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The multiple facets of Cajal-Retzius neurons

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Abstract

Cajal-Retzius neurons (CRs) are among the first-born neurons in the developing cortex of reptiles, birds and mammals, including humans. CRs' peculiarity lies in the fact they are initially embedded into the immature neuronal network before being almost completely eliminated by cell death at the end of cortical development. CRs are best known for controlling the migration of glutamatergic neurons and the formation of cortical layers through the secretion of the glycoprotein Reelin. However, they have been shown to play numerous additional key roles at many steps of cortical development, spanning from patterning and sizing of areas to synaptogenesis. The use of genetic lineage tracing has allowed the discovery of their multiple ontogenetic origins, migratory routes, expression of molecular markers and death dynamics. Nowadays, single-cell technologies enable us to appreciate the molecular heterogeneity of CRs with an unprecedented resolution. In this review, we discuss the morphological, electrophysiological, molecular and genetic criteria allowing the identification of CRs. We further expose the various sources, migration trajectories, developmental functions and death dynamics of CRs.

Finally, we demonstrate how the analysis of public transcriptomic datasets allows extraction of the molecular signature of CRs throughout their transient life and consider their heterogeneity within and across species.

Introduction

Cajal-Retzius cells (CRs) constitute a neuronal type that presents an array of very specific features. They are pioneer neurons of the cerebral cortex, migrate over very long distances and produce signals that are essential to coordinate brain development. Their most fascinating aspect is undoubtedly their transient lifetime: in rodents, the large majority of CRs undergo apoptosis once cortical layering has taken place. To date, it has been shown that CRs are required for multiple processes throughout development, ranging from cortical layering to functional area formation, dendritogenesis and radial migration of glutamatergic neurons, GABAergic neurons and oligodendrocyte progenitor cells (Barber et al., 2015; Caronia-Brown and Grove, 2011; D'Arcangelo et al., 1995; de Frutos et al., 2016; Griveau et al., 2010; Ogawa et al., 1995; Ogino et al., 2020; Supèr et al., 2000). Their timed death is also crucial for the proper wiring of cortical circuits (Riva et al., 2019). In parallel to the discovery of new developmental functions, accumulating evidence points at the heterogeneity of this neuronal population in terms of molecular profile, ontogenetic origins, migration routes and cell death fate. The recent development of single cell technologies now enables us to understand what is common and what distinguishes CR subpopulations, discover how they fulfill a repertoire of functions across development in a subtype-dependent manner, and tackle the question of their phylogenic conservation and implication in brain complexification.

In this review, we first summarise the morphological, physiological, molecular and genetic characteristics of CRs. We next describe the ontogenetic origins, migration routes, death dynamics and developmental functions of the various CR subtypes. In the final section, we highlight how public transcriptomic datasets from embryonic to adult brains allow better definition of the molecular signature of CR subtypes and address their contribution to brain development and evolution.

Morphology and physiology of Cajal-Retzius neurons

Location, birth and death of CR neurons

In the late 19th century, Santiago Ramón y Cajal and Gustaf Retzius observed that the outermost layer of the developing cerebral cortex of small mammals and humans is populated by a neuronal population with a dense axonal plexus of horizontal nerve fibers (Ramón y Cajal, 1909; Retzius, 1893). This neuronal population has been since then named after these two neuroscientists. CRs are found in the

marginal zone (MZ, the future layer I) of the neocortex, in the hippocampus and in the dentate gyrus (Figures 1A, B). They are identifiable by their peculiar morphology: they display an elongated soma, a thin axon stemming from one pole of the soma and one thick tapered dendrite from the opposite pole (Figure 1C) (Bradford et al., 1977; Del Río et al., 1995; Hestrin and Armstrong, 1996; von Haebler et al., 1993).

