Cerebral Cortex, 2017; 1–16

doi: 10.1093/cercor/bhx110
Original Article

ORIGINAL ARTICLE

The Subpial Granular Layer and Transient Versus Persisting Cajal-Retzius Neurons of the Fetal Human Cortex

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Abstract

Neurons of the subpial granular layer (SGL) in the human marginal zone (MZ) migrate tangentially from the periolfactory subventricular zone all over the neocortex. After an immature stage, from 14 to 18 gestational weeks (GW), the SGL attains maximum prominence around midgestation. At 20–25 GW, a transient miniature cell type in the MZ expresses glutamate decarboxylase (GAD) and calretinin, and extends a varicose plexus surrounding somata of large transient Cajal-Retzius neurons (tCRN), potentially modulating their activity. The compact Reelin+ horizontal axon plexus of tCRN forms a transient interface between cortical plate and MZ; it may serve as a migration substrate for cortical interneurons, and attracting NPY+ fibers from the subplate. Around 30 GW, after the disappearance of SGL and tCRN, a population of persisting Cajal-Retzius neurons (pCRN) appears and remains into adult life. pCRNs express Reelin, Tbr1, calretinin, nitric oxide synthase, and the cytokine receptor CXCR4. They are characterized by subpial location, closeness to blood vessels, and aggregation in the walls of developing sulci. Unlike tCRNs, pCRNs do not develop a compact axon plexus in the lower MZ. Occasional mitoses in the midgestation SGL suggest that CRN progenitor cells may give rise to late-appearing pCRNs populating the definitive molecular layer.

Key words: cell death, Cortex development, GABA, interneurons, migration

Introduction

The Cajal-Retzius neurons (CRN) of the developing cortex have been the subject of extensive studies and reviews in a variety of species (Molliver and Van der Loos 1970; Marin-Padilla and Marin-Padilla 1982; Marin-Padilla 1990; Meyer et al. 1999; Soriano and Del Rio 2005; Meyer 2010; Kirischuk et al. 2014; Martinez-Cerdeño and Noctor 2014). Human CRNs have more complex morphologies and developmental histories than their rodent counterparts, which may be related to the human accelerated region (HAR) RNA gene HARF1, specifically expressed in CRN (Pollard et al. 2006). Notably, large CRNs at human midgestation (Retzius 1891, 1893, 1894; Ranke 1910; Meyer and González-Hernández 1993, Meyer and Goffinet 1998) change their morphology and position in the marginal zone (MZ, according to the Boulder Committee, Bystron et al. 2008), while CRNs in newborn infants are smaller and closer to the pial surface (Ramon y Cajal 1890, 1911; Meyer and González-Hernández 1993). In previous publications from our group it was proposed that the different morphotypes represent distinct members of a wide CRN family which appear at specific timepoints at different birthplaces, and also have different fates (Meyer and Goffinet 1998; Meyer et al. 1999, 2002, Cabrera-Socorro et al. 2007; Meyer 2010). The most characteristic feature of the CRN family is the secretion of the glycoprotein Reelin, which through binding to its lipoprotein receptors VLDLP and APOER2 leads to phosphorylation of the adapter protein Dab1, and is

essential for inside-out gradient and laminar positioning of radially migrating excitatory cortical plate (CP) neurons (Ogawa et al. 1995; D'Arcangelo et al. 1997; Rice et al. 1998; Hiesberger et al. 1999; Trommsdorff et al. 1999) reviewed by Tissir and Goffinet (2003) and Frotscher (1998). The Reelin/Dab1 signaling pathway specifically acts on somatic translocation at the end of radial migration (Franco et al. 2011; Sekine et al. 2011, 2012). Since Reelin is also expressed in subsets of cortical interneurons (Schiffmann et al. 1997; Pesold et al. 1998; Perez-Garcia et al. 2001; Martinez-Cerdeño and Clascá 2002; Martinez-Cerdeño et al. 2003), additional markers to define members of the CRN family are the calcium-binding protein calretinin (del Rio et al. 1995; Verney and Derer 1995), the transcription factor Tbr1, characteristic of glutamatergic neurons of pallial origin (Hevner et al. 2003), the tumor protein p73 (Meyer 2011; Meyer et al. 2002, 2004), nitric oxide synthase (NOS) or NADPH-diaphorase (Duckett and Pearse 1968; Santacana et al. 1998; Judas et al. 1999); and the cytokine receptor CXCR4 (Stumm et al. 2003; Tissir et al. 2004; Borrell and Marín 2006; Paredes et al. 2006).

By contrast, the subpial granular layer (SGL) has received much less attention. One of the reasons for this neglect is certainly the apparent lack of animal models. Rodents do not have a clearly recognizable SGL, although interneurons born in the ganglionic eminences use the MZ as a transit zone to their destination in the CP (Wichterle et al. 2001; Ang et al. 2003; Hevner et al. 2004; Lopez-Bendito et al. 2008; Tanaka et al. 2009). However, a well developed SGL exists in non-human primates and is thus not human-specific (Zecevic and Rakic 2001). An additional difficulty for the study of this so far enigmatic cell layer is the fact that it easily peels off during dissection of a fetal brain, and is often lost in frozen and vibratome sections.

A number of exhaustive studies in the older literature described the structure and developmental time table of the human SGL, and are still valid after more than 50 or even 100 years (Ranke 1910; Schaffer 1918; Brun 1965). Schaffer (1918) suggested that the SGL might be involved in shaping the cortical folds. Brun (1965) as well as Gadisseux et al. (1992) proposed that the SGL originates in the periolfactory ventricle or retrobulbar area (Meyer and Wahle 1999). More recent work revealed the neuronal nature of the SGL (Gadisseux et al. 1992; Meyer and González-Hernández 1993; Zecevic and Rakic 2001), and established sublayers within the SGL (Kostovic et al. 2004; Tkachenko et al. 2016). SGL cells express the calcium-binding protein calretinin (Meyer and Goffinet 1998), the microtubule-associated protein MAP2 (Gadisseux et al. 1992; Tkachenko et al. 2016), and GABA or its synthetizing enzyme glutamate decarboxylase (GAD) (Zecevic and Rakic 2001; Rakic and Zecevic 2003; Zecevic et al. 2011). A close spatial interrelationship between CRN and SGL, with both changing their position in the MZ during fetal development, was described in a DiI-tracing study (Meyer and González-Hernández 1993). On the basis of the common expression of Reelin but different morphologies and times of appearance, Meyer and Goffinet (1998) distinguished between large polymorphic CRNs and smaller CR-like cells, and suggested that the latter differentiate from the SGL.

Recent work in rodent cortex and hippocampus has highlighted the intricacies and functional impact of the GABAergic innervation of CRNs in prenatal and early postnatal life, which may also lead to their eventual demise (Mienville 1998, 1999; Kilb and Luhmann 2001; Kolbaev et al. 2011; Myakhar et al. 2011; Cosgrove and Maccaferri 2012; Quattrocolo and Maccaferri 2013, 2014; Blanquie et al. 2016). We wondered whether a similar constellation might exist in the prenatal human cortex, where myriad GAD+ SGL neurons coexist with CRNs. Our results reveal a dense transient GAD+ fiber network that targets the equally transient polymorphic CRNs (tCRN, the dominant CRN population at midgestation), and potentially could modulate their functional activity through synaptically and non-synaptically released GABA (Demarque et al. 2002; reviewed by Owens and Kriegstein 2002; Represa and Ben-Ari 2005), and/or influence the migration of interneurons in the MZ (Inada et al. 2011; reviewed by Luhmann et al. 2014). We also re-define the small CR-like cells (Meyer and Goffinet 1998) as persisting Reelin+/calretinin+/Tbr1+ Cajal-Retzius neurons (pCRNs) of the peri- and postnatal human cortex, and show that they co-express the CRN markers NOS and CRCX4. We highlight the differences between the transient and the persisting CRN family members, most notably the tCR plexus, which breaks down just at the onset of cortical gyration, and thus before the subsequent dramatic expansion of the neocortical areas. The SGL emerges in our study as a complex admixture of various populations of GAD-expressing neurons, which are differentially related to the transient and persisting members of the CRN family.

Materials and Methods

The fetal human brains, between 14 and 40 gestational weeks (GW), were from our collection used in previous studies (Meyer et al. 2000, 2002; Meyer and Goffinet 1998), to which new cases have been added. They were obtained after legal abortions following national guidelines in Spain, under the supervision of the Ethical Committee of the University La Laguna. In accordance with the Declaration of Helsinki, 1964. The perinatal brains (31–40 GW) were from children without known neurological pathologies that died during or shortly after birth, mostly due to respiratory failure. The brains were fixed in Bouin (Panreac, Ref. 25 4102) or Carnoy, embedded in paraffin, and cut in a coronal plane into 10 μm-thick serial sections. Bouin fixative gave excellent results for cytoplasmic proteins, whereas Carnoy was preferred for nuclear staining. We also examined 5 adult brains, from donations to our Department of Anatomy, which were fixed in 10% formaldehyde, cut into 80 μm-thick sections using a freezing microtome, and processed for immunohistochemistry for calcium-binding proteins (Suarez-Sola et al. 2009).

Furthermore, we re-examined the archived photoconverted material from a previous DiI-tracing study (Meyer and González-Hernández 1993) and show unpublished data that support our new findings on the dynamics of cell populations in the human MZ. In particular, Golgi like staining by DiI tracing in 80 μm-thick sections revealed morphological traits not readily visible in our standard 10 μm sections.

