely coincident with the upper tier of DiI-traced axons (Fig. 3F). This demonstrated the thalamo-cortical nature of this axonal tract, and also that the tract of DiI-labeled axons in iOSVZ are early cortico-thalamic axons from layer 5 pyramidal neurons (Clasca et al. 1995). Interestingly, a small number of CTB-labeled axons extended radially from OSVZ to marginal zone (MZ), where they branched and further extended tangentially (Fig. 3G).

To characterize the full extent of the thalamo-cortical tract, without the technical limitations to our tracer injections, we performed immunostains against Calretinin (Calb2), previously reported to be expressed by this axonal projection (Morante-Oria et al. 2003). In ferret, Calb2 was detected as early as E30, labeling cells in the emerging cortical plate (CP) (Fig. 4A), in a manner similar to previous descriptions in rat (Fukumitsu et al. 1998). At E34, bundles of Calb2+ fibers were found running tangentially along the IZ and MZ, together with tangentially oriented cells resembling Cajal-Retzius cells. At E38 the abundance of Calb2+ fibers became more prominent and now they span from the oOSVZ through the MZ, where they overlapped with Pax6+ progenitor cells, but were largely absent from iOSVZ, ISVZ, and VZ (Fig. 4A–C'). In addition, many Calb2+ cell bodies were distinguishable in CP, subplate (SP), and IZ (Fig. 4A). At postnatal stages, thick bundles of Calb2+ processes were densely abundant throughout the cortical thickness, now including the entire OSVZ, but continued to remain absent from ISVZ and VZ (Fig. 4A,D–E'). Calb2+ fiber bundles had a characteristic radial alignment in oOSVZ and IZ, and continued to overlap extensively with Pax6+ cells (Fig. 4E,E'). At P14 (equivalent to mouse E18.5, human 18 gw), Calb2+ cells had the typical appearance of pyramidal neurons and distinctly populated deep portions of the CP, corresponding to the already distinguishable layer 5 (Fig. 4A). This was not surprising because also in the developing mouse cerebral cortex Calb2 is expressed by non-GABAergic pyramidal neurons (Alcantara et al. 1998).

Our Calb2 stains, together with the CTB and lipophilic dye tracing analyses, demonstrated that the combination of cortical and thalamic afferents defines 4 laminar subdivisions of the OSVZ: 1) lower iOSVZ containing only callosal axons; 2) upper iOSVZ containing both callosal and cortico-thalamic axons; 3) lower oOSVZ containing Calb2+ fibers that are not thalamic nor callosal; and 4) upper oOSVZ containing only thalamo-cortical axons. At late developmental stages, the OSVZ was packed with Calb2+ fiber bundles. Importantly, both thalamic and callosal axon tracts remained specifically outside of ISVZ and VZ. Taken together, these observations demonstrated that different

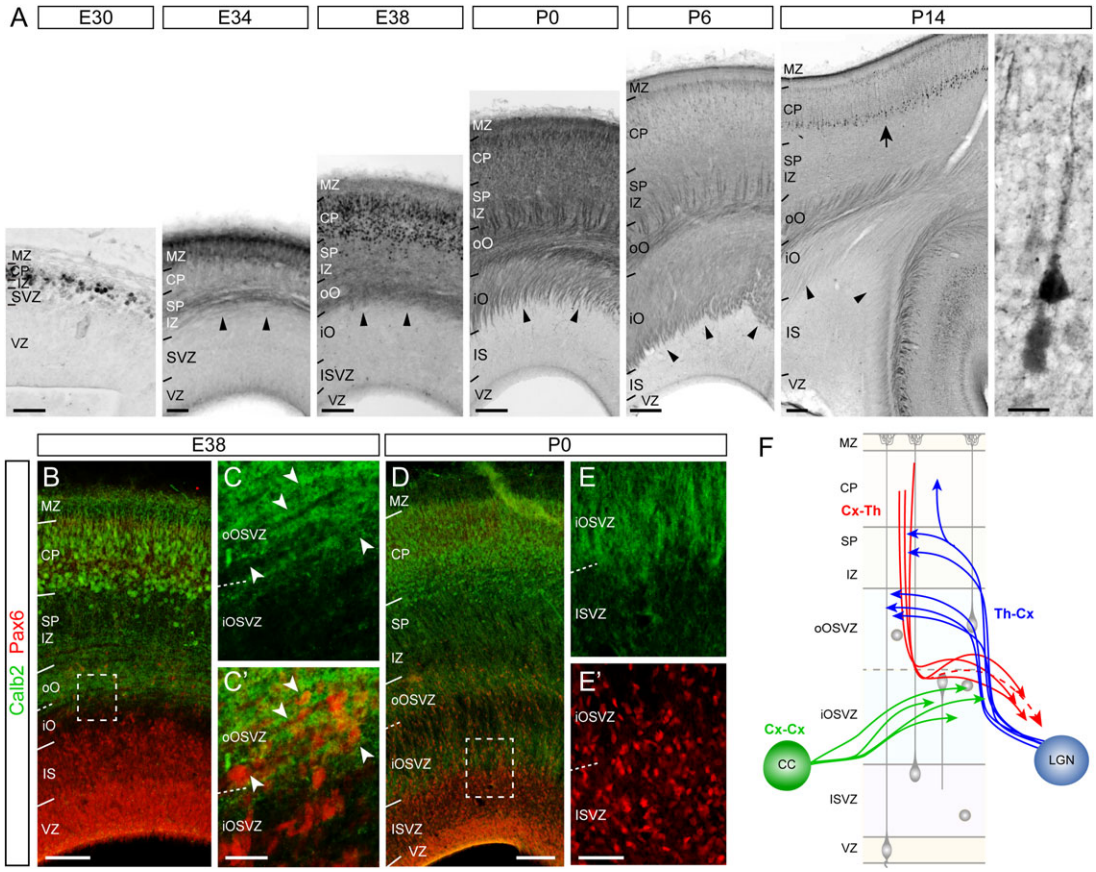

Figure 4. Calretinin distribution throughout development. (*A*) Immunostains against calretinin (Calb2) in the prospective visual cortex at the indicated ages. Calb2+ cells were present already at E30 in CP, and Calb2+ fibers were first seen at E34 in IZ/CP (black arrowheads). By E38 these populated the outer OSVZ, and starting at P0 they densely populate the entire OSVZ thickness (arrowheads). At P14 Calb2+ cells occupied layer 5 and displayed pyramidal morphology (black arrow and inset). Calb2+ cells and processes were never observed in VZ or ISVZ. (*B*) Bundles of Calb2+ fibers (green) populated the oOSVZ at E38, overlapping with Pax6+ progenitor cells (red). (*C*,*C'*) High magnification of the area boxed in (*B*) showing in detail the bundles of Calb2+ fibers (green) overlapping with Pax6+ progenitor cells (red) at the boundary between iOSVZ and oOSVZ. Arrowheads indicate Pax6+ cells surrounded by Calb2+ fibers. (*D*) At P0 Calb2+ fibers extended to the iOSVZ intermixing with Pax6+ progenitor cells. (*E*,*E'*) Detail of the bundles of Calb2+ fibers at the boundary between ISVZ and iOSVZ, as indicated by the boxed area in (*D*). (*F*) Summary diagram of the laminar distribution of the major axonal tracts. Scale bars: 50 μm (E30 and E34), 150 μm (E38 and P0), 300 μm (P14), 15 μm (P14 inset); 150 μm (*B*,*D*), 35 μm (*C*,*C'*), 50 μm (*E*,*E'*).

sets of axon fibers populate the OSVZ from early embryonic stages, and thus are in a position to directly influence progenitor cells in this layer via their cell body. In addition, because aRGCs in VZ and bRGCs in ISVZ and OSVZ extend long processes through these layers, extrinsic signals from these axons may also be sensed via the basal process (Fig. 4*F*).

Neuronal Cells and Processes Populate Germinal Layers from the Onset of Neurogenesis

The above analyses show that germinal layers of the developing cerebral cortex, particularly OSVZ, are densely populated by axon tracts from a variety of origins, which intermingle with progenitor cells. In addition to thalamic, callosal and cortico-fugal axon fibers, we also observed abundant Calretinin+ axon tracts from other, unidentified origins populating these germinal layers. To visualize all types of neuronal processes from any origin (axonal tracts, dendrites, migrating neurons) that overlap with cortical progenitor cells during development, we analyzed the patterns of distribution of Tau, TuJ1, MAP2, and Synaptophysin (Syp) proteins. Tau is a highly soluble microtubule-associated protein mostly found in the distal portions of axons but not present in dendrites. TuJ1 (βIII-tubulin) identifies the cytoskeleton of newborn and mature neurons. Microtubule-associated protein 2 (MAP2) is present specifically in the somatic and dendritic cytoskeleton of newborn and mature neurons (Li et al. 2010; Pinto et al. 2013). Synaptophysin (Syp) is a protein involved in synaptic transmission.