In the neocortex, their dendrites course parallel to the pia (Figure 1C) and often give rise to distal secondary and tertiary dendrites oriented vertically towards the pial surface (Del Río et al., 1995; Radnikow et al., 2002). The axons extend over long distances to form a dense network of horizontal processes covering the entire surface of the neocortex (Anstötz et al., 2014; Sun et al., 2019). A certain degree of confusion exists in the literature as one can find references to “Cajal cells”, “Retzius Cells” or “CR-like cells” (Meyer and Goffinet, 1998; Meyer and Wahle, 1999; Meyer et al., 1999). This is not only due to the scarcity of molecular markers initially available but also related to species- and stage-specific features of CRs (Meyer and González-Gómez, 2018a; Meyer et al., 1999). For example, unlike rodents, the human fetal MZ contains two kinds of morphologically distinct but molecularly undistinguishable CRs: an early born population, initially bipolar but possibly becoming polymorphic and vertically oriented that disappears around midgestation, concomitant with the appearance of a second subtype of smaller and more superficial CRs (Meyer and González-Gómez, 2018b; Meyer and González-Hernández, 1993). Since most of the experimental work on CR has been performed in mice, all subsequent discussion refers to this species unless specified otherwise.

In the hippocampal formation, CRs are mainly found in the MZ bordering the hippocampal fissure, the future stratum lacunosum moleculare (SLM) of the hippocampus and outer molecular layer (OML) of the dentate gyrus (Figure 1B) (Ceranik et al., 2000; Del Río et al., 1995; von Haebler et al., 1993). Their dendrites are confined in the same layer as the somata whereas their long-range axons sometimes cross the hippocampal fissure to reach sub-regions of the hippocampal formation such as the subiculum or the layer I of the entorhinal cortex (Anstötz et al., 2016; Ceranik et al., 2000).

Birth dating experiments conducted in rats indicated that CRs are among the first cortical neurons generated, between E12 and E14 (König et al., 1977; Lavdas et al., 1999; Raedler and Raedler, 1978; Valverde et al., 1995). In mice the peak of CR neurogenesis occurs at E10.5-E12.5 (Hevner et al., 2003; Takiguchi-Hayashi, 2004). In humans, CRs are observed as early as 5 gestational weeks (GW) (Meyer et al., 2000). CRs therefore appear to be an exception to the "inside-out" model of neocortical development stipulating that early-born neurons occupy deeper positions (Angevine and Sidman, 1961). In rodents, the majority of CRs disappear rapidly after cortical lamination has taken place, during the first three postnatal weeks in mice (Causeret et al., 2018). In humans, massive CR demise is observed around GW 23 to 28, although subtype specificities exist (Meyer and González-Gómez, 2018a; Meyer and González-Gómez, 2018b). The early generation and early disappearance of CRs led scientists to

refer to them as “transient early-born” or “transient pioneer neurons”. Whether their disappearance is due to cell dilution in an expanding cortex (Martin et al., 1999), morphological transformation (Parnavelas and Edmunds, 1983) or cell death (Chowdhury et al., 2010; Del Río et al., 1995; Derer and Derer, 1990) was unclear for decades. It is only relatively recently that cell death has been validated as the primary fate of CRs and quantified using genetic tracing (Ledonne et al., 2016).

Neuronal properties

Establishing the neuronal nature of CRs was initially challenging given the difficulty of distinguishing their thin axon, a morphological characteristic essential to define a cell as a neuron. Immunological and electrophysiological studies later allowed verification of their neuronal identity: CRs are negative for glial markers and have the ability to fire action potentials (Hestrin and Armstrong, 1996; König and Schachner, 1981). Patch clamp experiments provided an additional tool to characterize the electrophysiological properties of CRs. They display a high input resistance (1 to 3 GΩ) and a depolarized threshold for action potentials (-20 to -40 mV) (Anstötz and Maccaferri, 2020; Ceranik et al., 2000; Luhmann et al., 2000; Mienville and Pesold, 1999; Radnikow et al., 2002; Sava et al., 2010; Sun et al., 2019). Although all these studies reported a membrane potential of -40 mV to -60 mV, these values do not take into consideration the short circuit effects of the leak conductance between cell membrane and patch pipette. After correction, Achilles et al., (2007) reported a resting membrane potential of -80 mV. Upon depolarization, CRs fire repetitive action potentials with a particularly long duration, small amplitude, low discharge frequency and a prominent voltage sag upon hyperpolarization (Ceranik et al., 2000; Kilb and Luhmann, 2000; Kilb and Luhmann, 2001; Radnikow et al., 2002). Several studies have reported a slight shift towards more mature values of these properties after birth: hyperpolarization of resting membrane potential, decreasing input resistance and threshold for action potential (Sun et al., 2019; Zhou and Hablitz, 1996). CR excitability seems to increase during postnatal life until the time of their demise (Kirmse et al., 2005; Sun et al., 2019) although some groups have failed to observe any maturation of their electrophysiological parameters (Mienville and Pesold, 1999; Radnikow et al., 2002), a discrepancy possibly due to differences in experimental set-up.