Immunohistochemistry

Sections were deparaffinized, hydrated, and boiled in 10 mM citrate buffer (pH 6) for 20 min for antigen retrieval, rinsed in Tris-buffered saline (TBS, pH 7.6, 0.05 M), and incubated in the primary antibodies overnight in a humid chamber. After rinsing, they were incubated in the corresponding biotinylated secondary antibodies (rabbit anti-mouse IgG or goat anti-rabbit IgG; Dako, Glostrup, Denmark), diluted at 1:200 in TBS, followed by incubation with avidin-biotin complex (ABC, DAKO) in TBS. Bound peroxidase was revealed using 0.04% 3,3-diaminobenzidine (Sigma, USA), 0.05% ammonium nickel (II) sulfate, and 0.03% hydrogen peroxide in TBS, pH 7.6. Sections were dehydrated, cleared, and coverslipped using Eukitt (O. Kindler, Freiburg, Germany). Negative controls omitted the primary antibodies.

The following primary antibodies were used: a polyclonal antibody anti-p73α (against amino acids (aa) 427–636 of full length p73, 1:300, gift of D. Caput); a monoclonal anti-Reelin antibody 142, raised against aa 164–405 (De Bergeyck et al. 1998), (gift of A. Goffinet); a rabbit monoclonal anti-vimentin ([EPR3776], ab92547, immunogen: synthetic peptide within Human Vimentin aa 400 to the C-terminus (acetyl), 1:200, Abcam); a polyclonal rabbit antibody against Glial Fibrillary Acidic Protein (anti-GFAP, ab7260, full length native protein (purified) corresponding to Human GFAP as immunogen, 1:500, Abcam); a rabbit polyclonal against the enzyme GAD (ab49832, 1:1000, Abcam); a mouse monoclonal anti-PCNA (proliferating cell nuclear antigen) ([PC10], ab29, 1:500, Abcam); a rabbit polyclonal Tbr1 antibody (Millipore, 1:100, AB2261); a rabbit polyclonal antibody against the full length of Neuropeptide Y (porcine) (ab10980, 1:800, Abcam); a rabbit antibody against the residues 300 to the C-terminus of Human Doublecortin (DCX) (ab18723, 1:200, Abcam); rabbit polyclonal antibodies against calretinin (1:3000) and calbindin (1:7000) (Swant, 7699/4 and CB-38a, respectively); a rabbit monoclonal anti-nNOS, against residues around serine 1417 of human nNOS (neuronal) protein ([EP1855Y] ab76067, 1:200, Abcam); a mouse monoclonal anti-Human Ki67 (Clone MIB-1, DAKO); a rabbit polyclonal anti-CXCR-4 (AB425, 1:50, Chemicon); and a rabbit polyclonal anti-synaptophysin, against synthetic peptide corresponding to Human Synaptophysin aa 41–62 (ab14692, 1:200, Abcam).

Sequential 2-Color Immunostaining

This method was used because the mouse monoclonal anti-calretinin antibody gave only faint staining in the younger embryos. Antigens were immunolabeled sequentially by using primary antibodies (Tbr1 and CR) generated in rabbit. The first antibody was developed using DAB/nickel as chromogen. Thereafter, sections were rinsed in TBS and incubated overnight with the second antibody. After incubation with the biotinylated secondary antibodies and ABC as described above, sections were developed by using DAB alone as chromogen. Sections were dehydrated, cleared in xylene, and coverslipped with Eukitt (Freiburg, Germany). Photographs were taken with a Zeiss Axio microscope equipped with an AxioCamMRc5 digital camera and AxioVision LE 4.6 software. Images were processed using Adobe Photoshop CS2 for adjustment of brightness and contrast.

Double Immunofluorescence

Mouse monoclonal anti-Reelin antibody 142 was mixed with rabbit polyclonal anti-Tbr1 or anti-GAD antibodies and sections were incubated overnight at room temperature. Then the secondary biotinylated anti-rabbit IgG antibody (1:400, Amersham, Arlington Heights, IL) and Alexa Fluor 488 goat anti-mouse IgG (H+L) antibody (1:500, A11001, Invitrogen) were incubated for 1 h at room temperature in the dark, followed by incubation with streptavidin Cy3 conjugate (1:500, SA1010, Invitrogen) for 1 h. Nuclei were detected by DAPI staining (Chemicon International, Temecula, CA, USA). Slides were rinsed in TBS and then, were coverslipped with DABCO (1%) and glycerol-PBS (1:1). Negative controls were performed in the absence of primary antibodies. Fluorescence immunosignals were obtained using a Fluoview 1000 laser scanning confocal imaging system (Olympus Optical).

Results

The Immature SGL (14–17 GW)

Previous studies showed that the SGL appears between 11 and 13 GW (Rakic and Zecevic 2003; Kostovic et al. 2004) at the ventrolateral aspects of the hemispheres, beginning in the insular cortex; at 16 GW, it extends all over the cortex (Brun 1965; Gadisseux et al. 1992; Meyer and González-Hernández 1993). In line with these reports, from 14 to 17 GW the SGL was a compact layer of densely packed small, immature cell somata in the upper tier of the MZ (Fig. 1*A*). CRN lay mostly in a subpial position (Fig. 1*B*), within the SGL, or along its lower border (Fig. 1*A′*). They displayed the bipolar morphology and horizontal orientation characteristic of early fetal life (Meyer and Goffinet 1998), (Fig. 1*A′*,*B*), and expressed their distinctive markers, Reelin combined with transcription factors p73 (Fig. 1*B*) and Tbr1 (not shown; see Meyer et al. 2002). CRN sent their axonal process into a dense, compact horizontal plexus which at 14 GW filled the MZ between SGL and cortical plate (CP) (Fig. 1*A*,*A′*). This plexus remained as long as the population of large transient CRN (tCRN) persisted (see below). It was faintly Reelin+ (Fig. 1*B*) and thus formed a continuous Reelin+ sheet contacting with the apical dendritic tufts of pyramidal cells all over the CP. From 14 to 16 GW, this was the site of highest density of synaptophysin+ synaptic vesicles (Fig. 1*D*). Furthermore, the lower margin of the plexus provided a signal for radial glia fibers to divide before entering the MZ and terminate at the pial surface (Fig. 1*C*).

At 16 GW, the initially cell-sparse MZ region between SGL and the tCR plexus increased in width during and became invaded by SGL granules (Fig. 1*E*). Virtually all granule cells expressed calretinin and GAD (not shown); immunostaining appeared to be nuclear, perhaps due to the scant cytoplasm and the almost absence of dendritic processes characteristic of immature migrating neurons (Fig. 1*E*). Calretinin was also in tCRN and their plexus. Of note, the association between the highly vascularized meninges and the pial surface was very close, and the tCRN appeared more closely attached to the pia and meninges than to the underlying MZ; in some instances, CRN migrated out of the brain into the subarachnoid space (Fig. 2*G*). This may explain why at this age the SGL/tCRN are so easily torn off when the meninges are removed during brain dissection, while the plexus remains in its place.

Origins and Mitotic Activity of the SGL

In keeping with Brun (1965), Gadisseux et al. (1992) and Meyer and Wahle (1999), the origin of the SGL was in the subventricular zone (SVZ) of the lateral ventricle extension in the periolfactory basal forebrain, or retrobulbar area (Stephan 1975), which showed high mitotic activity well into the second half of gestation (Fig. 2*B*). Figure 2 shows the distribution of mitotic markers PCNA and Ki67 at distinct sites of a 25 GW telencephalon. A high density of PCNA+ cells was found in the periolfactory SVZ, and in a proliferating cell layer that extended along the pial surface of the anterior perforated substance (Fig. 2*C*), in close contact with the meninges, which were also proliferating intensely. The anterior perforated substance is continuous with the ventral insular cortex via the *limen insulae*, as well as with the rostromedial aspect of the temporal lobe (Fig. 2*A*). High mitotic activity was also present in the SGL of the rostroventral insula, in contrast to the adjacent temporal neocortex limiting

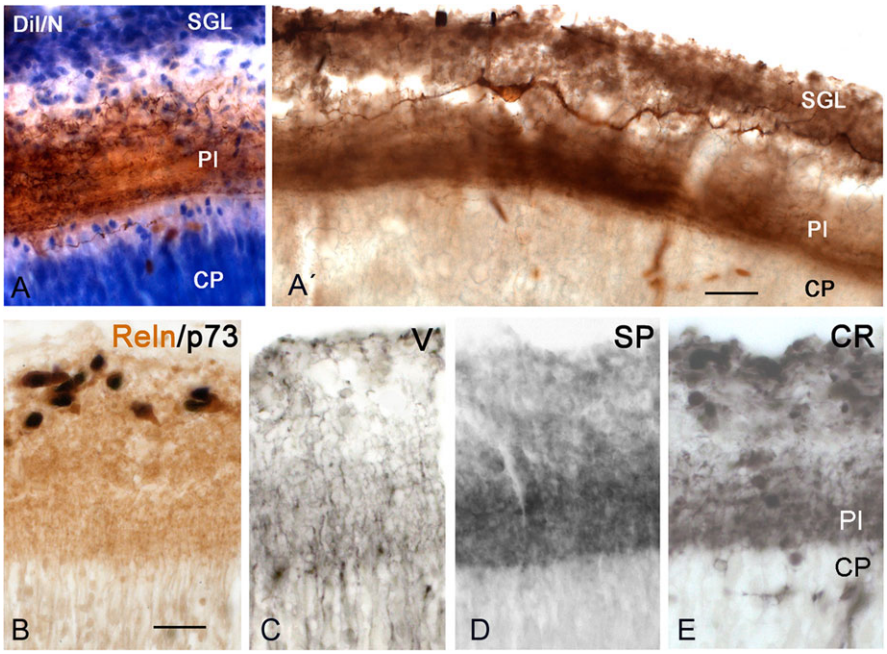

Figure 1. The immature SGL and CRN. (*A* and *A′*) 14 GW, show the CR axon plexus (Pl), traced with DiI, occupying the lower half of the MZ. In *A*, CRN are hidden within the SGL, counterstained with cresyl violet. Notice the cell-sparse band between the plexus and the cortical plate (CP). The sections in (*A* and *A′*) are much thicker than those in *B–E*. At 16 GW (*B–E*), the MZ widens, and CRN co-express Reelin (Reln) in the cytoplasm and p73 in the nucleus (*B*), whereas calretinin (CR) is in both CRN and their plexus, and the SGL (*E*). Vimentin (V) + radial glia fibers branch at their entrance into the plexus (*C*), which contains high density of synaptophysin (SP) + vesicles. Scale bars: In (*A*, *A′*) 25 μm, in (*B–E*) 20 μm.