At the onset of cortical neurogenesis (E30, equivalent to mouse E12) we observed numerous Tau+, TuJ1+ and MAP2+ processes in the upper part of the cortex, including MZ, CP, and IZ, but not in VZ (Fig. 5*A*–*D*). Importantly, the incipient SVZ was also densely stained with Tau, TuJ1, and MAP2, at similar levels as the overlying postmitotic layers (Fig. 5*B*–*D*), indicating an important spatial overlap between neurons and progenitor cells in this basal layer, already at the earliest stage. At E34 the overall patterns of distribution of Tau, TuJ1, and MAP2 remained similar to E30, with the addition of a few scattered TuJ1+ processes and cell bodies in the VZ (Fig. 5*F*–*I*). TuJ1+ cells in the VZ adopted horizontal and oblique orientations, suggestive of tangentially migrating neurons as in mouse (Fig. 5*H'*) (Menezes and Luskin 1994). The intensity of MAP2 expression in the SVZ was higher at E34 than E30, representing the highest level across cortical layers except MZ (Fig. 4*I*). At this stage, similar to E30, Tau stain was densest in 2 bands, one in the lower part of the IZ and the other in the CP. At E38, the localization of these

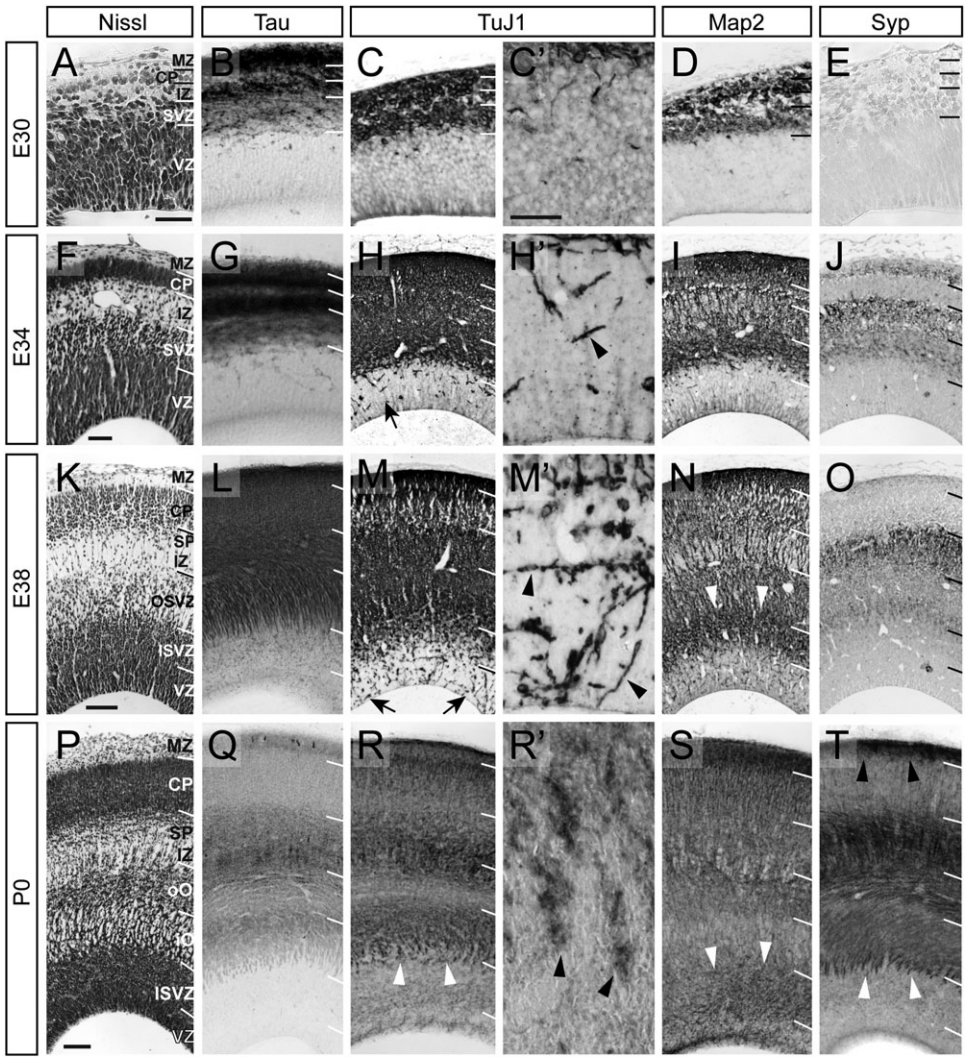

Figure 5. Ontogeny of distribution of neuronal cells and processes in the prospective ferret visual cortex. Labeling patterns after Nissl stain, Tau, TuJ1, MAP2 and Synaptophysin (Syp) stains in the prospective visual cortex at the embryonic (E) and postnatal (P) stages indicated. Nissl stains distinguish the different cellular layers across the cortical wall (*A*, *F*, *K*, and *P*). Tau is found in the distal portions of axons, but not dendrites (*B*, *G*, *L*, and *Q*). MAP2 is present specifically in the somatic and dendritic cytoskeleton of newborn and mature neurons (*D*, *I*, *N*, *S*). Syp is a protein involved in synaptic transmission (*E*, *J*, *O*, *T*). Images in (*C'*, *H'*, *M'*) are high magnifications from VZ in (*C*, *H*, *M*), and (*R'*) is a high magnification of the ISVZ/OSVZ boundary in (*R*). Black arrows in (*H*, *M*) and arrowheads in (*H'*, *M'*) indicate TuJ1+ cells and processes in the VZ. White arrowheads indicate the ISVZ/OSVZ border, where abrupt changes in TuJ1, MAP2 and Syp expression occur. High levels of Syp were also detected in the postnatal MZ (black arrowheads in T). ISVZ, inner subventricular zone; iO/oO, inner/outer OSVZ; SVZ, subventricular zone. Scale bars: 50 μm (E30 and E34), 150 μm (E38 and P0), 25 μm (*C'*–*R'*).

proteins remained similar to E34 (Fig. 5*K*–*N*). Concomitant with the subdivision of the SVZ, TuJ1 stain density decreased significantly in ISVZ while remaining strong in OSVZ (Fig. 5*M*,*M'*). MAP2, in contrast, was stronger in ISVZ (Fig. 5*N*). In VZ the abundance of randomly oriented TuJ1+ and MAP2+ cells increased further compared to E34 (Fig. 5*M*,*N*). Altogether, these results demonstrate the physical proximity between different parts of differentiating neurons and progenitor cells during cortical development.

Next we asked at which point in development cortical progenitors may start being influenced by these neurons and axonal projections. Neurotransmitters have been shown to directly modulate cortical progenitor proliferation, and these are usually secreted via synaptic vesicles, so next we investigated the expression of Synaptophysin (Syp), an essential component of the pre-synaptic machinery that has been previously used as indicator of synapse development (Li et al. 2010; Pinto et al. 2013). We detected Syp only starting at E34, primarily in the SVZ, IZ, and MZ, but not in VZ (Fig. 5*E*,*J*). The fact that Syp was not detected within the CP at this stage suggests that either Syp+ processes do not originate in CP neurons at this stage, or this protein is differentially distributed between axon and soma in these neurons. At E38 (onset of neurogenesis for layer 4) (Jackson et al. 1989) with the splitting of SVZ, Syp labeling was evident in OSVZ but quite low ISVZ, and continued undetectable in VZ (Fig. 5*O*).

To determine if the presence of neuronal cells and processes in germinal layers was transient embryonically or it persisted postnatally, we analyzed 3 postnatal stages, spanning through the completion of cortical neurogenesis. At P0 (onset of neurogenesis for layer 2/3), ISVZ and oOSVZ exhibited a relatively low density of Tau and TuJ1+ processes and, reciprocally, a high density of MAP2+ processes (Fig. 5*P*–*S*). Of note, this was the earliest stage when the VZ was populated by a significant

density of TuJ1+ and Map2+ cells and processes (Fig. 5R,S). Conversely, iOSVZ displayed high levels of TuJ1 and Tau stain and relatively low levels of MAP2. Because MAP2 is exclusive to cell somas and dendrites, we interpreted the MAP2+ processes in VZ and ISVZ as corresponding to the leading process of migrating neurons, which have not yet grown a Tau+ axon. Likewise, we interpreted the high density of Tau+ and TuJ1+ processes in iOSVZ, combined with low levels of MAP2, as indicative of a high density of axon bundles with few neuronal cell bodies or dendrites (Fig. 5Q–R'). Syp expression was remarkably similar to Tau and TuJ1 across all germinal layers, including the thick bundles in iOSVZ, which was consistent with a high density of axon bundles but few neuronal cell bodies or dendrites (Fig. 5T).

Between P6 and P14 (equivalent to mouse E16.5 to E18.5), corresponding to the last stages of cortical neurogenesis (Jackson et al. 1989), the patterns of TuJ1 and MAP2 distribution remained similar to P0, with a gradual increase in relative TuJ1 content in ISVZ and oOSVZ, and concomitant reduction of MAP2 in all germinal layers (Fig. 6 and Supplementary Fig. 1). Syp levels also showed a remarkable transient reduction at P6 across all cortical layers except the MZ, which by P14 were high again in all neuronal layers and very high in MZ, but not in the germinal layers (Supplementary Fig. 1).