CRs are glutamatergic neurons (Del Río et al., 1995; Hevner et al., 2003; Quattrocolo and Maccaferri, 2014), express a variety of receptors including $GABA_A$, NMDA, AMPA/Kainate, mGlu and serotoninergic receptors (de Frutos et al., 2016; López-Bendito et al., 2002; Martínez-Galán et al., 2001; Schwartz et al., 1998) and receive synaptic contacts from both GABAergic and non-GABAergic neurons onto their somata and dendrites, as demonstrated by electron microscopy and immunohistology (Anstötz et al., 2014; Radnikow et al., 2002). While CRs undeniably exhibit GABAergic post-synaptic currents (PSCs), the existence of functional glutamatergic synapses remains unclear. Several groups performing whole cell recordings in the mouse postnatal neocortex have reported that evoked excitatory PSCs are insensitive to the NMDA and AMPA/Kainate receptor antagonists but fully abolished by $GABA_A$

receptor blockade, indicating that evoked PSCs are strictly GABAergic (Kirmse et al., 2007; Soda et al., 2003; Sun et al., 2019). In terms of spontaneous PSCs, hippocampal and neocortical CRs have only been reported to display spontaneous GABAergic PSCs, confirming that glutamatergic synapses are most likely physiologically silent (Kilb and Luhmann, 2001; Kirmse et al., 2007; Marchionni et al., 2010; Quattrocolo and Maccaferri, 2013; Soda et al., 2003; Sun et al., 2019). However, it should be noted that some studies performed in the mouse, rat or human neocortex reported the existence of weak glutamatergic inputs (Ceranik et al., 2000; Lu et al., 2001; Radnikow et al., 2002; Sun et al., 2019). In these studies, discrepancies further appear as to whether these excitatory PSCs are mediated via NMDA- or both NMDA- and AMPA/Kainate-receptors. It seems that these differences are due to the fact that the expression of functional NMDA and AMPA/Kainate receptors is highly dependent on the species or the genetic background, as demonstrated by comparative studies between rat and humans or between C57BL/6J and ICR mouse strains (Chan and Yeh, 2003; Lu et al., 2001).

Connectivity of CRs

Many experiments have addressed the question of CRs integration into the developing cortical network but their input-output connectivity remains poorly understood. In the neocortex, monosynaptic retrograde tracing experiments using Frizzled10-CreERT2 mice (see Table 1) have revealed that layer VI $RELN^+$ neurons, layer V $Ctip2^+$ pyramidal neurons and layer I inhibitory neurons establish synaptic inputs on CRs (Cocas et al., 2016). Functionally, electrical stimulation of the transient subplate GABAergic neurons and pharmacological activation of mGluR1/5-expressing interneurons, possibly Martinotti cells, elicit monosynaptic PSCs in neocortical CRs (Cosgrove and Maccaferri, 2012; Myakhar et al., 2011).