with the inferior circular sulcus, where mitoses were rare (Fig. 2*D*). The neocortical SGL, including that of the dorsal insula, was not proliferating, although in older fetuses occasional mitoses were observed surrounded by numerous DXC+ neurons (Fig. 2*F*). The PCNA-labeled nuclei were often larger than those of adjacent granules.

The human olfactory forebrain undergoes extensive developmental changes. With the progressing closure of the rhinencephalic ventricle, the origin of the SGL in the rostral extension of SVZ became separated from the surface of the anterior perforated substance. The sagittal section in Figure 2*H*, of a 27 GW-old fetus, illustrates a cell stream from the SVZ of the ganglionic eminences toward the SGL in the anterior perforated substance, just caudal to the rostral migratory stream that leads into the olfactory bulb. At this age, the SGL in the cortex was in the process of regression; it remains to be determined to what extent this late migration route is used by interneurons on their way to the cortex.

The Mature SGL and the Transient CR Plexus

The SGL as a compact cell layer reached a peak in width around midgestation (20–22 GW), with individual granule cells still being immature (Meyer and González-Hernández 1993).

The age of 25 GW marked the onset of dissolution of the SGL and differentiation of distinct SGL components, which created a substratification of the MZ. While remnants of the SGL still formed a cell-dense band just below the pial surface (Fig. 3*C*), many granule cells had descended and distributed throughout the MZ. The majority of granule cells were GAD+ (Fig. 3*A*) and calretinin+ (Fig. 3*B*). A few granule cells expressed calbindin (CB); since their proportion remained minor, we neglected this CB+ population. DCX was expressed in the most superficial tier of the SGL (Fig. 3*D*). High expression of Reelin was characteristic of tCRN somata, whereas their axonal plexus was only moderately Reelin+ (Fig. 3*E*). tCRN were often close, or directly apposed, to blood vessels descending in the MZ. This age corresponded to the "polymorphic stage" of CRN (Meyer and Goffinet 1998), and many tCRN displayed a vertically elongated soma. Faint Reelin positivity was also present in small interneurons located at the level of the tCR axon plexus.

We sought to define the organization of the deep MZ stratum at 23–25 GW, when it was more complex than at any other stage. In 80 μm-thick DiI-labeled sections, the outstanding element was the tCR axon plexus (Fig. 3*I,J*), which displayed the characteristic triangular dilatations known from the Golgi-stained CRNs of the classic literature. The tCR plexus still formed the boundary between MZ and CP; however, in contrast to earlier stages, the plexus stratum became densely cellular around migestation (best recognizable in the 80 μm Nissl-stained sections in Fig. 3*F, I*), representing the deep granular layer (DGL) of Kostovic et al. (2004) and Tkachenko et al. (2016). The majority of these small cells were calretinin+ (Fig. 3*G*), and rather few, slightly larger neurons expressed Reelin (Fig. 3*H*), which contributed to the Reelin positivity of the plexus stratum (for GAD, see below). This was the last stage when the axon plexus was visible; with the disappearance of the tCRN the plexus disappeared as well, and the cells within this stratum may become integrated in the CP.

SGL-Derived Short-Lived Interneurons Innervate Transient CRN

DiI tracing revealed the complex morphology of tCRN at midgestation (23 GW; Fig. 4*A*), their often vertical soma, ascending dendritic processes and the axon projecting into the horizontal tCR plexus. Nissl counterstaining (Fig. 4*A*), 2-color immunostaining (Fig. 4*B*) and confocal microscopy (Fig. 4*C–F*) showed that the somata and ascending processes of tCRN were closely surrounded by SGL granule cells. This closeness was particularly

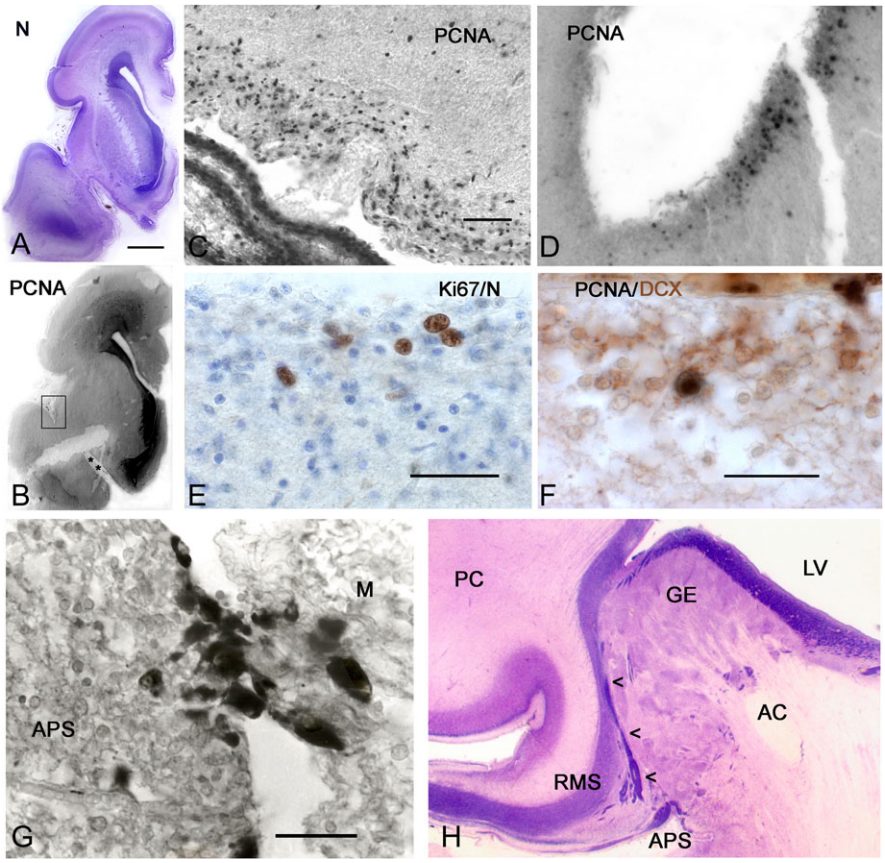

Figure 2. Origin and mitotic activity of the SGL at 25 GW. (*A*) (Nissl, N) and (*B*) (PCNA) show a coronal section near the *limen insulae* at the level of the olfactory ventricle, which is intensely proliferating and continuous with a mitotic SGL extending along the anterior perforated substance ((*C*), marked with 2 asterisks in (*B*)). A mitotic SGL exists also in the ventral insula (the boxed area in (*B*) is shown in (*D*)), but not in the adjacent superior temporal lobe and dorsal insula. (*E*) (Ki67 counterstained with Nissl) and (*F*) (PCNA, black, and DCX, brown): Occasional mitoses are present in the superficial SGL in the neocortex and surrounded by DXC+ immature neurons (*F*). Notice the large size of dividing nuclei (*G*) Reelin+ CRN in the anterior perforated substance (APS) suggesting migration out of the brain into the meninges (M), indicating strong affinity between CRN and meninges. (*H*) Sagittal Nissl-stained section at 27 GW, showing the rostral migratory stream (RMS) and just caudal to it, the migration stream of the SGL indicated by arrowheads, ending in the APS. PC: prefrontal cortex, GE: ganglionic eminence, AC: anterior commissure. Scale bars: In (*A*) 5 mm, (*C*) 50 µm, (*D*) 50 µm, (*E*) 20 µm, (*F*) 15 µm, (*G*) 20 µm.

striking at 21/22 GW (Figs. 4*B* and 5*A*), when calretinin+/GAD+ granule somata and profuse axon-like processes appeared directly attached to Reelin+/calretinin+ tCRN somata and main ascending dendrites.

At 25 GW, many granule cells had descended to a deeper position in the MZ and appeared more differentiated (see below for GAD). Numerous calretinin+ boutons contacted the tCRN somata (Fig. 4*C*). These boutons were also observed with synaptophysin staining (Fig. 4*D*,*E*), suggesting synaptic contacts between granule axons and tCRN. In turn, DCX was expressed in the scant cytoplasm of the most superficial SGL cells (Fig. 4*F*) and in fibers and varicosities in the middle tiers of the MZ, suggesting that the most superficial level of the SGL was the less mature one. We want to point out that the extreme verticalization at 25 PCW represented the final stage in the life of a tCRN, with often vacuolated cytoplasm and shrunken nucleus (Fig. 4*D*,*F*) (see also Meyer et al. 2002).