Migrating and Differentiating Neurons Closely Intermingle with Progenitor Cells

The above observations suggested the possibility that various progenitor cell populations may interact with, or receive the influence of, axonal tracts and migrating neurons. To demonstrate the physical proximity between progenitor cells and neurons, we performed double immunostains for the pan-neuronal markers TuJ1 and MAP2, and the transcription factors Pax6 and Tbr2, which at early stages identify RGCs and Intermediate Progenitor Cells (IPCs), respectively (Englund et al. 2005; Kowalczyk et al. 2009; Reillo et al. 2011; Reillo and Borrell 2012; Betizeau et al. 2013). At E30, when layer 5 neurons are being generated but still most RGCs self-amplify, Pax6+ cells in the VZ were virtually isolated from postmitotic neurons, but in contrast TuJ1+ processes and MAP2+ cells overlapped closely with Tbr2+ cells and some cells weakly labeled for Pax6+ in the SVZ (Fig. 7A–C, Supplementary Fig. 2). Between E34 and E38, Pax6+ cells in VZ intermingled with an increasing number of TuJ1+ and MAP2+ processes and cells with migratory morphology, although these were most abundant around Tbr2+ cells in ISVZ (Fig. 7B,C and Supplementary Fig. 2). The substantial overlap of Pax6+ or Tbr2+ cells with neuronal cells and processes persisted postnatally (not shown). These results demonstrated that neurons and neuronal processes are

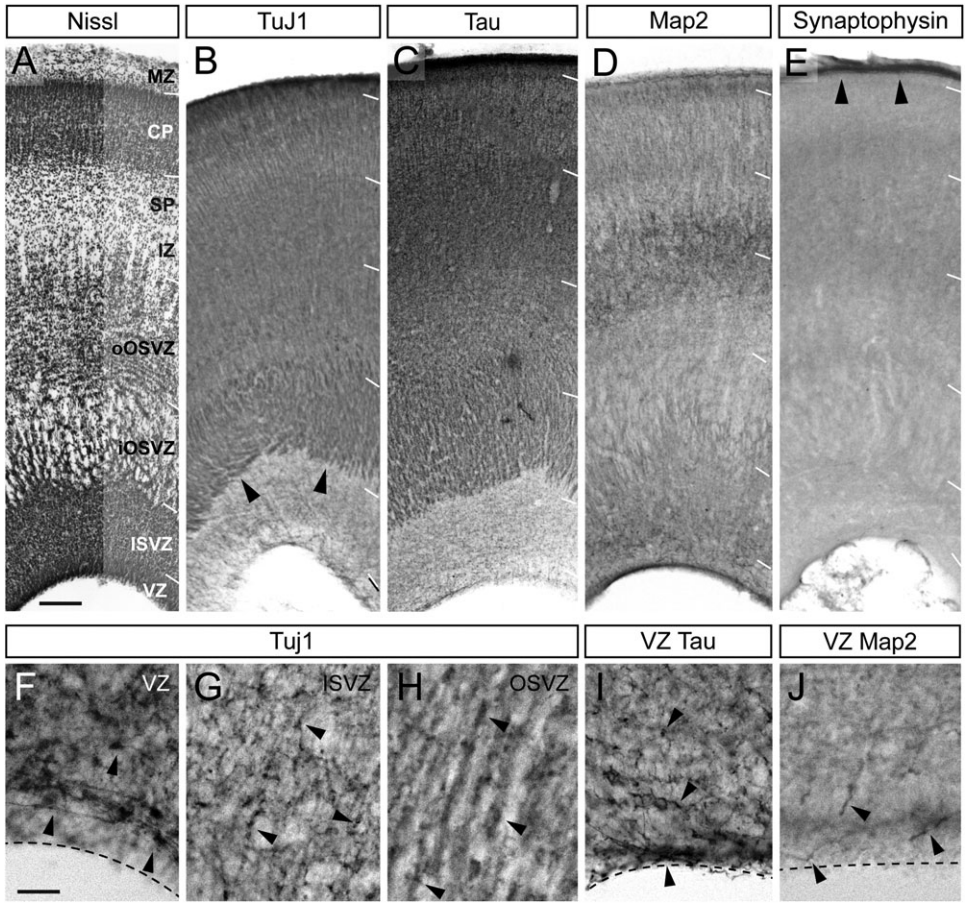

Figure 6. Distribution of neuronal cells and processes in the prospective ferret visual cortex at P6. (*A–E*) Labeling patterns for the indicated stains. The patterns of TuJ1 (*B,F,G,H*), Tau (*C,I*) and MAP2 (*D,J*) distribution remained similar to P0, with a gradual increase in relative TuJ1 content in ISVZ and oOSVZ (*B,F,G,H*) and concomitant reduction of MAP2 in all germinal layers. Syp levels showed reduction at P6 across all cortical layers except the MZ (*E*). (*F–J*) High-magnification images of the indicated stains and germinal layers. Arrowheads indicate positive cells or processes, in each case. iO/oO, inner/outer OSVZ. Scale bars: 200 μm (*A–E*), 25 μm (*F–J*).

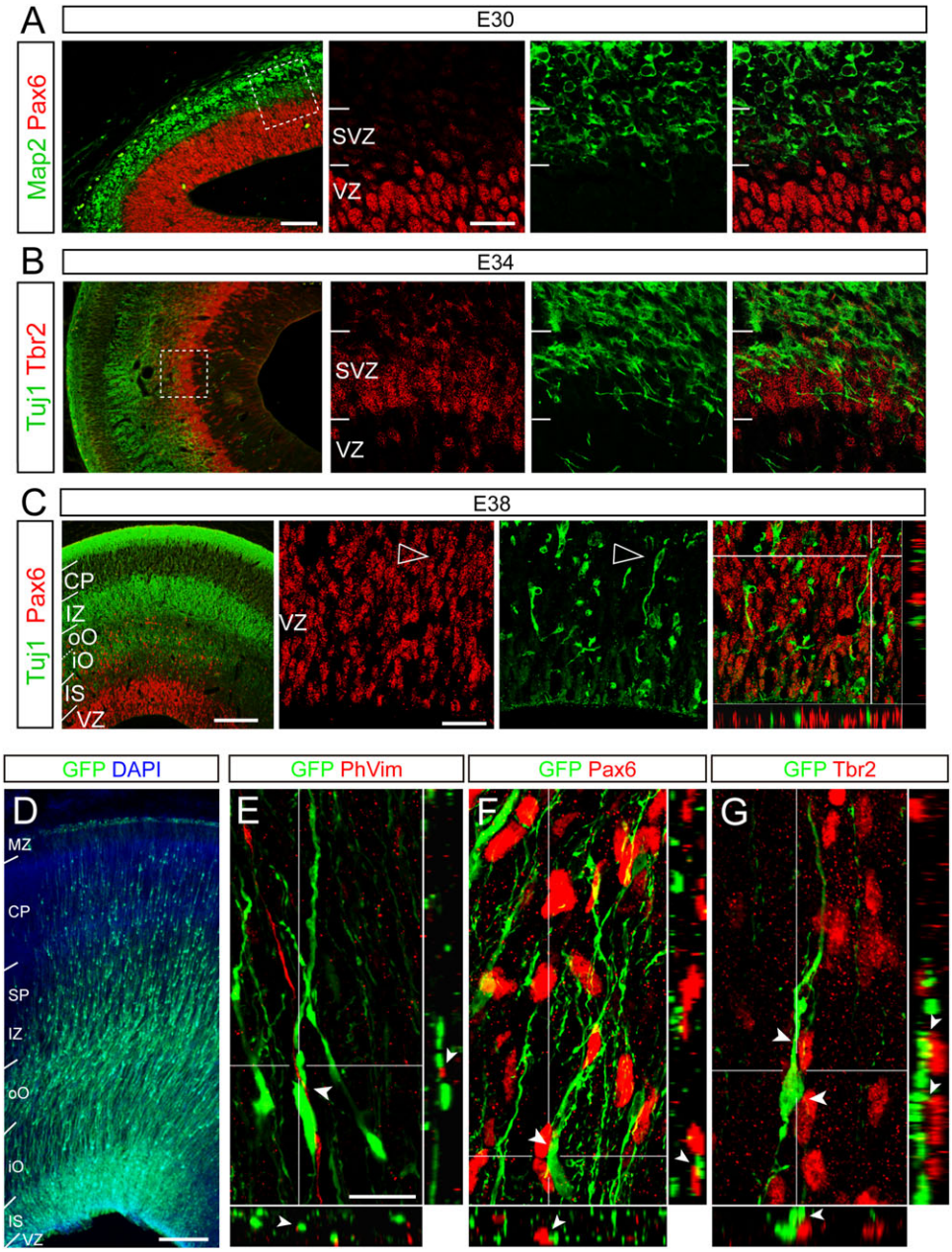

Figure 7. Immature and migrating neurons populate germinal layers in the developing ferret cortex. (*A–C*) Patterns of immunostain for the marker combinations and developmental stages indicated. At E30 (*A*), MAP2-expressing cells and processes populate the SVZ but do not intermix with Pax6+ cells in VZ. At E34 (*B*), a similar pattern of expression is shown for TuJ1, but now with some neuronal processes and cells in VZ, and many in SVZ overlapping with Tbr2+ progenitors. At E38 (*C*), neuronal processes and migrating neurons (TuJ1+) are present in all germinal layers, including ISVZ and VZ. The orthogonal projection of a Tuj1+ cell (open arrowheads) surrounded by Pax6+ nuclei is shown. (*D*) Migrating excitatory neurons labeled with GFP at P8 upon electroporation at P1. (*E–G*) High magnifications of GFP-labeled migrating neurons in OSVZ co-stained with antibodies against phosphovimentin, Pax6 and Tbr2, as indicated. Shown are confocal images, and orthogonal projections of z-stacks at the level indicated in each case. Arrowheads indicate the leading process of migrating neurons, in very close proximity with progenitor cells as revealed by the double stains. Scale bars: 100 μm (*A*, *B*), 300 μm (*C*), 50 μm (magnifications in *A–C*), 200 μm (*D*), 15 μm (*E–G*).

densely intermingled with the different progenitor cell populations throughout cortical development.