Regarding their outputs, labeling experiments suggest that neocortical CRs preferentially innervate layer II-III and V pyramidal neurons and, to a lesser extent, other layer I neurons (Anstötz et al., 2014; Radnikow et al., 2002). When CR death is impaired, for example in *Bax*-conditional mutants (discussed further below), their increased survival promotes an activity-dependent exuberance of dendrites in layer II/III pyramidal neurons, consistent with a functional link between CRs and upper layer pyramidal neurons (Riva et al., 2019). However, the mechanism involved remains elusive as the photoactivation of CRs in these mutants does not appear to lead to the stimulation of neurons in cortical layers II-III (Riva et al., 2019).

In the hippocampus, optogenetics and electrophysiological experiments have established SLM neurogliaform and oriens-lacunosum-moleculare interneurons as functional presynaptic inputs for CRs (Quattrocolo and Maccaferri, 2013) and SLM interneurons as post-synaptic targets (Anstötz et al., 2018a; Anstötz et al., 2018b; Quattrocolo and Maccaferri, 2014). Nevertheless, despite recent advances in understanding the hippocampal and neocortical circuits involving CRs, the synaptic function of these cells remains poorly understood and will require further investigation.

Molecular characteristics of CRs

CRs gained significant attention after the discovery that they express high levels of Reelin (RELN), a secreted extracellular matrix protein necessary for the lamination of the neocortex (D'Arcangelo et al., 1995; Ogawa et al., 1995). This discovery shed light on an important developmental role of CRs and provided scientists with a sensitive immunological marker. RELN has since then been widely used to immunolabel CRs. At embryonic stages, the onset of expression appears slightly later in certain subpopulations of CRs than others (Griveau et al., 2010). In addition, although all CRs express *RELN*, it is important to underline that RELN expression is not specific to CRs (Anstötz et al., 2016; Chowdhury et al., 2010; Frade-Pérez et al., 2017; Moreau et al., 2020). For example, *Reln* mRNA can be detected in the cortical plate below the MZ from E12.5 onwards (Yoshida et al., 2006). In addition, from late embryonic stages, the number of interneurons positive for *Reln* within the MZ itself progressively increases (Alcántara et al., 1998; Anstötz and Maccaferri, 2020), which makes it inappropriate to rely on RELN positivity and localization in the MZ to ascertain CRs identity (see Figure 1C).

Two additional markers have been widely used. The calcium binding protein Calretinin has been found to label CRs throughout their life (Del Río et al., 1995; Ogawa et al., 1995; Soriano et al., 1994). However, Calretinin expression varies depending on the ontogenetic origins of CRs, developmental stages and species (Bielle et al., 2005; Martinez-Galan et al., 2014; Meyer and Goffinet, 1998; Meyer et al., 1998; Moreau et al., 2020). In rat for instance, it was reported that virtually no neurons coexpress RELN and Calretinin at neonatal stages (Martinez-Galan et al., 2014). The transcription factor p73, a paralog of the tumor-suppressor gene p53, is another marker that has been commonly used to label CRs and arguably represents one of the best CRs marker available (Cabrera-Socorro et al., 2007; Meyer et al., 2002; Yang et al., 2000; Yoshida et al., 2006). However, not all CR subpopulations are $p73^+$ (Griveau et al., 2010; Hanashima et al., 2007; Moreau et al., 2020) (see below for the detailed molecular signature of CR subtypes).

Despite the variety of tools available to distinguish CRs, it is striking that none is sufficient to unequivocally identify all of them when used alone, pointing to the existence of multiple subtypes - if not cell types - of CRs. In the following two sections, we will describe how CRs can be distinguished in subpopulations according to their origins, migratory behaviors and cell death fate.

CR subtypes differ in their origins, migration routes and distribution

The origins of CRs long remained enigmatic, or at least debated. Although never formally proven, the idea that CRs could be generated in the neocortical VZ and find their marginal position by radial migration has always been present, perhaps simply because CRs are glutamatergic and the dogma stipulated that glutamatergic cells migrate radially whereas GABAergic interneurons follow tangential