We analyzed in more detail the expression of GAD in the MZ, which is largely overlapping, though not identical, with that of calretinin. Unfortunately, the antibodies suitable in our material were all raised in rabbit, and we were unsuccessful in determining the degree of co-localization by confocal microscopy. At 22 GW, most GAD+ SGL cells were still concentrated below the pial surface whereas others had their soma apposed to a tCRN (Fig. 5*A*), similar to calretinin-staining. Also GAD-immunoreactivity appeared nuclear, again probably due to the scant cytoplasm. At 25 GW (Fig. 5*B–H*), a distinct type of miniature neuron (max. soma diameters 4–6 µm) had differentiated in the middle tier of the MZ, at the same level as the somata of tCRN. The tiny granule somata gave rise to an axon-like plexus of fine varicose fibers and terminals densely surrounding the somata of tCRN and distributing within the neuropil of this level of the MZ (Fig. 5*B*,*C*,*E*). Their morphology was better recognizable near the walls of microvessels (Fig. 5 *D*), where the neuropil was less dense. On the basis of morphology, we considered this type of SGL-derivative a differentiated, though miniature, local-axon neuron of the MZ. They were no longer present at older stages (30 GW) and are thus extremely short-lived. The complex cell composition of the MZ at this age is summarized in a semi-diagrammatic form in Figure 6.

At the level of the tCR plexus, GAD staining (Fig. 5*F*) was different from that of calretinin (Fig. 3*G*); it appeared less cellular and was rather a loose plexus of varicose fibers (Fig. 5*F*). These GAD+ fibers may have their origin outside the MZ or even outside the cortex. Tbr1, a marker of excitatory pallial neurons, showed that the only excitatory elements of the MZ were the tCRN (Fig. 5*H*); there was no Tbr1 or Tbr2 expression in any derivative of the SGL.

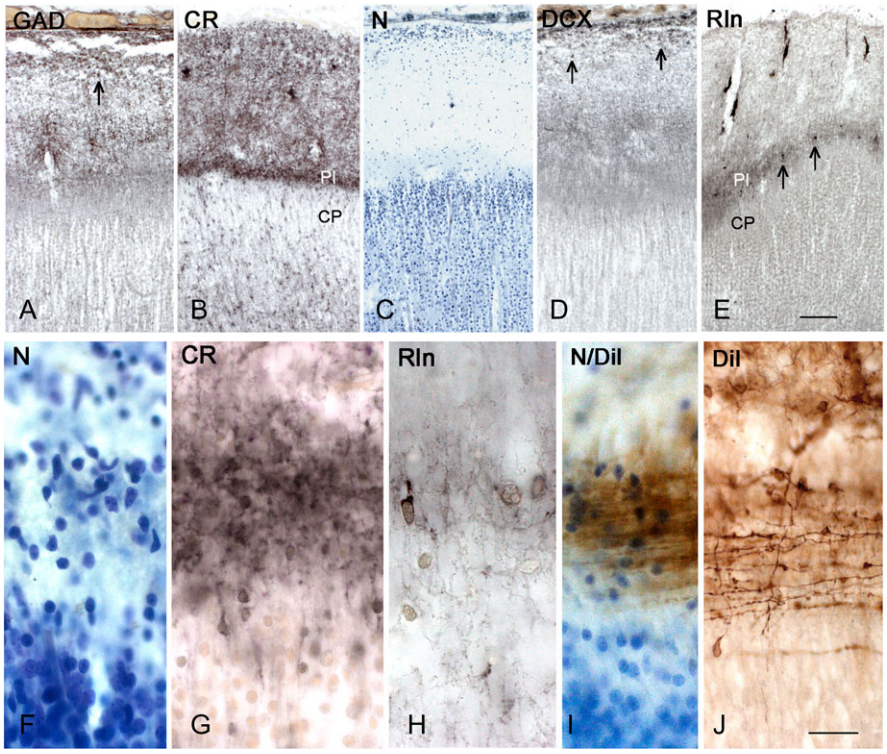

Figure 3. (*A–E*) SGL and tCRN after midgestation (25 GW), when a compact SGL is restricted to the uppermost tier of the MZ. (*A–E*) MZ and upper cortical plate (CP), immunostained with GAD (*A*), calretinin (CR; *B*), Nissl (N, *C*), DCX (*D*) and Reelin (Rln). GAD staining is diffusely distributed all over the MZ and much more intense than in the CP; calretinin is in CRN and granule cells, DCX in cells below the pia and in the neuropil, and Reelin in the vertical tCRN characteristic of this stage, which are often adjacent to blood vessels. Note the Reelin+ and calretinin+ CR plexus just above the CP in (*B*) and (*D*). Pl: CR plexus. Arrows in (*A*), (*D*) and (*E*) point to examples of small neurons. (*F–J*) The CR plexus at midgestation. (*F*), (*I*) and (*J*) are from 80 μm-thick sections used for DiI tracing, (*F*) and (*I*) are counterstained with Nissl. The CR plexus shows characteristic dilations and is now populated with small granular neurons, many of them being calretinin+. Reelin+ interneurons are slightly larger and may contribute Reelin+ axons to the CRN plexus. (*A–E*) 40 μm, (*F–J*) 15 μm.

To determine whether GABAergic subplate neurons were sending their axons into the MZ, we examined the expression of NPY, which at the age of 25 GW was expressed by numerous subplate interneurons displaying the morphology of the so-called axonal-loop cells which send their fibers into the MZ (Wahle and Meyer 1987; Chun and Shatz 1989). In fact, an NPY+ axonal plexus had reached the MZ at 25 GW and was most prominent at the level of the Reelin+ tCR plexus (Fig. 5*G*). A few NPY+ fibers had entered the upper MZ, although they did not appear to specifically target tCRN.

Disappearance of SGL and Transient CRN and Appearance of Persisting CRN

The SGL as a distinct sublayer of the MZ disappeared around 27–31 GW, with local, region-dependent and even individual differences. Occasional remaining clusters of granule cells often displayed shrunken nuclei indicating ongoing degeneration (Fig. 7*A*). Importantly, most tCRN also disappeared between 25 and 30 GW, and the tCR plexus in the deep MZ was no longer present (Fig. 7*C*), in keeping with the death of calretinin+ tCRN. At this age, a new population of Reelin+ neurons appeared in the MZ, just below the pial surface. They were much smaller than the large polymorphic tCRNs, and displayed variably triangular, piriform or horizontal somata (Fig. 7*A,D*). Meyer and Goffinet (1998) termed these cells CR-like cells, and in fact their characteristic expression of p73 and Reelin (Fig. 7*C*), and the negativity for GAD, indicated that they belonged to the CRN family. In the perinatal brains, the small CRN persisted, which is why we term them "persisting (p) CRNs". In fetuses of 30–34 GW, the MZ was narrower, and a diffuse plexus of GAD+ and calretinin+ axon terminals was in the subpial tier of the MZ, less varicose than the plexus present at 25 GW, suggesting that the population of miniature neurons had disappeared concurrently with tCRNs. GAD+ and calretinin+ neurons were much less numerous and resembled "regular" interneurons (Fig. 7*B,C*). It was also evident that GAD and calretinin were not necessarily co-expressed in the same cells at this age. This was particularly striking in the deep MZ and upper layer II, where the numerous calretinin+ interneurons would be expected to be also GAD+. Whereas it was quite common that groups of calretinin+ interneurons seemed to travel along a blood vessel from the MZ to layer II (Fig. 7*C*, asterisk), a similar passage of GAD+ neurons was not observed. However, GAD-expression in cortical interneurons may be upregulated later in development.

With advancing gestation, radial glia fibers no longer reached the MZ, and the glial cells of the MZ became more and more prominent: Large vimentin+ and GFAP+ astrocytes were attached to the pial surface, and smaller ones lay dispersed throughout the MZ and cortical plate (Fig. 7*E,F*). Subpial astrocytes are thus not a component of the SGL, but rather develop after its disappearance. We want to point out that we used markers for microglia such as Iba1 y CD68, which were negative in the MZ and thus not shown here.

Persisting CRN in the Perinatal and Adult Molecular Layer

In perinatal brains, the definitive molecular layer (ML) was established. The pCRN were now the dominant neuronal population