Cortical excitatory neurons migrate radially from the germinal layers to the CP following the fiber scaffold of RGCs (Rakic 1972; Sidman and Rakic 1973; Marin and Rubenstein 2003; Lukaszewicz et al. 2005; Borrell 2010; Hansen et al. 2010; Reillo et al. 2011). During this process, migrating neurons are in intimate apposition to RGCs and IPCs (Rakic 1972; Sidman and Rakic 1973), and hence they may influence the proliferation of these progenitors via diverse mechanisms, including glutamate release (LoTurco et al. 1995). To verify the extent of this overlap in the developing cortex in vivo, we labeled migrating neurons by electroporation of GFP-encoding plasmids into the cortical VZ of newborn (P1) ferret kits. As expected, one week later (P8) GFP+ neurons were migrating radially through ISVZ and OSVZ (Fig. 7*D*). Careful examination showed that GFP+ radially migrating neurons and their leading process were frequently apposed onto, and aligned with, the basal process of bRGCs in

OSVZ, as identified with PhVim antibodies (Fig. 7E). Additional marker analyses demonstrated the similarly close overlap of migrating neurons with Pax6+ and Tbr2+ cells in ISVZ and OSVZ (Fig. 7F,G), markers of bRGCs and IPCs.

Taken together, our analyses demonstrate that, from early embryonic stages, a large amount and variety of developing neuronal cells and processes overlap extensively and intimately with cortical progenitor cells in multiple germinal layers, especially the basal process of aRGCs and bRGCs. Importantly, the germinal layers involved, and the types of neurons and axon tracts overlapping with them, vary significantly and in a highly dynamic manner during development.

Inhibitory Neurons Migrate Along Specific Germinal Layers in the Cortex

In addition to excitatory projection neurons, cortical inhibitory interneurons have also been proposed to potentially influence progenitor cells. It has been shown that the inhibitory neurotransmitter GABA, secreted by these cells, is a potent modulator of various developmental processes, including cell migration and progenitor proliferation (Blanton et al. 1990; LoTurco et al. 1995; Marin and Rubenstein 2001; Owens and Kriegstein 2002; Tiveron et al. 2006). So next we investigated the timing and distribution of this specific population of cortical neurons, and their potential overlap with cortical progenitor cell subpopulations.

In mice and ferrets cortical inhibitory interneurons are born in the MGE and migrate tangentially from there along the cortical SVZ, until they switch to radial migration and finally populate the CP (Anderson et al. 1997, 2002). In order to elucidate to which extent tangentially migrating interneurons populate the different cortical germinal layers in ferret, we studied their distribution in the developing cortex using anti-GABA stains. We observed a large amount of GABA+ cells throughout the cortical thickness between E34 and P0, which had a typical morphology of migrating cells (Fig. 8A–G) but their laminar distribution changed significantly between stages. At E34, GABA+ cells were particularly dense in SVZ and MZ, less so in VZ, IZ, and SP, and virtually absent in CP (Fig. 8A). In contrast, at P0 GABA+ cells populated most abundantly the MZ, then the CP and oOSVZ, and much less the iOSVZ, ISVZ, and VZ (Fig. 8B–G). This might reflect that interneurons had been migrating mostly tangentially at E34, and many had switched to radial migration by P0.

Importantly, the abundant stream of GABA+ cells observed in the MZ suggested that these cells may have a strong influence on aRGCs and bRGCs via the distal branches and endfeet of their basal process, which form within this layer (Fig. 8C–E). Indeed, it has been previously suggested that extrinsic signals may be sensed through this distal portion of the basal process of RGCs, including for example the secreted glycoprotein Reelin (Hartfuss et al. 2003) or other factors in MZ derived from Cajal-Retzius cells (Super et al. 2000; Griveau et al. 2010). In addition to Cajal-Retzius, located in MZ from the onset of cortical development (Marin-Padilla 1998; Soriano and Del Rio 2005; Borrell and Marin 2006), and GABA+ cells migrating along the MZ, other sources of distal signals onto RGCs may come from blood vessels. The basal fiber of RGCs may occasionally not reach the pial surface, but terminate short onto blood vessels. In these cases, influences from the MZ and other superficial layers are lost, and instead RGCs may gain the influence from those vessels (Takahashi et al. 1990; Javaherian and Kriegstein 2009; Stubbs et al. 2009; Errede et al. 2014; Tan et al. 2016). We have analyzed the presence and distribution of blood capillaries in the developing ferret cortex, and found these to be abundant in the cortical germinal layers during the entire embryonic and early postnatal neurogenetic period, being at a particularly high density in VZ and ISVZ (Supplementary Fig. 3).

By the end of neurogenesis (P6), the germinal layers were densely populated by GABA+ processes (Fig. 8H). This made it very difficult to determine if individual cells continued to migrate and intermingle with progenitor cells, and which layers they might prefer for tangential versus radial migration. To overcome this limitation, we labeled the lineage of MGE progenitor cells postnatally by injecting GFP-encoding retroviruses (rv::*Gfp*) locally in the MGE of newborn ferrets (Anderson et al. 2002), and analyzed the distribution of GFP-expressing cells in the cortex at later stages. After 2 days of survival, we confirmed that our injections were local and specific to the MGE, labeling cells with the typical appearance of migrating interneurons (Fig. 8I,K). Eight days after rv::*Gfp* injection, we found a large number of GFP+ cells distributed along the entire rostro-caudal and latero-medial extent of the cerebral cortex. The majority were found in ISVZ, plus some in VZ, but they remained specifically absent from OSVZ (Fig. 8J,L).

Our observations in the E34 ferret were in agreement with previous descriptions in mouse, where most interneurons migrate tangentially along the SVZ and MZ (Marin and Rubenstein 2001; Lopez-Bendito et al. 2008). However, at later stages, with the distinction of the OSVZ, ferret interneurons did not overlap with it, hence bypassing a substantial part of basal progenitors. Altogether, our analysis of migrating neurons demonstrates that both excitatory neurons migrating radially, and GABA+ inhibitory neurons migrating tangentially, overlap extensively with cortical progenitor cells, but in specific spatial and temporal patterns. Specifically, radially migrating neurons are in intimate proximity with the basal process of RGCs, potentially from aRGCs in VZ and bRGCs in ISVZ/OSVZ. In contrast, migrating GABA+ interneurons overlap abundantly with VZ and ISVZ during tangential migration, while specifically avoiding the OSVZ until they migrate radially (Fig. 8M).

Diverse Populations of Interneurons Intermingle with Cortical Progenitors

The mature cerebral cortex contains a remarkable diversity of inhibitory interneurons (DeFelipe et al. 2013), some of which start to differentiate early in development. One of the most abundant groups expresses the calcium-binding protein Calbindin (CalB), so we next studied the temporal and spatial distribution of CalB+ cells and processes in the developing ferret cerebral cortex. CalB was first detected at E38 and P0, labeling a high amount of axonal fibers that crossed the oOSVZ and IZ (Fig. 9A). At E38, CalB+ cell bodies were frequent in ISVZ, iOSVZ, upper CP, and MZ, but not in oOSVZ or IZ (Fig. 9A,C). Four days later, at P0, CalB+ cells were markedly more abundant than before and their distribution was more homogeneous across layers, although with a clear tendency to accumulate in ISVZ, upper CP and MZ (Fig. 9A,D). At P6 the density of CalB+ cells decreased compared to previous stages, while CalB+ processes became more abundant in MZ, lower CP and, prominently, in the outer aspect of ISVZ (Fig. 9A,E,F). At P14 the density of CalB+ cells and processes increased significantly in all layers including ISVZ and OSVZ, but not in VZ which remained largely free. This increase was particularly prominent in oOSVZ and IZ/SP, where cells displayed the irregular multipolar morphologies typical of mature SP neurons (Fig. 9A,B) (Valverde and Facal-Valverde 1988; Allendoerfer and Shatz 1994), and also in the upper ISVZ where the band of CalB+ fibers became quite conspicuous (Fig. 9A,G).