routes (Rubenstein and Rakic, 1999). Currently, human is the only species in which there is some support for this model with the observation of dispersed RELN-positive columns at early stages (Meyer and González-Gómez, 2018a; Meyer et al., 2000). The retrobulbar area was proposed as a possible origin in humans as CRs appear to migrate tangentially in the cortex from this region (Meyer and Wahle, 1999; Meyer et al., 1998). The medial ganglionic eminence (MGE) was initially suggested to be a source of CRs based on DiI tracing in rats (Lavdas et al., 1999), while another study concomitantly concluded the opposite after analyzing MGE-deficient *Nkx2.1* mutants (Sussel et al., 1999). Now, the consensus is that CRs have a pallial origin, at least in rodents. In support of this view, mice lacking *Emx2* (required for normal pallial development) display a loss of both Reln and Calretinin expression in the MZ, suggesting that CRs derive from the *Emx2*-positive pallial anlage (Mallamaci et al., 2000). Moreover, almost all Calretinin-expressing cells located in the neocortical MZ of P4 mice derive from *Emx1*-expressing pallial progenitors (Gorski et al., 2002), and CRs express the pallial marker Tbr1, even when located in subpallial regions of the embryonic brain (Hevner et al., 2001; Hevner et al., 2003).

It is the use of genetic fate-mapping in mice in combination with *in utero* electroporation that has allowed the precise sites of CRs production to be pinpointed. In Table 1 we provide a list of all the transgenic mouse lines currently available which can be used to follow CRs through the expression of Cre recombinase, GFP or β-galactosidase. To date, four sources at the borders of the pallium have been precisely described (Figure 2): the cortical hem (Takiguchi-Hayashi, 2004), the ventral pallium (VP, also referred to as PSB, pallial-subpallial boundary) (Bielle et al., 2005), the pallial septum (Bielle et al., 2005), and the thalamic eminence (TE) (Meyer et al., 2002; Ruiz-Reig et al., 2017; Tissir et al., 2009). It is generally accepted that the hem provides the majority of CRs, especially in the dorsal pallium. From these focal sites at the border, but within the pallial anlage, CRs reach the neocortical surface by tangential not radial migration, at least in rodents.

In humans, analysis of p73 expression (which labels all but VP-derived CRs) suggests that the hem, septum and TE are conserved sources among mammals (Meyer et al., 2002). In sauropsids (birds, turtles and lizards), *Reln*-positive cells have been observed at the pallial surface, but little is known regarding the origins and migratory behavior of these putative CRs (Bar et al., 2000; Goffinet, 2017). The pattern of p73 expression in lizards suggests that most CRs are located in the ventral telencephalon and derive from the septum and TE, whereas only few hem-derived CR invade the pallium dorsally (Cabrera-Socorro et al., 2007). In birds, whether CRs populate the cortex by tangential migration remains a matter of debate. Although electroporation experiments in quails initially suggested that both the hem and septum give rise to dorsally migrating CRs (Nomura et al., 2008), a recent report challenged this view after failing to detect any tangentially migrating glutamatergic neurons in the developing chick pallium (García-Moreno et al., 2018). It remains to be clarified whether discrepancies between these studies are related to the avian species used, the timing of electroporation (as CR progenitors can only be targeted at very early developmental stages) or any other difference in the experimental paradigm. A possible

breakthrough in understanding the evolution of CRs origins and migration among sauropsids and mammals could come from the recent development of single-cell technologies (see below).

Once generated, CRs migrate tangentially following a complex choreography (Figure 2): VP and septum-derived CRs (identified by the *Dbx1-Cre* mouse line) follow both dorsal and ventral flows (Bielle et al., 2005). Hem-derived CRs (identified by the *Wnt3a-Cre* mouse line) migrate anteriorly and laterally but seem to encounter a semi-permeable lateral boundary, possibly corresponding to the lateral olfactory tract (LOT), resulting in most of them being eventually distributed above hippocampal and neocortical territories (Yoshida et al., 2006). TE-derived CRs (identified by the *ΔNp73-Cre* mouse line together with septum- and hem-derived CRs) follow an anterior stream along the LOT together with other neurons migrating towards the olfactory bulb (Ruiz-Reig et al., 2017).