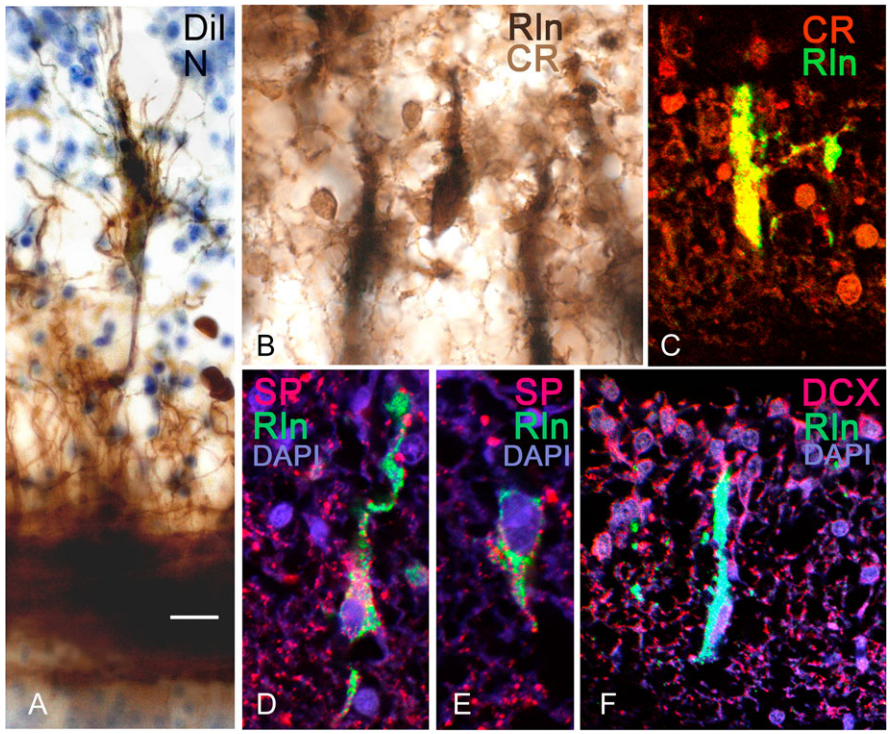

Figure 4. The vertical tCRN and their relationship with the SGL. (*A*) A tCRN at 23 GW, photoconverted after DiI tracing and Nissl counterstained, showing the ascending dendritic processes, densely surrounded by SGL cells, and the descending process that contributes to the CR plexus. In the thin paraffin sections, usually only the soma can be distinguished. (*B*) 21 GW, calretinin (CR, brown) and Reelin (Rln, black). At this stage, granule cell somata are often directly attached to a CRN soma, which is also contacted by many calretinin+ processes and endfeet. (*C–F*) Confocal images showing Reelin+ tCRN (in green) at 25 GW, and their contacts with calretinin (CR)+ cells and fibers (in red) (*C*), synaptophysin (SP, *D,E*) + terminals, and DCX+ fibers (*F*). DCX+ somata are restricted to the upper tier of the SGL, with terminal fibers predominating at deeper levels. Scale bars: In (*A*), for (*A–F*) 10 μm.

(Fig. 8*B,F*). They were usually found in a superficial position, often immediately below the pial basement membrane (Fig. 7*J*), and even outside the neuropil of the ML (Fig. 8*A–E, J*). We did not succeed in immunostaining using the CRN- marker p73, which may be downregulated at this age, and the identification as a CRN was based on co-expression of other CRN molecules such as Tbr1 (Fig. 8*O*), NOS (Fig. 8*E,K,L*), and CXCR4 (Fig. 8*P,Q*). Notably, they were GAD-negative, but surrounded by GAD+ axon terminals (Fig. 8*M,N*). pCRN were particularly common along narrow sulcal walls (Fig. 8*F*) and near the entrance of blood vessels and their branching points in developing microsulci, particularly in the depths of the Sylvian fissure (Fig. 8*G–I*). The appearance of binucleated cell somata was not exceptional (Figs 7*D* and 8*K,Q*). At term, the ML contained numerous GAD+ and calretinin+ interneurons, which are also a feature of the adult cortex (Ma et al. 2014; Gabbott 2016). Strikingly, the more superficial ones had piriform somata in a subpial position similar to the pCRN (Fig. 8*B,D*), with which they could be easily confused, although they tended to be slightly smaller. The upper cortical plate (layer II) was populated by many small calretinin+, GAD+, and Reelin+ interneurons (Fig. 8*B,D,F*). Reelin-expression was usually more intense in pCRN than in interneurons.

The expression of the cytokine receptor CXCR4 has been reported in rodent CRN (Stumm et al. 2003; Tissir et al. 2004; Borrell and Marín 2006). We visualized CXCR4 immunoreactivity in some tCRN at midgestation (not shown), and in pCRN in newborns (Fig. 8*P,Q*). Note the absence of a calretinin+ or Reelin+ CRN axon plexus in the deep ML (Fig. 8*B,C,F*). Unlike their transient counterparts, pCRN do not give rise to a compact axon plexus. Reelin positivity was more intense at the level of the many Reelin+ interneurons of layer II (Fig. 8*F*).

In adult tissues, immunostaining of CRN markers was more difficult. Calretinin, one of the characteristic markers of pCRN, revealed in young adults (Fig. 9, 28 years) the presence of numerous horizontal or oblique cells with pCRN-like bipolar or monopolar morphologies mostly in narrow sulci, preferentially near the sulcal bottom. They extended their dendrites sometimes over long distances. This result indicates that pCRN survive into adulthood, although they were less common in older brains.

Discussion

Origin and Cell Components of the SGL

The various SGL components derive at specific time windows from distinct sites within the basal forebrain SVZ. In the early stages, its origin is in the paleocortical or rhinencephalic ventricle (Brun 1965; Gadisseux et al. 1992, Meyer and Wahle 1999) from where it spreads through the insula over the entire neocortex. In later stages, the SGL descends from the SVZ of the ganglionic eminences to the anterior perforated substance. The SGL exists also in the monkey, where Zecevic and Rakic (2001) identified the GABAergic signature of SGL granules and detected expression of transcription factor Dlx-1 mRNA, consistent with an origin from ganglionic eminences. Furthermore, based on autoradiographs in animals injected with (3H)thymidine, they found a major source of the SGL in the olfactory primordium.

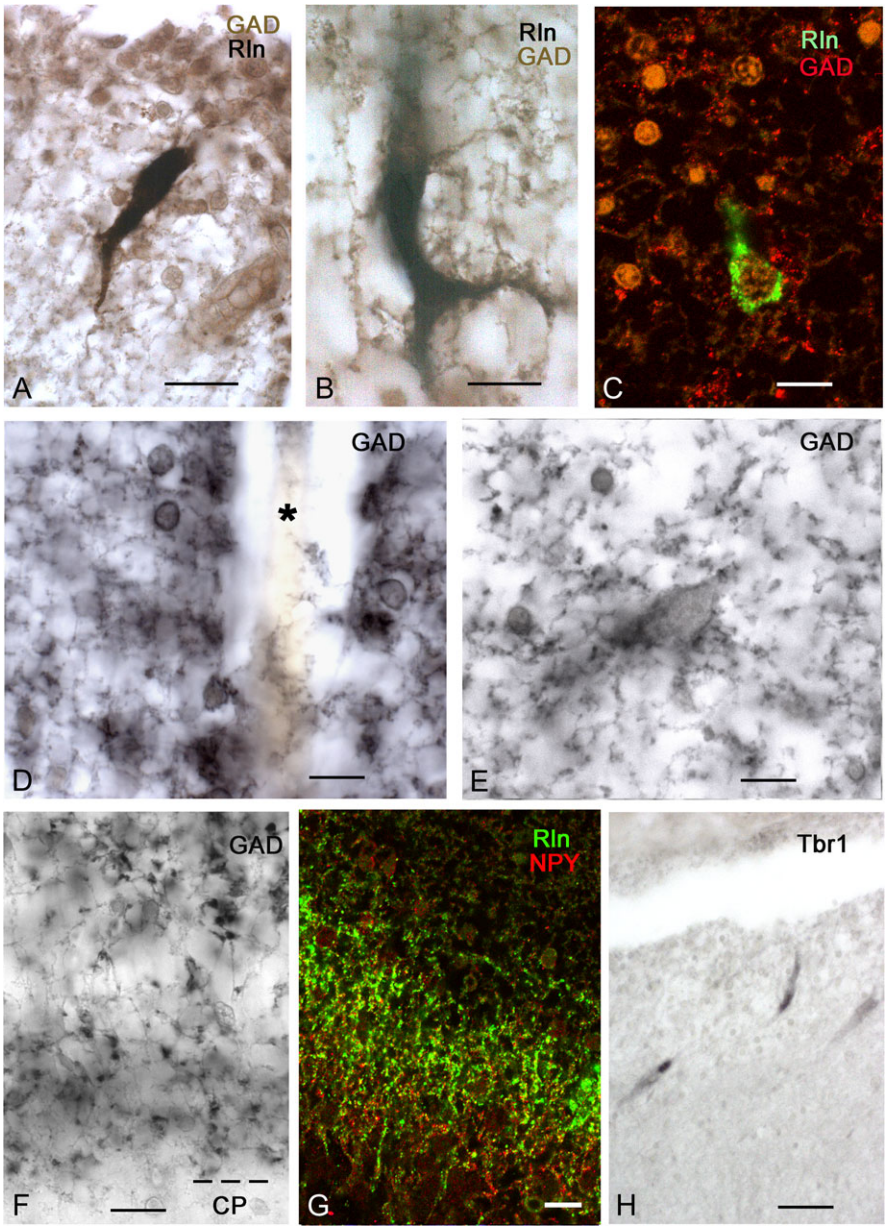

Figure 5. The GAD+ innervation of tCRN. (*A*) 22 GW. Reelin (black), GAD (brown). GAD+ granule cell somata and varicose fibers surround the soma of a tCRN. (*B–H*) 25 GW. (*B*) High power view of the dense GAD+ plexus (brown) densely innervating a Reelin+ tCRN (black). (*C*) 25 GW, confocal image with a tCRN (Reelin, green) and GAD+ SGL cells and fibers (red). The nuclear GAD staining is possibly an artifact due to the scant cytoplasm of these cells. A dense GAD+ plexus surrounds the tCRN soma. (*D*) Miniature interneurons in the MZ. The vicinity of a blood vessel (asterisk) allows visualization of the local, varicose axon-like plexus, otherwise obscured by the dense neuropil. (*E*) GAD+ miniature neurons close to a GAD-negative tCRN soma, which is densely surrounded by GAD+ varicose fibers. (*F*) The area of the CR plexus also contains a GAD+ axon plexus, although GAD+ cells are rare. (*G*) Confocal image showing the Reelin+ CRN and their plexus (green), NPY+ terminal fibers originating from axonal-loop cells in the subplate, and the position of the cell-dense plexus area (DAPI, blue). CP: cortical plate. (*H*) Tbr1 in tCRN, which are the only cell type in the MZ expressing this pallial marker. Scale bars: (*A*) 15 μm, (*B*) 8 μm, (*C*) 15 μm, (*D–F*) 10 μm, (*G*) 15 μm, (*H*) 25 μm.