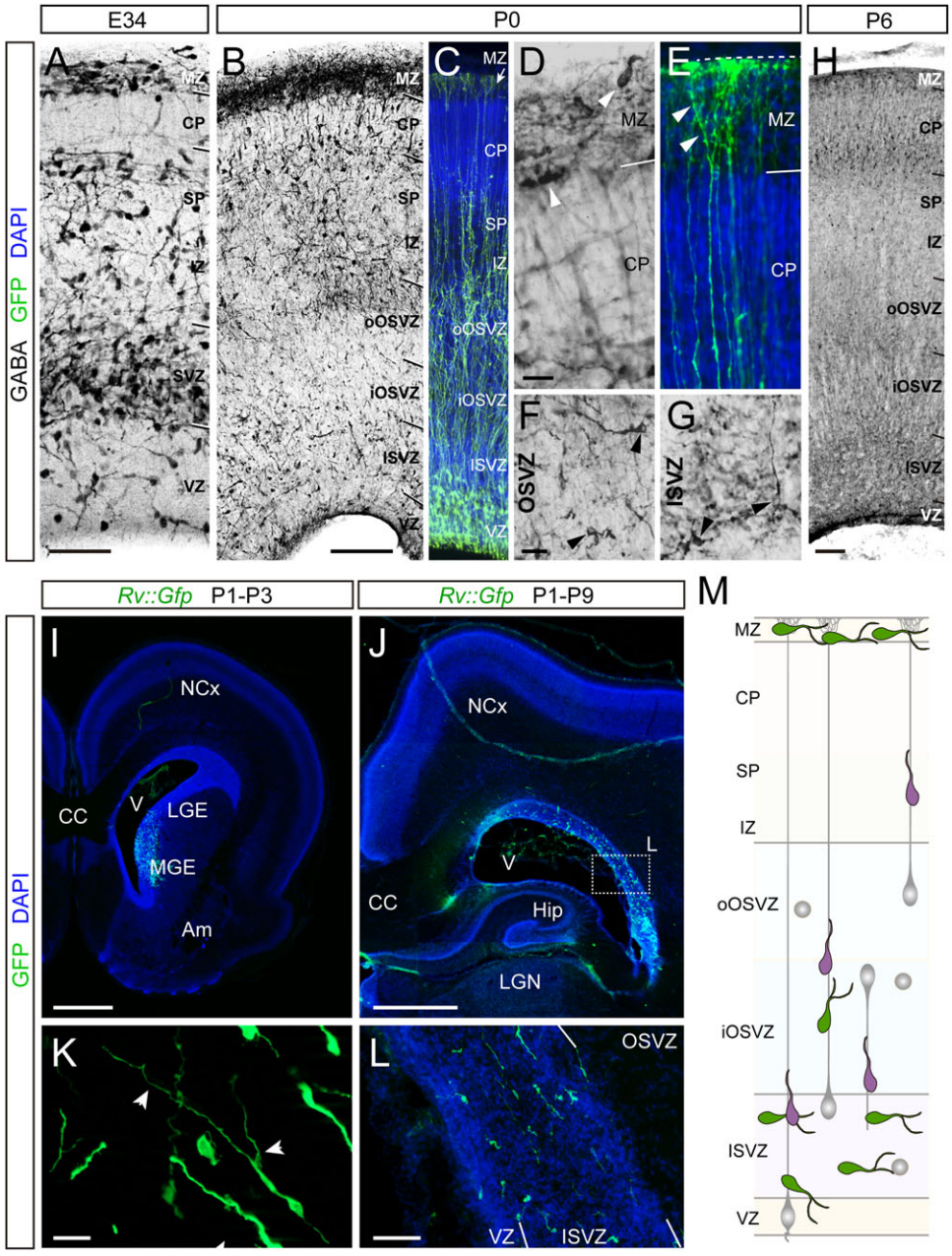

Figure 8. Inhibitory neurons migrate along cortical germinal layers in specific spatial and temporal patterns. (*A–H*) Patterns of GABA staining at E34 (*A*), P0 (*B,D,F,G*) and P6 (*H*), and GFP labeling of RGCs at P1 (*C*, *E*). The migratory morphology of GABA+ cells is seen at high magnification (*D,F,G*; arrowheads) in the indicated layers at P0. The basal process of RGCs arborizes profusely within the MZ (*E*, arrowheads) extensively overlapping with the MZ stream of migrating GABA+ cells (*D*, arrowheads). (*I–L*) Patterns of distribution and morphology of GFP+ cortical interneurons labeled upon rv::*Gfp* injection in MGE at P1 and analysis at P3 (*I*, *K*) or P9 (*J*,*L*). Boxed area in (*J*) indicates the region shown in (*L*). Most cells exiting the GE at P3 displayed a bifurcated leading process, typical of migrating interneurons (*K*, arrowheads). (*M*) Summary schema of the laminar location of migrating interneurons in the developing ferret cortex. Am, amygdala; LGE, lateral ganglionic eminence; NCx, neocortex; V, ventricle. Scale bars: 50 μm (*A*,*L*), 150 μm (*B*,*C*), 20 μm (*D–G*), 100 μm (*H*), 1 mm (*I*), 2 mm (*J*), 15 μm (*K*).

It has been described in mice and primates, as well as other species, that in the developing cerebral cortex CalB is expressed by populations of cells that are not inhibitory interneurons, including pyramidal neurons (Del Rio et al. 1996; Hof et al. 1999). To better characterize CalB+ cells and processes in the developing neocortex, we co-stained for the inhibitory neurotransmitter γ-aminobutyric acid (GABA) at P0 and P6, the earliest stages with high abundance of both markers. At both stages, we found cells co-expressing GABA and CalB across the cortical thickness (Fig. 9*H–N'*, solid arrowheads). However, we also found a proportion of CalB+ processes located in ISVZ, OSVZ, and MZ that did not co-express GABA (Fig. 9*I–N'*, open arrowheads). This indicated that the developing ferret cerebral cortex contains 2 distinct populations of CalB+ cells, some GABA+ and others GABA−. Taken together, our data demonstrated the presence of multiple populations of neurons distributed differently across the cortical thickness, including germinal layers, where the ISVZ was densely populated by CalB+ processes and the OSVZ was packed with Calb2+ fiber bundles.

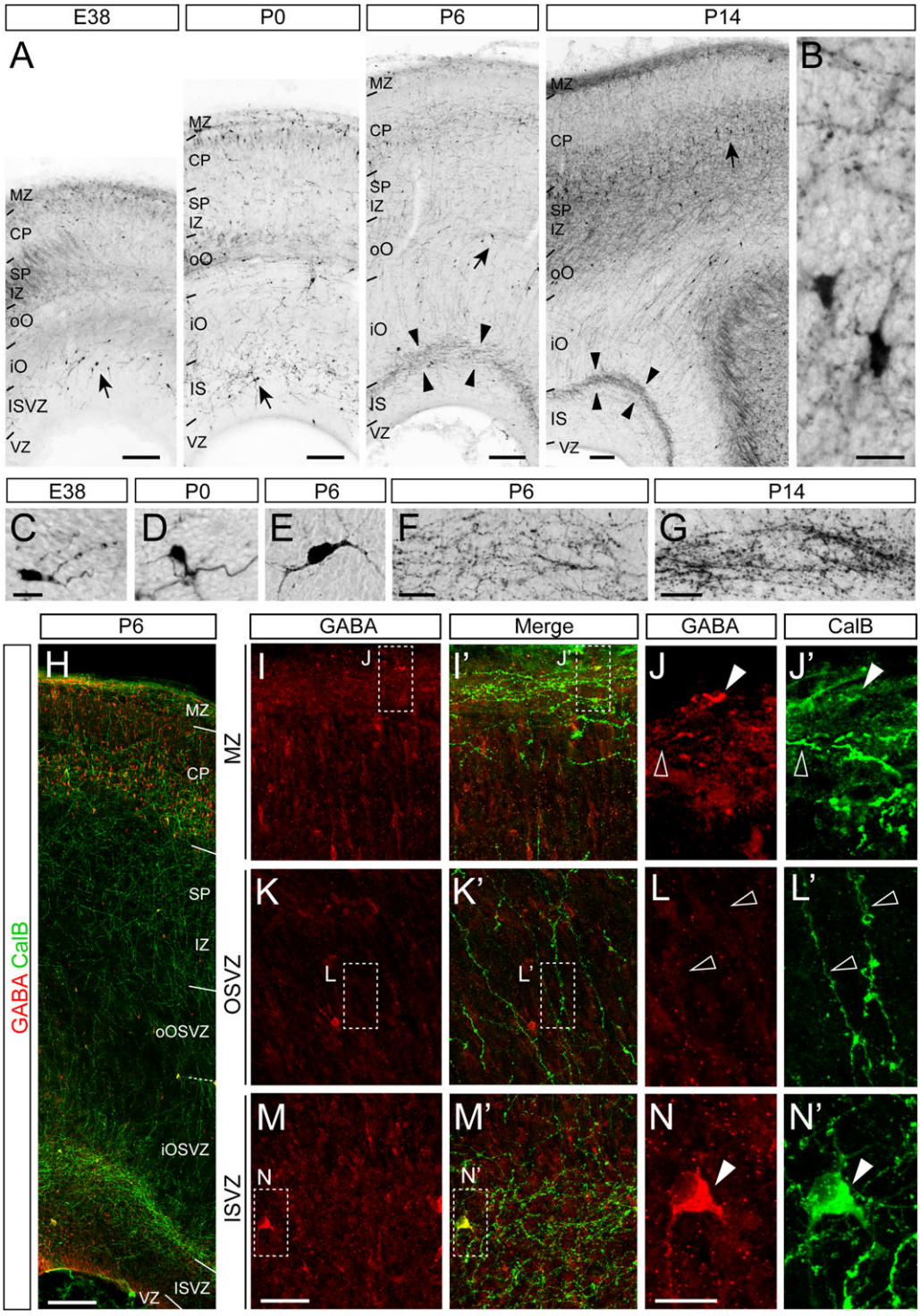

Figure 9. Expression pattern of CalB during development and differential expression of GABA in subsets of CalB+ cells. Views of CalB immunostains at the indicated stages (*A*) and corresponding high magnifications from CP (*B*), ISVZ (*C*,*D*), OSVZ (*E*) and top of ISVZ (*F*,*G*). Black arrows point at CalB+ cell somas. Black arrowheads indicate CalB+ cell processes. (*H*–*N'*) Co-stains of GABA and CalB at P6, showing overlapped distribution of labeled cells and processes across the cortical thickness. (*I*–*N'*) Details of the indicated layers, showing overlapped expression of GABA and CalB. Boxed areas are shown in higher magnification, demonstrating some CalB+ processes negative for GABA (*L*, *L'*; open arrowheads), as well as a CalB+ cell soma and some processes positive for GABA (solid arrowheads in *J*, *J'*, *N*, *N'*). High-magnification images are single confocal planes. Scale bars: 150 μm (E38 and P0), 200 μm (*P6*, *H*), 10 μm (*C*–*F*), 300 μm (P14), 15 μm (*B*), 10 μm (*L*–*O*), 50 μm (*G*), 50 μm (*I*, *I'*, *K*, *K'*, *M*, *M'*), 20 μm (*J*, *J'*, *L*, *L'*, *N*, *N'*).