During their migration, CRs remain confined to the marginal zone through the chemoattractive action of Cxcl12 (formerly known as SDF-1) secreted from the meninges and cognate receptors Cxcr4 and Cxcr7 on CRs (Borrell and Marín, 2006; Paredes, 2006; Trousse et al., 2015). Furthermore, it was recently shown that CRs from the TE and/or septum accumulate in the LOT region during early steps of corticogenesis to eventually undergo a second migratory phase directed towards the neocortex a few days later (de Frutos et al., 2016). The formation of such a reservoir of cells followed by their redistribution would allow the density of CRs at the surface of the cortex to be maintained and would compensate for its increase in size during development without the need to generate additional CRs.

CRs are highly motile cells, and eventually cover the entire telencephalon. Their distribution, however, is not homogenous but rather highly reminiscent of their place of birth and migration path. Thus VP-derived CRs are mostly located in the rostrolateral pallium, septum-derived CRs in the rostromedial pallium, hem-derived CRs in the caudomedial and dorsal pallium, and TE-derived CRs in the caudal ventral telencephalon and the prospective piriform cortex (Bielle et al., 2005; Griveau et al., 2010; Ruiz-Reig et al., 2017; Takiguchi-Hayashi, 2004; Tissir et al., 2009; Yoshida et al., 2006). Such a preferential distribution has been shown to be maintained at postnatal stages (Ledonne et al., 2016). This is achieved by contact repulsion between migrating CRs (Borrell and Marín, 2006), a process modulated by Eph/ephrin interactions and extracellular Pax6 (Kaddour et al., 2020; Villar-Cerviño et al., 2013) that ensures complete coverage of the telencephalic vesicle. Consistent with this, modulation of the number or speed of migration of some CRs subsets has been reported to result in the redistribution of the others (Barber et al., 2015; Griveau et al., 2010).

The diversity of CRs does not end in their origin, migration pattern and function. Cell death occurs in CRs at a surprisingly early time and is differentially regulated depending on their origin, molecular identity and localization (Ledonne et al., 2016). In the following section, we describe in detail the last advances in cell death of CRs and its underlying mechanisms.

CR death: sub-type specific mechanisms and timing

Apoptotic cell death is a physiological process that eliminates about a third of all immature cortical neurons (Wong and Marín, 2019). This process depends on neuronal activity: silencing neuronal networks increases apoptosis whereas intensifying the firing frequency promotes neuronal survival (Blanquie et al., 2017b; Ikonomidou, 1999; Priya et al., 2018). Accumulating evidence indicates that this process serves to select functional circuits (Blanquie et al., 2017b) and to match the number of inhibitory neurons to that of excitatory neurons, thus preserving a physiological excitation/inhibition balance (Denaxa et al., 2018; Wong et al., 2018). As noted above, CRs are transient: the vast majority disappear at the end of cortical development and only a small fraction survive until adulthood in rodents, mostly in the hippocampus and to a lesser extent in the neocortex. In humans, the situation is slightly different as a transient subpopulation disappears around GW 23 to 28, concomitant with the appearance of a second subpopulation that persists until postnatal stages, and to a certain extent in adults (Meyer and González-Gómez, 2018a; Meyer and González-Hernández, 1993).

Morphological studies and permanent labeling in mice have formally and clearly established in a quantitative manner that CRs do not disappear because of migration, transformation or dilution in an expanding cortex, but through programmed cell death (Chowdhury et al., 2010; Derer and Derer, 1990; Ledonne et al., 2016). Quantification of CR apoptosis in different cortical areas shows heterogeneities in their cell death fate. In the mouse hippocampus, which is populated almost exclusively by hem-derived CRs (Louvi et al., 2007; Yoshida et al., 2006), up to 85% of CRs undergo apoptosis (Anstötz and Maccaferri, 2020; Anstötz et al., 2016) whereas this proportion reaches more than 95% in the neocortical MZ (Anstötz and Maccaferri, 2020; Chowdhury et al., 2010; Ledonne et al., 2016). The kinetics of disappearance are also temporally shifted between the two regions: neocortical CRs, even hem-derived ones, are mostly eliminated during the first two postnatal weeks whereas hippocampal CRs predominantly disappear during the third and fourth postnatal weeks (Anstötz et al., 2016; Del Río et al., 1996; Ledonne et al., 2016).