The expression in human SGL of DLX/GABA and more rarely of NKX2.1/GABA indicates a dominant contribution of the lateral ganglionic eminence and a minor contribution of the medial ganglionic eminence (Rakic and Zecevic 2003). With the closure of the olfactory ventricle and the maturation of the diverse structures of the basal forebrain, the origin the SGL may shift from ventral to successively more dorsal levels, so that an initial rhinencephalic source is compatible with a later origin in the GE.

Gadisseux et al. (1992) first identified SGL cells as neurons, and Zecevic and Rakic (2001) and Zecevic et al. (2011) described their GABAergic signature. We propose that the SGL, after an initial stage of immaturity from 14 to 18 GW, develops around midgestation into heterogeneous cell populations, comprising in a first place an MZ-specific, transient miniature interneuron that reaches maximum differentiation at 25–27 GW to disappear thereafter , and which has not been described previously. The death of this cell type is consistent with the apoptotic nuclei in the MZ observed by Spreafico et al. (1999) and Rakic and Zecevic (2000). The miniature neurons do not represent early members of the definitive interneuron population of the ML. Although their dendritic tree was poorly developed or even unrecognizable, the varicose local axon-like plexus, and above

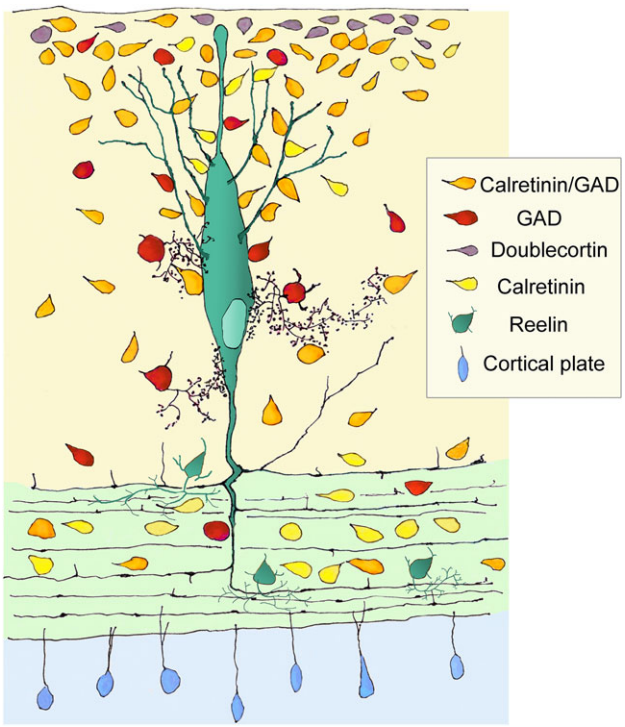

Figure 6. Semi-diagrammatic representation of the cell populations in the 25 GW MZ. Color code: Yellow background, MZ; light green background: the CR plexus area; light blue background: cortical plate. The color code for the different cell populations is explained in the inset. Notice that calretinin and GAD are expressed in a largely overlapping fashion. The miniature interneurons were represented as GAD+ cells, since they were more clearly identified with this marker.

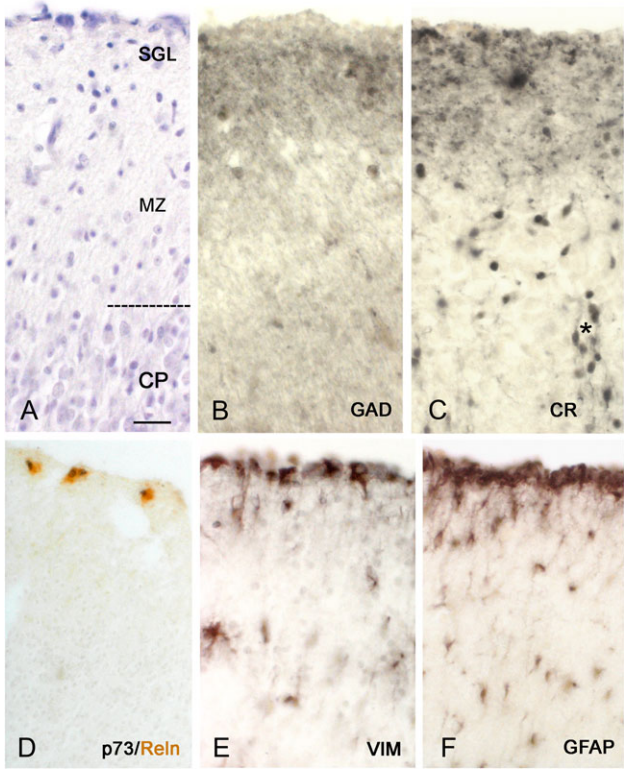

Figure 7. The disappearance of SGL and tCRN. (*A–F*) 31 GW. (*A*) Nissl stain of the MZ. Remnants of the SGL below the pia have dark shrunken nuclei, and absence of tCRN with occasional subpial pCRN (asterisk). GAD (*B*) and calretinin (CR, (*C*)) immunoreactivity is decreased compared to previous stages. Note the absence of the calretinin+ CRN plexus in (*C*). GAD and calretinin label different interneuron populations. Asterisk in (*C*) marks a microvessel used as migration substrate. (*D*) 2-color staining of pCRN with Reelin (yellow) in the cytoplasm and p73 (black) in the nucleus. Note that pCRN may be binuclear. (*E*) (Vimentin, V), and (*F*) (GFAP) show that radial glia fibers do no longer reach the MZ, which is instead populated by astrocytic elements. Scale bars: In (*A*), for (*A–F*) 15 μm.

all, the small soma size, were rather unique and did not conform to the known interneurons of the postnatal layer I. The varieties of layer I calretinin+ interneurons and putative CRN of the adult monkey cortex were described in detail (Gabbott 2016) and do not resemble our miniature neurons. Also the interneuron types of the postnatal and adult rodent layer I have been characterized morphologically and functionally (Hestrin and Armstrong 1996; Ma et al. 2014). They include late-spiking neurogliaform cells, cells with descending axons, and bipolar/bitufted burst-spiking neurons, all of which persist into adult life.

Furthermore, the SGL gives rise to future interneurons of layer I and the upper CP. In rodents, the MZ represents one of several migration routes taken by cortical interneurons from their origin in the ganglionic eminences to the cortex (Wichterle et al. 2001; Ang et al. 2003; Hevner et al. 2004). Subgroups of migratory interneurons en route to their final destination enter the MZ where they may stay for a sojourn of up to 48 h. Within the MZ, they disperse tangentially by a random walk movement before they switch to a radial migration mode and descend into the CP (Lopez-Bendito et al. 2008; Tanaka et al. 2009). Long-distance tangential migration may be crucial for human interneurons which have to travel over increasingly longer distances as the cortex grows. In fact, at human migestation, radially oriented DLX+ interneurons were observed descending from the MZ into the CP (Rakic and Zecevic 2003; see also Zecevic et al. 2011), confirming the contribution of the SGL to cortical interneurons also in human.

Our morphological data suggest that the mostly calretinin+ granules aggregating around midgestation within the tCR plexus will migrate down into the CP. The tCR axon plexus, which becomes increasingly populated with reelin+, calretinin+ and/or GAD+ neurons, that form the "dense granular layer" of Kostovic et al. (2004), emerges as a key structure of the human MZ. The plexus represents a transient compartment which may allow interneurons to wait and/or migrate tangentially to far away regions, while the upper cortical layers are still migrating. The final disintegration of the plexus would remove any obstacle for downward migration. Although most SGL-derived prospective CP interneurons within the plexus expressed calretinin, and a few ones calbindin, it is quite possible that also calretinin-negative peptidergic interneurons travel within the SGL, and express their respective neurochemical phenotype only at their destination in the CP (Ma et al. 2014). In rodents, calretinin+ interneurons derive from the caudal ganglionic eminence (Xu et al. 2004), which is far removed from the periolfactory forebrain. Nevertheless, a novel migration pathway from the ventral sector of the human caudal ganglionic eminence to the anterior cortex via the lateral ganglionic eminence was described for COUP-TFII expressing interneurons (Alzu'bi et al. 2016) and might be related to the calretinin+ SGL cells. On the other hand, calretinin+ bipolar/bitufted interneurons of layers II/III (Fig. 8) are much more numerous in humans/primates than in rodents (Gabbott et al. 1997; Dzaja et al. 2014; Gabbott 2016). This evolutionarily advanced cell type may thus originate from sources, and migrate via routes not present in the rodent, and we may be