Next, we sought to determine if there was some specificity or developmental variation in the populations of GABA+ cells overlapping with cortical germinal cells, beyond those expressing CalB. To cover the majority of cortical interneuron subclasses we immunostained against markers of the major interneuron populations: Parvalbumin (Parv), Somatostatin

(Sst) and Neuropeptide Y (NPY) (Petilla Interneuron Nomenclature Group et al. 2008; DeFelipe et al. 2013). Parv was only detected in the cerebral cortex after P14 and never in germinal layers (not shown), consistent with the late expression of this protein in differentiating cortical interneurons in rodents (Alcantara et al. 1996). In contrast, cells positive for Sst and NPY were detected starting at P0, although at this age only very few and scattered cells were found in CP (Sst) and IZ (NPY) (data not shown). The abundance of Sst+ and NPY+ cells increased by P6, when Sst+ cells were frequent in CP and NPY+ cells were abundant in IZ/SP (Supplementary Fig. 4*A*,*B*). Importantly, at P6 we also observed a small number of cells and processes positive for Sst and NPY in the ISVZ and OSVZ, which persisted until P14 (Supplementary Fig. 4*E*–*J*). Stains for Sst and NPY also labeled processes filled with varicosities and that were tangentially oriented along the MZ (Supplementary Fig. 4*C*,*D*), consistent with the incipient axonal arborizations of Martinotti cells in the prospective layer 1 (Marin and Rubenstein 2001; Wang et al. 2004; Tanaka and Nakajima 2012). These results show that interneurons positive for Sst and NPY send axons through the germinal layers, hence being in a position to exert a paracrine influence onto progenitor cells. Altogether, our analysis of interneuron populations indicates that, at least in gyrencephalic species, several of these may be also a relevant source of extrinsic influences, both in their immature state during tangential migration, and during differentiation via their dendritic and axonal arborizations (Supplementary Fig. 4*K*).

Influence of Developing Axons on Cortical Progenitor Cell Biology

Our above analyses indicated that subsets of axons in the developing cortex are in a position to influence progenitor cells. Next, we investigated in some detail the nature of the cellular interaction between axons and progenitor cells. We focused on the CalB+ axons decorated with varicosities that we observed densely populating the outer portion of the ISVZ at P6 and P14 (Fig. 9*A*,*F*,*G*). To visualize the potential contact between CalB+ axons and cortical progenitors, we labeled progenitor cells by electroporation of GFP-encoding plasmids in the cortical VZ at P1, and examined GFP+ cells at P6 (Fig. 10*A*). We found that CalB+ axons in ISVZ closely overlapped with GFP+ basal fibers of RGCs (Fig. 10*B*,*C*). Three-dimensional reconstruction of high-magnification confocal images revealed the very intimate apposition of CalB+ axons and their varicosities onto the shaft of RGC basal processes (Fig. 10*D*).

Next we sought to gain insight into the molecular nature of these contact points. The gap junction protein Connexin 43 (Cx43) has been shown to concentrate in RGC varicosities contacting blood vessels in the human cortex (Errede et al. 2014), and it is involved in RGC-mediated neuronal migration in mouse (Elias et al. 2007). In the developing ferret cortex, we found abundant Cx43 puncta along the basal process of RGCs, particularly within varicosities (Fig. 10*E*–*G*), strongly suggesting their potential implication in the communication between RGCs and migrating neurons, axons or blood vessels.

In order to determine whether some of the axon tracts that we identified in the developing ferret cortex may indeed exert a significant influence on the behavior or fate of cortical progenitor cells, we next performed binocular enucleation in newborn ferrets. This manipulation is known to impair the development of the LGN (Williams et al. 2002) and, as a result, to impair the thalamic projection to the primary visual cortex (Fig. 3) (Reillo et al. 2011). We performed identical manipulations in newborn ferret kits, and then analyzed at P6 the abundance of GFAP+ astrocytes. We have previously shown that P6 is the age at which GFAP+ astrocytes become first detectable in the ferret visual cortex under normal development (Reillo and Borrell 2012). We found that upon binocular enucleation at P1, the abundance of GFAP+ astrocytes at P6 was 2-fold greater than in control kits (Fig. 10*F*,*G*). Intriguingly, this enhancement of astrocytogenesis was specific to the ISVZ, whereas the OSVZ remained unaffected. This demonstrated that the LGN thalamo-cortical axons running through the OSVZ (Fig. 2) exert a selective influence on the fate of ISVZ but not OSVZ progenitors.

Discussion

In this study we have identified multiple populations of axonal fibers and neurons, both immature and differentiated, that closely overlap with several populations of cortical progenitor cells throughout embryonic and postnatal development of the gyrencephalic ferret. Axonal projections from distinct sources, including thalamo-cortical, callosal and cortico-fugal, populate very specific subdivisions of the germinal layers, particularly OSVZ but also ISVZ. Similarly, inhibitory interneurons migrate tangentially along specific subdivisions of these germinal layers. Because progenitor cells extend processes outside the layer of residence of their cell body, they may come in close proximity with a combination of several of these neuronal elements. We propose that the exposure of each specific progenitor cell subtype to a different combination of layer-specific elements defines a complex code of potential extrinsic influences onto these cortical progenitor cell populations (Fig. 11). Remarkably, the presence and precise laminar position of all these neuronal elements changes very dynamically during development, from early to late stages, so we propose that this code of extrinsic influences is highly dynamic in nature (Fig. 11*A*).

Relevance of Progenitor Cell Morphology

Progenitor cells in the developing mammalian cerebral cortex come in a variety of morphologies (Fig. 11). Previous studies show that the basal process of RGCs is highly sensitive to extrinsic stimuli (Weissman et al. 2004), and that internalized signals such as calcium level oscillations travel along this basal process and regulate neurogenesis (Rash et al. 2016). We and others have shown that the basal process of RGCs is abundantly decorated with varicosities and short lamellate expansions (Fig. 1) (Rakic 1972; Sidman and Rakic 1973). This suggests the possibility that these subcellular specializations may serve progenitor cells to detect extrinsic signals (Errede et al. 2014), in a similar way as dendritic spines are specialized to detect neurotransmitters released from synaptic vesicles. Once these signals are detected, second messengers of the signaling cascade may travel along the radial process down to the cell nucleus, to modify gene expression and/or regulate cell proliferation and neurogenesis (Rash et al. 2016).

We have shown that migrating neurons and axonal tracts overlap with cortical progenitor cells at 2 levels: cell somas and radial processes. Different progenitor cell types extend apical and/or basal processes (or none) and have the cell body in different layers, so their cellular processes (basal or apical) extend through different sets of layers in the developing cortex. Consequently, the sources of extrinsic signals that may potentially influence cortical progenitor cells vary completely