Within the hippocampal formation, the time course of cell death of CRs appears delayed in the hippocampal fissure and dentate gyrus compared to the subiculum and entorhinal cortex, which display neocortical-like kinetics (Anstötz et al., 2016). The differences go further than a simple variation between the neocortex and hippocampal formation. Comparing the density of ΔNp73-derived (hem/septum) versus Dbx1-derived (VP/septum) CRs along the rostrocaudal axis of the neocortex reveals that the extent of cell death depends on the cortical territories but also on the subtype of CRs: Dbx1-derived CRs decline in numbers as early as P4, especially in rostral regions, whereas ΔNp73-derived CRs mostly decline between P4 and P10 (Ledonne et al., 2016). Moreover, CRs in the medio-rostral cortex undergo death with delayed kinetics with respect to their ontogenetically related equivalents in the dorso-lateral neocortex. These heterogeneities raise the question of the mechanisms

underlying CR cell death. Which environmental cues and/or genetic programs control the fate of CRs? Can we predict cell death fate based on the molecular signature, the embryonic origin or the identity acquired throughout development?

The time course and rate of apoptosis of hem-derived CRs depends on their final location (hippocampus vs cortex) (Anstötz and Maccaferri, 2020), indicating that the environment represents an important factor controlling their cell death. Although the mechanisms are not yet fully understood, experimental data indicate that electrical activity is a major factor. In the 1990s, *in vitro* pharmacological studies revealed the pro-apoptotic role of electrical activity on CRs (Del Río et al., 1996; Mienville and Pesold, 1999), an effect that contrasts with its pro-survival role on other cortical neurons during the same period. Another line of evidence is the observation that CRs survive when cultured in pure hippocampal explants but degenerate in entorhino-hippocampal cocultures, a model that preserves the afferent inputs into CRs (Del Río et al., 1996). These data led to the hypothesis that CR death results from an overload of intracellular calcium via the direct stimulation of their glutamatergic receptors or a global increase in neuronal network activity.

CRs receive $GABA_A$ mediated inputs (Kilb and Luhmann, 2001; Soda et al., 2003), express the chloride inward transporter NKCC1 throughout their life but do not express the chloride outward transporter KCC2 (Achilles et al., 2007; Pozas et al., 2008). This expression profile, typical of immature neurons, renders $GABA_A$R-mediated inputs excitatory. Further, it has been shown that the excitatory component of $GABA_A$ inputs on cultured neocortical CRs triggers their cell death (Blanquie et al., 2017a). This $GABA_A$-mediated depolarization leads to an increase in expression of the p75 neurotrophic receptor ($p75^{NTR}$), a member of the TNF receptor superfamily usually leading to cell death upon binding of pro-neurotrophins (Dekkers et al., 2013). *In vivo*, deletion of the NKCC1 transporter diminishes the rate and speed of CR death but does not allow a full rescue of CRs (Blanquie et al., 2017a), indicating that additional mechanisms further tune their fate. Whether the increase in $p75^{NTR}$ receptor upon $GABA_A$ stimulation is differentially modulated according to the ontogenetic origin of CRs or a particular molecular signature is a question that remains to be addressed. Increasing evidence indicates that intrinsic mechanisms further underlie the heterogeneities in the cell death of CRs too.