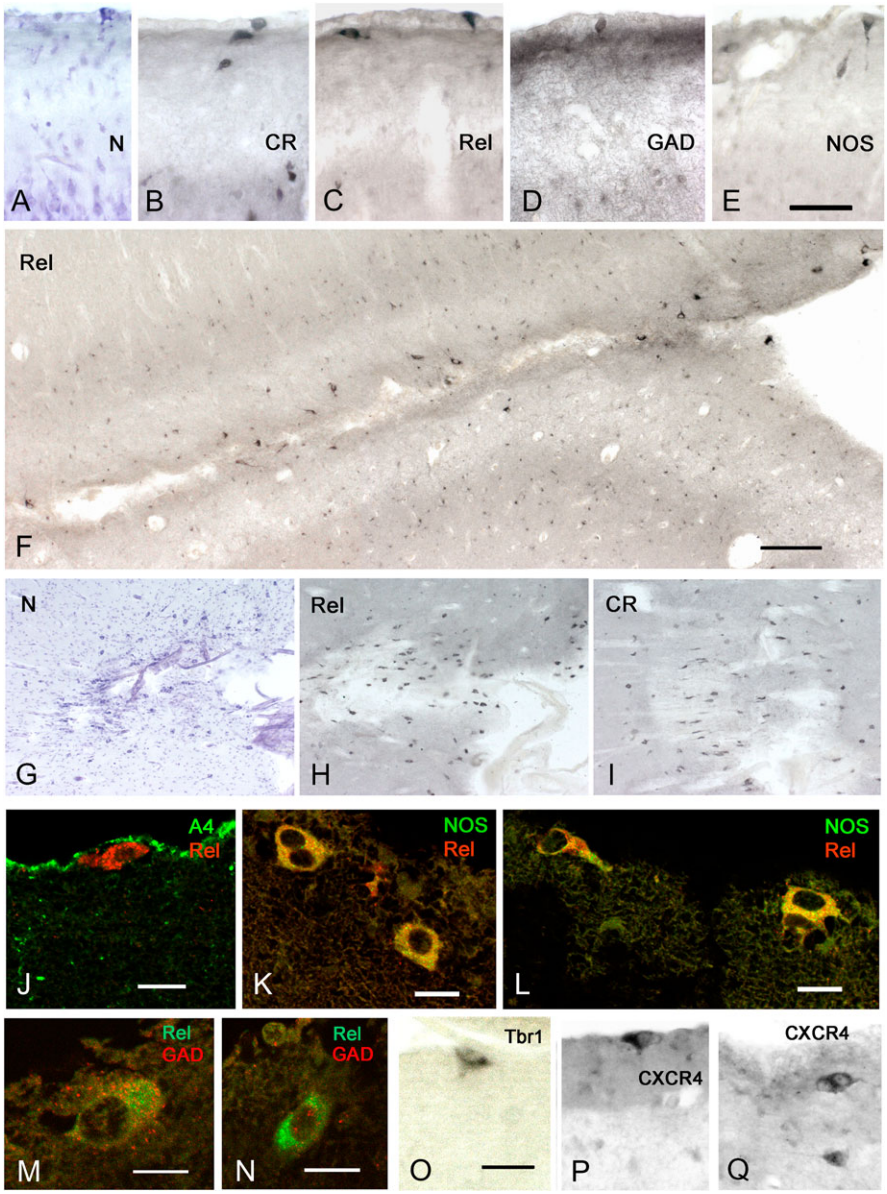

Figure 8. 40 GW. The molecular layer (ML) at term. (*A–E*), (*O–Q*) Comparative views of cell populations in the ML with similar morphologies (subpial position, piriform shape), but which express CRN markers ((*B*) calretinin, (*C*) Reelin, (*E*) NOS, (*O*) Tbr1, (*P*,*Q*) CXCR4) and interneuron markers ((*B*) calretinin, (*C*) reelin, (*D*) GAD). Morphology alone is thus not the defining criterion, although pCRN tend to be larger than the small interneurons of layer I. (*F*) shows a panoramic view of the distribution of Reelin+ pCRN and interneurons of layers I and II. pCRN are common in sulci and often aggregate close to the entrance of blood vessels. The numerous Reelin+ interneurons in layer II (*F*) give a more intense background staining of the neuropil; the tCRN plexus of the deep MZ is no longer present (see *B* and *C*). (*G*,*I*) Adjacent sections stained with Nissl (*G*), Reelin, (*H*) and calretinin (*I*) show a blood vessel creating a microsulcus surrounded by numerous pCRN. (*J*:) A Reelin+ pCRN (red) just below the outer limiting membrane, stained with Aquaporin 4 (A4) (green). (*K*,*L*) NOS (green) and Reelin (red) co-localize in pCRN. Note the binucleated cell in (*K*). (*M*,*N*) pCRN (Reelin, green) are GAD-negative, though surrounded by GAD+ terminals (red), consistent with their expression of Tbr1 (*O*). (*P*,*Q*) CXCR4 in pCRN, one of them (in *Q*) also binucleated. Scale bars: In (*E*), for (*A–E*) 20 μm, in (*F*), for (*F–I*) 40 μm; (*J*) 15 μm, (*K*,*L*,*M*,*N*) 10 μm, in (*O*), for (*O–Q*) 10 μm.

confronted here with an important species-dependent difference, in keeping with a general trend of diversification of origins and migration pathways of GABAergic cells in human (Letinic et al. 2002; Petanjek et al. 2009; Jakovcevski et al. 2011; Hladnik et al. 2014; Al-Jaberi et al. 2015; Clowry 2015).

The Transient CRN

The large polymorphic CRN of human midgestation do not seem to exist in rodents, and comparative studies show that in most species CRN conform to the archetypical Cajal-Retzius morphology of a bipolar or monopolar, horizontally oriented neuron (Fox and Inman 1966; Sas and Sanides 1970; Perez-Garcia et al. 2001; Cabrera-Socorro et al. 2007). The drawings by Retzius (1893) of CRN in dogs and cats illustrate that even in larger, gyrencephalic brains they display horizontally oriented somata with one main dendrite. Of note, human CRN express the RNA gene HAR1 (Pollard et al. 2006), which sets them apart from the rodent and monkey CRN, and suggests that they may display features not present in other species.

The fact that the human tCRN at midgestation are not the same cells as the pCRN at term may be another confusing factor.

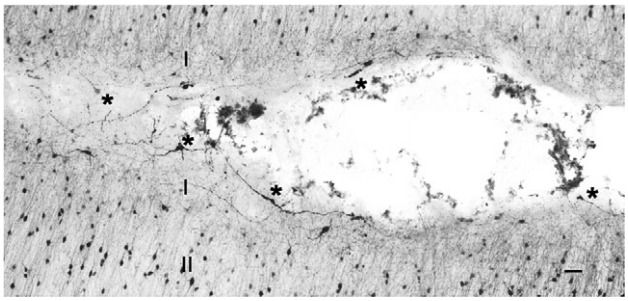

Figure 9. Adult, 28 years. Calretinin in 80 µm-thick section, which provides a view of several bi- or monopolar pCRN (marked with asterisks) in the ML of a narrow sulcus in the prefrontal cortex. Scale bar: 15 µm.

Cajal and Retzius studied human fetuses and infants at different stages and thus described distinct age-specific members of the Cajal-Retzius cell family. Despite their different morphological features and fates, they have in common a quite unique neurochemical profile, with expression of transcription factors Tbr1 (Hevner et al. 2004) and p73 (Meyer et al. 2002), negativity for GABA, positivity for Reelin (Ogawa et al. 1995; Meyer and Goffinet 1998), and calretinin (Verney and Derer 1995), Perhaps more variable is the co-expression with NADPH-diaphorase/NOS (Duckett and Pearse 1968; Meyer and González-Hernández 1993; Santacana et al. 1998, Judas et al. 1999), and CXCR4 (Stumm et al. 2003; Tissir et al. 2004; Borrell and Marín 2006; Paredes et al. 2006). In rodents, CRN express a large variety of molecules (reviewed by Barber and Pierani 2016) and it remains to be seen whether these molecules are also expressed in human tCRN and pCRN.

The morphological features of tCRN were described previously (Meyer and González-Hernández 1993; Meyer and Goffinet 1998). Their most distinctive feature is the massive horizontal plexus which from early stages on forms a physical barrier between CP and MZ, establishing the first synaptic contacts with pyramidal cells of the CP (Molliver and van der Loos 1970; Molliver et al. 1973; Larroche 1981; Marín-Padilla 1998; Zecevic 1998), as also shown here with the expression of synaptophysin at 14 GW. According to classical Golgi studies (Retzius 1891, 1893; Ramon y Cajal 1911; Marin-Padilla and Marin-Padilla 1982; Marín-Padilla 1998) this plexus extends over long distances; even in rodents, where it is less dense, it may be as long as 2 mm (Radnikow et al. 2002; Anstötz et al. 2014). The main effort of the tCRN during development seems to consist in extending more and more parallel axon collaterals at increasingly deeper levels. This is in keeping with the surprising observation (Derer and Derer 1990; Meyer and González-Hernández 1993; Anstötz et al. 2014) that degenerating CRN still had axonal growth cones, which creates an impression of "persisting immaturity" (Derer and Derer 1990; Mienville 1999). Since the outstanding role of tCRN and their plexus is secretion of Reelin (Derer et al. 2001), growth and extension of the plexus increase the Reelin+ surface acting on the myriad cohorts of CP neurons which have to reach this plexus at their final stage of migration, and anchor on top of the CP. The appearance of Reelin+ interneurons within the plexus at 25 GW provides additional Reelin when the tCR plexus begins to break down.