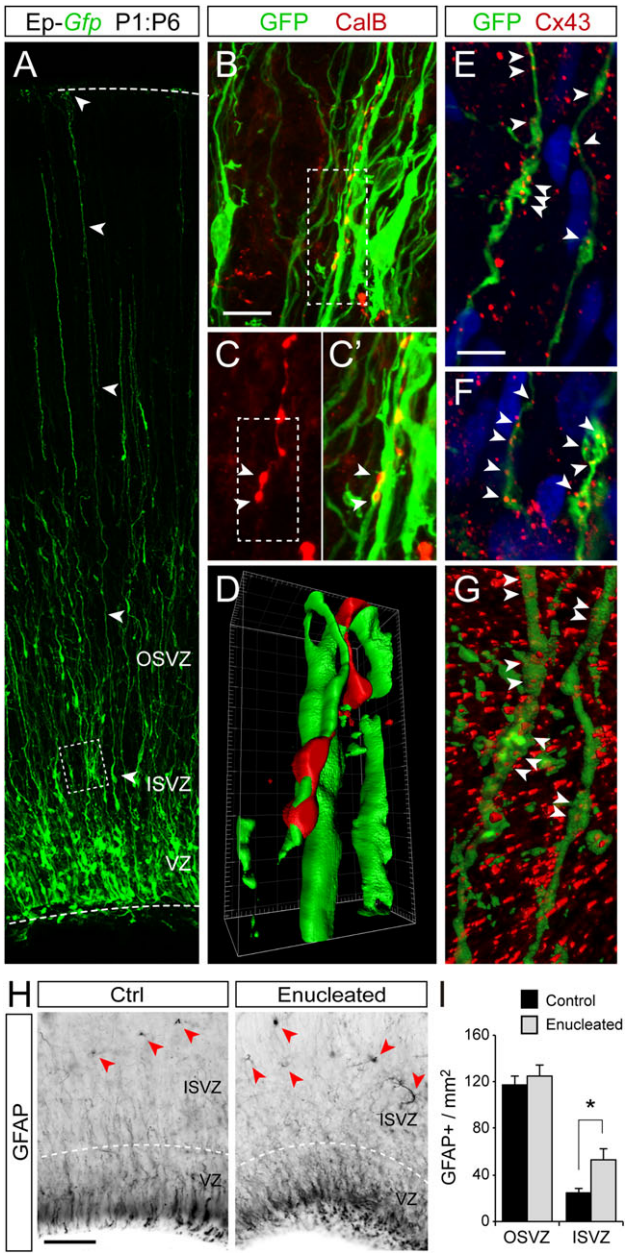

Figure 10. Interaction between axons and RGCs in the developing ferret cerebral cortex. (*A–D*) GFP labeling of RGCs (green) at P6 after electroporation at P1, co-stained for CalB (red). Low magnification in (*A*) shows the basal fibers of RGCs (arrowheads) from the cell body in VZ/ISVZ to the surface of the cortex. Dashed lines indicate the ventricular and pial surfaces. Dashed boxes in (*A*), (*B*) and (*C*) indicate the areas shown in (*B*), (*C*) and (*D*), respectively. Arrowheads in (*C*) indicate 2 varicosities of a CalB+ axon (red). Images in (*D*) show a single confocal plane and orthogonal views of the. Image in (*E*) is a surfaced 3-D reconstruction of a z-stack of confocal images, rotated to demonstrate the intimate association between the radial fiber of a GFP + RGC and the CalB+ axon and varicosities. (*E–G*) GFP labeling of RGCs (green) at P3 after electroporation at P1, co-stained for Cx43 (red) and DAPI (blue). Images in (*E*,*F*) are single confocal planes, demonstrating the presence of Cx43+ puncta (arrowheads) along the RGC basal fiber shaft and varicosities. Image in (*G*) is a surfaced 3-D reconstruction of a z-stack of confocal images as in (*E*), rotated to demonstrate numerous Cx43+ puncta on the surface of GFP + RGC fibers. (*H*,*I*) Representative images of GFAP stains in the visual cortex VZ and ISVZ at P6, from a ferret enucleated binocularly at P1 and control littermate (*F*), and quantification (*G*). The density of GFAP+ astrocytes (red arrowheads) was significantly higher in ISVZ of enucleated animals than controls, but similar in OSVZ. Data are mean + S.E.M.; $n = 3$ animals per group; *t*-test, $^{*}p < 0.05$. Scale bars: 10 μm (*B*), 100 μm (*F*).

depending on these factors (Fig. 11*B*). Similar to the specific targeting of inhibitory synapses onto subcellular compartments of the recipient neuron (soma, dendrites or axon initial segment) (Petilla Interneuron Nomenclature Group et al. 2008), the laminar overlap between migrating neurons and axons with progenitor cell bodies and radial processes will define the specific targeting of the former onto the latter.

IPCs only extend short processes (Noctor et al. 2004; Betizeau et al. 2013), so they may be sensitive only to signals from within their layer or sublayer of residence. Basal RGCs may receive influences from their layer of residence plus any layer crossed by their basal fiber; and aRGCs may potentially receive information from all cortical layers via their apical and basal process, which span from the ventricular through the pial surfaces. Occasionally, the basal fiber of RGCs may not reach the pial surface but terminate short onto blood vessels. In these cases, influences from axons running along the MZ and other superficial layers are lost, and these cells may now be particularly influenced by blood vessels (Takahashi et al. 1990; Javaherian and Kriegstein 2009; Stubbs et al. 2009; Tan et al. 2016), for example via Cx43 channels (Errede et al. 2014). In addition to the constraints imposed by cell morphology and position, the response of progenitor cell subtypes to extrinsic cues is likely to depend on the expression of specific receptors to extracellular factors and cell-adhesion molecules (Dehay et al. 2001; Dehay and Kennedy 2007; Fietz et al. 2010, 2012; Ayoub et al. 2011; Stenzel et al. 2014), thus adding one more layer of signaling specificity and progenitor cell regulation complexity.

Developing Axons and their Laminar Distribution

Previous studies have shown that binocular enucleation of newborn ferret kits causes a significant reduction of progenitor cell proliferation in the primary visual cortex at P2, specifically in bRGCs of the OSVZ but not ISVZ (Reillo et al. 2011), demonstrating an effect of the thalamo-cortical pathway on the division rate of OSVZ progenitor cells. Here we have used the same manipulation to analyze the potential influence of thalamic axons on the fate of progenitor cells, and found that the formation of GFAP+ astrocytes at P6 is increased upon enucleation, but in this case specifically in the ISVZ but not OSVZ. Hence, the same manipulation of the thalamo-cortical pathway caused 2 very different effects on 2 different progenitor cell populations. Given our findings that the thalamo-cortical axons extend through the upper part of OSVZ but not through the ISVZ, these results prompt us to speculate that these axons selectively influence bRGCs in ISVZ or OSVZ via their basal process. Moreover, this influence has a very selective impact on the biology of these cells (rate of cell division versus cell fate of the progeny), so it may be mediated by different and specific signaling mechanisms.

Our observations of CalB+ axons and their varicosities intimately contacting the basal process of RGCs suggest one potential mechanism for cellular interaction and communication between progenitor cells and passing axons. At the molecular level, we have identified Cx43 puncta in the basal process of RGCs and its varicosities, supporting the possibility that extrinsic signals may be conveyed onto RGCs via gap junctions. Fietz and colleagues (Fietz et al. 2010) have shown the presence of β3-integrin in the basal process of RGCs and its varicosities, and that blockade of such integrin function impairs proliferation of a specific subset of OSVZ progenitors, demonstrating that extracellular signals may be presented by passing axonal

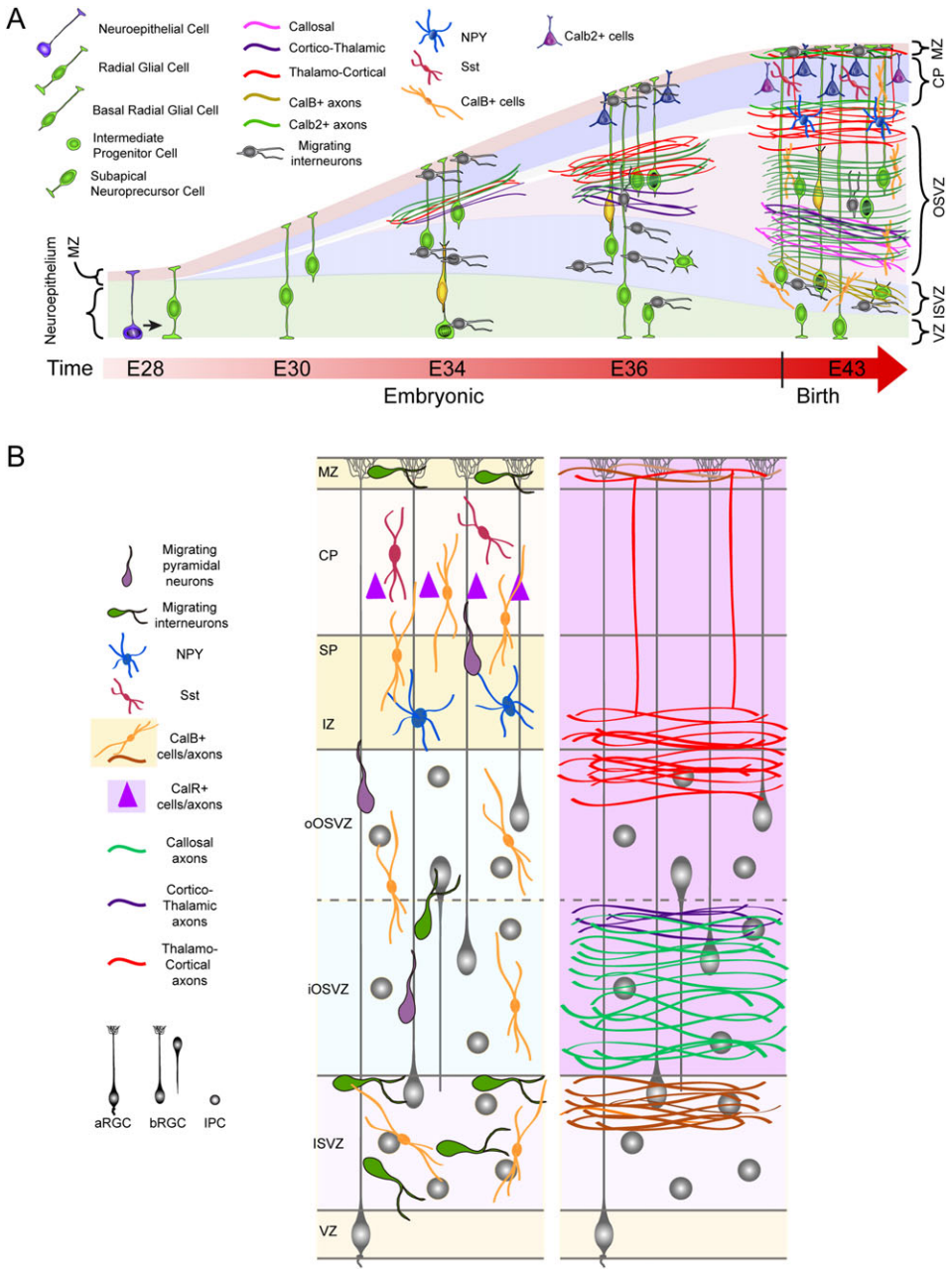

Figure 11. Summary schemas of the code of extrinsic influences on cortical progenitor cells. (*A*) Our findings demonstrate that complex sets of axon fiber tracts and cell populations progressively populate the developing ferret cerebral cortex, from early embryonic stages and in a highly laminated fashion. (*B*) Detailed layer-specific distribution of different types of postmitotic and migrating neurons (left) and axonal populations (right), overlapping with the multiple types of cortical progenitor cells in the early postnatal ferret. Apical Radial Glia Cells (aRGC) extend their basal process through the entire cortical thickness. Basal Radial Glia Cells (bRGC) located in different layers and positions along the apico-basal axis of the cortex extend their basal process through different sets of layers. Intermediate Progenitor Cells (IPCs) are confined to their particular germinal layer. Because neuronal and axonal populations have very specific laminar distributions, each of the progenitor subtypes are exposed to a unique combination of extrinsic influences. CalB, calbindin; Calb2, Calretinin; NPY, neuropeptide Y; Sst, somatostatin.

fibers in the form of cell contact-dependent molecules, and that these signals have cell-type specificity.

Previous studies already proposed the notion of a direct influence from thalamic axons onto cortical progenitor cells, and some interesting candidate molecules and signaling pathways were identified, including FGF2 (Dehay et al. 2001; Dehay and Kennedy 2007; Reillo et al. 2011). Some of these signaling pathways may be conserved in ferret, as some of their key signaling elements are expressed in germinal layers of the embryonic and newborn ferret cortex, including FGF receptors and ligands, but also EphA receptors (Supplementary Fig. 5), which bind EphrinA ligands expressed by thalamic neurons (Huberman et al. 2005).

Here we provide evidence not only supporting the influence of thalamic axons onto cortical progenitor cells, but further extending it to the potential action of additional axonal systems, including cortico-thalamic and callosal axons, and groups of CalB+, Sst+, and NPY+ axons populating the upper aspect of the ISVZ. Moreover, we show that axons from these different origins extend along the developing cortex in a strict

layer-specific and mostly non-overlapping manner, exquisitely distinguishing fine subdivisions of the OSVZ and ISVZ. This remarkable laminar specificity of developing axonal tracts during early cortical development establishes important distinctions in their potential influence over progenitor cell populations. Considering that RGCs may sense extrinsic factors via the cell soma as well as the basal radial process, subpopulations of RGCs will be exposed to different sets of extrinsic signals depending on the precise laminar location of their cell soma, which defines the cortical layers and sublayers crossed by their radial process (Fig. 11*B*). For example, CalB+ axons running along the outer part of ISVZ may influence IPCs and bRGCs whose cell body is located in that position, and also aRGCs, whose radial process crosses through the ISVZ. However, these axons have no opportunity to influence IPCs in other locations, bRGCs whose cell body and basal process are more superficial than ISVZ, or SNPs whose basal process does not reach the ISVZ. Nevertheless, CalB+ axons may influence those bRGCs located in OSVZ that extend an apical process through the ISVZ (Fig. 11*B*).

Some of the sets of axons that we identify in the developing ferret are highly reminiscent of axonal tracts previously identified only in primate embryos. Namely, these are axons populating the upper ISVZ or iOSVZ, reminiscent of the primate Inner Fiber Layer, and also thalamo-cortical axons populating the upper oOSVZ and lower IZ, reminiscent of the primate Outer Fiber Layer (Smart et al. 2002; Kawasaki et al. 2013). These 2 sets of axonal tracts are absent in mice and were not previously distinguished in ferrets, and whether they are homologous to the primate counterparts (Dehay and Kennedy 2007) remains to be elucidated.

Recent studies in human and non-human primates have related the significant expansion of the SP in these species (5 to 10-fold thicker compared to rodents) to a dynamic interaction of progenitor cells and migrating neurons with an increased number of afferent axons (Duque et al. 2016). Considering their potential role as sources of extrinsic influence onto cortical progenitor cells, this is an important possibility with further implications in cortical expansion and folding (Rakic 1988; Dehay et al. 1996a, 1996b; Reillo et al. 2011). Extrinsic influences on cortical progenitors may be particularly relevant in the development of large brains, frequently gyrencephalic, where morphogenetic centers may be too distant to impose the fine regional control over progenitor proliferation observed across the developing telencephalon of these species (Dehay et al. 1993, 1996a; Borello and Pierani 2010; Reillo et al. 2011).

Influence from Migrating Neurons

We have shown here that in the developing ferret cortex, GABA+ interneurons migrate tangentially along the SVZ, VZ, and MZ, similar to mouse (Marin and Rubenstein 2001). Using retroviral lineage tracing we have also shown that at later stages, when the SVZ is split into ISVZ and OSVZ, the tangential migration of late-born MGE-derived interneurons is limited to the ISVZ, while the radial migration of their early counterparts takes place mostly through more superficial layers of the developing cortex, as in mouse (Lopez-Bendito et al. 2008). Previous studies demonstrate that GABA modulates the proliferation of cortical progenitor cells in cortical cultures in vitro (LoTurco et al. 1995; Wang and Kriegstein 2009), so the abundant populations of GABA+ interneurons migrating tangentially along the germinal layers, and also MZ, are candidates to influence those progenitor cell populations and subtypes with which they overlap, either via the cell body or basal process. Interestingly, GABA and also Glutamate have been previously shown to reciprocally modulate the radial migration of neurons (Manent et al. 2005, 2006).

Intriguingly, this situation may be quite different in mouse, where most cortical neurons are born from IPCs in the SVZ and migrate straight away to the CP, with only a brief overlap with the SVZ and even less with VZ (Tabata and Nakajima 2003; Kowalczyk et al. 2009). Paradoxically, the situation in primates may be closer to mouse than ferret, since much cortical neurogenesis at late stages takes place in OSVZ rather than in deeper layers (Lukaszewicz et al. 2005; Hansen et al. 2010; Betizeau et al. 2013), suggesting that a majority of radially migrating neurons may never encounter VZ or ISVZ progenitor cells. Nevertheless, even if migrating neurons never contact the soma of progenitor cells, they do interact very closely with RGCs during radial migration, as this process relies on the molecular cross-talk between migrating neurons and the radial fiber of RGCs (Anton et al. 1997; Elias et al. 2007; Valiente et al. 2011).

Regarding excitatory neurons, born from cortical progenitor cells, we have shown that they migrate radially in very close apposition to Pax6+ RGCs and their radial process, and Tbr2+ IPCs. The intimate association between radially migrating neurons and the radial process of RGCs has been known for decades, but essentially considered under the perspective of this interaction influencing the radial migration of neurons (Rakic 1971, 1972; Sidman and Rakic 1973; Anton et al. 1997, 1999; Elias et al. 2007; Valiente et al. 2011). Here we have considered this association under the new perspective that migrating neurons may be a potential source of extrinsic influence onto RGCs and their neurogenic activity. Indeed, the excitatory neurotransmitter glutamate has a strong influence on cortical progenitor proliferation in vitro (LoTurco et al. 1995; Yoon et al. 2008; Nelson et al. 2013), and even a small amount of glutamate released by migrating neurons onto their companion radial fiber may be sufficient to modulate the proliferative dynamics of progenitor cells. Whether it is GABA, glutamate or other molecules, genetic analyses of mutant mice demonstrate the strong influence of feedback from postmitotic neurons onto cortical progenitor cells to regulate neurogenesis in vivo (Seuntjens et al. 2009).

A Dynamic and Complex Code of Extrinsic Influences

All of the above arguments prompt the Dynamic Extrinsic Code concept, where the combination of a variety of layer-specific cellular components in the developing cerebral cortex defines a laminar arrangement of extrinsic signals. This extrinsic code changes significantly over developmental time (it is dynamic) and is read by cortical progenitor cells, modulating their proliferation and fate decisions. Depending on the layer and sublayer of progenitor cell residence, combined with their extension of an apical process, basal, both or none (Betizeau et al. 2013), each progenitor cell subpopulation may read or have access to a different segment of this laminar code, and hence to a different combination of extrinsic influences. This combinatorial design may be further refined depending on the expression of the relevant molecular components (receptors or downstream signaling elements), which will enable responding to a particular set of such extrinsic influences. A corollary of this hypothesis are 2 alternative but compatible possibilities: the extension, retention or retraction of apical and basal processes may be regulated by layer-specific extrinsic influences. Alternatively, this dynamics of cellular processes may be a cell-autonomous and genetically programmed mechanism to regulate the number

and nature of extrinsic influences that a progenitor will be exposed to at a given time in development. Further investigations on cortical progenitor cell regulation will be key in providing answers to these questions and validating this framework, which are fundamental to our understanding of cerebral cortex development and evolution.

Supplementary Material

Supplementary data are available at *Cerebral Cortex* online.

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Notes

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