We addressed the possible interactions between CRN and the subplate be examining the maturation of NPY+ axonal-loop cells (Wahle and Meyer 1987; Chun and Shatz 1989), which are dominant interneurons in the kitten subplate. The human subplate attains its maximum extension in the third trimester (Kostovic and Rakic 1990), when most tCRN have died. The subplate is a transient compartment containing excitatory and inhibitory neurons and serves as a waiting compartment for thalamo-cortical fibers (for review, Allendoerfer and Schatz 1994). At midgestation, NPY+ neurons are still immature, in keeping with the mostly immature functional properties of subplate neurons at this age (Moore et al. 2011, 2014), and their plexus in layer I appears massively only around 25 GW at the level of the tCR plexus. This suggests that the plexus, or the granule cells within it, may play a guiding or attracting role for NPY+ axons from the subplate; however, their target are probably not the tCRN but the definitive cell elements in the ML.

The period after midgestation is a turning point in the development of the cortex. Even though the cortical surface expands enormously from 8 to 25 GW, the tCR plexus still seems to be able to match this growth. However, after 26/27 GW, when the first primary sulci appear (Chi et al. 1977; Alif et al. 2015), the cortical surface expands exponentially (Blinkow and Glezer 1968). The extreme verticalization of tCRN at 22–25 GW, shortly before their death, may reflect the traction exerted by their plexus, stretched beyond its limits. The rupture of the plexus by mechanical stress may be one of the factors that contribute to the death of tCRN.

The GABAergic Innervation of Transient CRN

GABAergic neurons form part of the preplate and developing MZ from the earliest stage of corticogenesis (Zecevic and Milosevic 1997; Meyer et al. 2000; Gonzalez-Gomez and Meyer 2014; Al-Jaberi et al. 2015). However, the proportion of GABAergic cells in the MZ dramatically increases with the appearance of the SGL. During the initial SGL period, tCRN lie immersed in a layer of immature granule cells, and slowly change their early fetal horizontal phenotype into the polymorphic forms at midgestation (Meyer and González-Hernández 1993; Meyer and Goffinet 1998). The migrating granule cells of the immature SGL may be electrically silent. As in other places of the developing cortex, GABA is the main transmitter in the SGL, and may be released by paracrine or volume transmission (Owens and Kriegstein 2002; Represa and Ben-Ari 2005), and exert a trophic influence on tCRN. The onset of differentiation of miniature SGL interneurons is in parallel with the morphological transformation of tCRN, which may reflect an increase of electrophysiological activity. The observation by Chen and Kriegstein (2015) that neurons in human layer I had GABAergic responses by 20 GW which were not evident at 16 or 18 GW, supports the hypothesis of a concurrent functional maturation of SGL-derivatives and tCRN.

The GAD+ miniature interneurons described here establish numerous synaptophysin+ terminals on tCRN somata and may thus provide vesicle-mediated innervation of tCRN. Furthermore, both GABA and glutamate are known for acting through volume transmission on tangentially and radially migrating neurons (Demarque et al. 2002; Inada et al. 2011; Luhmann et al. 2015), which may point to a role of SGL (GABA) and CR plexus (glutamate) in influencing migration within the MZ or in the upper CP.

Very little is known about the GABAergic innervation of human CRN, in contrast to the large body of work on the impact of GABA on the functional activity of rodent CRN during embryonic and early postnatal life, up to their death during the second postnatal week (Mienville 1998; Radnikow et al. 2002; Chowdhury et al. 2010; Anstötz et al. 2014). As pointed out by Mienville (1999), despite their early appearance, rodent CRN attain a rather late maturation of their biophysical properties, with excitability and spontaneous spiking activity developing postnatally (Zhou and Hablitz 1996; Mienville and Pesold 1999). CRN permanently express high levels of the chloride inward transporter NKCC1 (Achilles et al. 2007) but

fail to express the chloride outward transporter KCC2 (Pozas et al. 2008), which is developmentally upregulated in most cortical neurons (Rivera et al. 1999). Because of high intracellular Cl- concentration maintained by an active chloride transport mechanism, GABA acts as a depolarizing transmitter in immature neurons (Dammerman et al. 2000; Ben-Ari 2002; Owens and Kriegstein 2002; Represa and Ben-Ari 2005). CRN receive a predominantly GABAergic input mediated by GABA A receptors (Kilb and Luhmann 2001; Radnikow et al. 2002; Soda et al. 2003), which derives from several sources, such as layer I interneurons (Radnikow et al. 2002; Soda et al. 2003; Anstötz et al. 2014), GABAergic subplate neurons (Myakhar et al. 2011), and Martinotti-like interneurons in the CP (Cosgrove and Maccaferri 2012). Furthermore, depolarizing GABAergic afferents from the zona incerta reach the MZ and promote the development of distal apical dendrites of pyramidal neurons in rodents and humans (Dammerman et al. 2000; Chen and Kriegstein 2015). Rodent CRN, in turn, through their glutamatergic excitatory influence on pyramidal cells, may integrate into early cortical circuits (Del Rio et al. 1995; Anstötz et al. 2014; Luhmann et al. 2014; Quattrocolo and Maccaferri 2014) and play a role in the formation of cortical columns, together with GABAergic cells in the MZ (Alcántara et al. 1998).

Recent work has shown that NKCC1-dependent depolarizing GABAergic signaling leads to the death of CRN through a pathway involving the p75 neurotrophin receptor (NTR) (Blanquie et al. 2016), a member of the tumor necrosis factor receptor family. Importantly, TAp73 is a direct transcriptional activator of $p75^{NTR}$ (Niklison-Chirou et al. 2013), which may shed light on the significance of p73 expression in CRN (Meyer et al. 2002; Meyer 2011). P73, a transcription factor involved in death or survival of neurons (Jacobs et al. 2004), is necessary for the generation of CRN, which fail to develop in p73 KO mouse embryos (Meyer et al. 2002, 2004). p73 may also execute their programmed death through the NKCC1-$p75^{NTR}$ pathway at a specific moment of development when upregulation of KCC2 would be required but fails.

In this context, the functional impact of CXCL12/CXCR4 signaling may also be important. During cortex development, CXCR4 is involved in multiple activities. Together with CXCR7, it is the receptor for the cytokine CXCL12 aka SDF1. (Schönemeier et al. 2008). CXCL12 is highly expressed in the meninges (Reiss et al. 2002; Tissir et al. 2004), whereas CXCR4 is in CRN as well as in interneurons in the intermediate zone (Stumm et al. 2003; Tiveron et al. 2006; Li et al. 2008; Lopez-Bendito et al. 2008). This signaling pathway also controls the subpial position of CRN and their tangential migration (Borell and Marín 2006; Paredes et al. 2006). In hippocampal CRN, which display features similar to their neocortical counterparts, activation of CXCL12/CXCR4 signaling hyperpolarizes CRN and strongly suppresses their spontaneous activity (Marchionni et al. 2010; Quattrocolo and Maccaferri 2014). Taking into account that in early fetal human cortex CRN are immediately below the pia, it may be inferred that CXCL12/CXCR4-mediated inhibition is much stronger then than around midgestation, when CRN descend to deeper MZ levels and lose their pial contact.

In sum, we can state that various factors combined may contribute to the demise of tCRN, whose functions are required during a specific period of corticogenesis, but may not be desired or even be counterproductive at later developmental timepoints.

The Persisting CRN

Persisting CRN are a common finding in many species (Fox and Inman 1966; Sas and Sanides 1970; Marin-Padilla and Marin-Padilla 1982; Chowdhury et al. 2010). Even in adult human they often conform to the stereotypical CRN morphology (mono- or bipolar dendritic tree, horizontal orientation), Reelin/calretinin expression, and it is not readily apparent that they are different from tCRN. The sequence of transient and persisting populations may occur only in large, folded brains where mechanical forces put a limit to the growth of the axonal plexus. It may also be argued that the persistence of a common, unifying input to all pyramidal cells by the CR plexus (Marin-Padilla and Marin-Padilla 1982; Marin-Padilla 1990) would be incompatible with area specification and the emergence of later maturing association areas. Development of the human cortex is protracted, migration extends into the third trimester of gestation (Malik et al. 2013), and the cortical surface expands dramatically after birth (Blinkow and Glezer 1968). pCRN may be adapted to this period when new sulci are emerging and a close interplay between the cortical gray matter, meninges and perfusing microvessels is required. Even though the pCRN do not form a compact axonal plexus, their axons branch extensively (Ramon y Cajal 1911; Meyer and González-Hernández 1993). In postnatal rodent cortex, CRN receive an excitatory synaptic input via serotonin 5-HT3 receptors and control the maturation of apical dendritic complexity through the N-terminal fragment of Reelin (Chameau et al. 2009). pCRN may also be involved in Reelin-mediated synaptic plasticity (Weeber et al. 2002; Beffert et al. 2005; Ramos-Moreno et al. 2006; Bosch et al. 2016).

Human CRN derive from several origins at different moments of corticogenesis (Meyer 2010). Where do the pCRN come from? We suggest that they derive from committed CRN progenitor cells that migrate with the SGL, distribute over the entire cortex, and divide when and where the decreasing density of tCRN and/or levels of extracellular Reelin reach a critical limit after midgestation. The occasional mitoses in the SGL are not sufficient to explain the masses of granule cells; however, they may be responsible for the late appearance of pCRN all over the cortex. This would also explain their subpial location, and even the occasional presence of binucleated pCRN, which suggests that the neurogenic potential of these progenitors is exhausted. The exact origin of the postulated CRN progenitor cells should be addressed experimentally in species that have an SGL and pCRN. Certainly this class of CRN deserves further studies in large-brained, gyrencephalic species.

Funding

This work was supported by none.

Notes

We thank Petra Wahle for stimulating discussions on Cajal-Retzius cells, and André Goffinet for generously providing the Reelin antibody. We thank Francisco Trujillo for help with Figure 6. *Conflict of Interest*: None declared.

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