ΔNp73, the N-terminally truncated version of p73, is specifically expressed by CRs from the septum, TE and cortical hem (Meyer et al., 2002; Tissir et al., 2009). Whereas full-length p73 expression is associated with apoptotis, ΔNp73 is necessary for maintaining neuronal survival (Pozniak et al., 2000) and seems to control the lifetime of CRs since its genetic inactivation leads to a strong reduction in CR numbers at birth (Tissir et al., 2009). This could explain the apparent faster decline of VP-derived CRs as these cells never express ΔNp73 (Ledonne et al., 2016). More recently, the observation that septum-derived CRs - but not hem-derived CRs - survive upon genetic overexpression of the hyperpolarizing potassium channel Kir2.1 demonstrates that a similar input can differentially affect the survival fate of

CRs depending on their ontogenetic identity (Riva et al., 2019). The downstream pathway also distinguishes the two subpopulations: genetic inactivation of the pro-apoptotic factor Bax rescues septum-derived CRs from apoptosis but does not affect the survival of hem-derived CRs (Ledonne et al., 2016). Altogether, these data demonstrate that a combination between environmental cues and intrinsic factors ultimately control when CRs die, and that subtype-specific mechanisms of death mediate CRs demise.

Programmed cell death of CRs has been shown to be crucial for cortical wiring. Indeed, the survival of septum-derived CRs by genetic inactivation of *Bax* leads to an exuberance of dendrites and spine density in layer II/III pyramidal neurons, resulting in an excitation/inhibition imbalance due to increased excitatory drive (Riva et al., 2019). Thus, proper cortical arealization and wiring depends not only on a specific density of CR subtypes in the neocortex and hippocampus during embryonic and early postnatal development (Barber et al., 2015; de Frutos et al., 2016; Griveau et al., 2010), but also on the appropriate cell death dynamics (Riva et al., 2019).

Two major questions in the future will be to disentangle which environmental cues affect the survival of which subpopulations and better understand whether the subtype-specific mechanisms of cell death are associated with distinct developmental functions and/or alterations in neurodevelopmental disorders.

CR functions during development

The principal function attributed to CRs is the control of radial migration through secretion of RELN (D'Arcangelo et al., 1995; Ogawa et al., 1995). RELN is a large glycoprotein of about 400 kDa. Upon binding to one of its two cognate receptors, the very low density lipoprotein receptor (VLDLR) and the apolipoprotein-E receptor type 2 (ApoER2), the disabled-1 (Dab1) adaptor is phosphorylated and subsequently activates various downstream pathways (D'Arcangelo et al., 1999; Hiesberger et al., 1999). Mouse mutants for RELN (reeler) or Dab1 (scrambler), present an abnormal layering in the hippocampus, neocortex and cerebellum (D'Arcangelo et al., 1995; Howell et al., 1997; Sheldon et al., 1997). Consistent with this, CR depletion in the hippocampus by genetic manipulation of p73 leads to severe morphological defects (Amelio et al., 2020; Meyer et al., 2004; Meyer et al., 2019).

CRs further regulate neurite outgrowth, neurite complexity, proliferation and distribution of oligodendrocyte progenitor cells via the RELN/Dab1 signaling pathway (Borrell et al., 2007; Del Río et al., 1997; Niu et al., 2004; Niu et al., 2008; Ogino et al., 2020). In humans, the preferential localization of persistent CRs in sulci raised the hypothesis that they might be involved in cortical folding (Meyer and González-Gómez, 2018a; Meyer and González-Gómez, 2018b). A variety of additional developmental functions have been uncovered, including regulation of the identity and function of radial glia (Supèr et al., 2000), timing of GABAergic interneurons migration (Caronia-Brown and Grove,

2011), dendritogenesis of cortical pyramidal neurons (de Frutos et al., 2016). Furthermore, CR subtype-specific presence and distribution at the surface of the developing cortex participates in early patterning and in the control of the size of cortical areas (Barber et al., 2015; Griveau et al., 2010). This function does not rely on a RELN-dependent mechanism but rather subtype-specific signaling (see below). Importantly, decreased CR density at embryonic stages affects cortical circuits even after CRs have disappeared, supporting a long-term function for CRs despite their transient lifespan (de Frutos et al., 2016).

The diversity of CRs in terms of ontogenic origins, migration routes, cell death and survival fate raises the question whether each subtype fulfills a specific function. Patch-clamp experiments followed by biocytin labelling in *Dbx1*^{Cre} mice failed to demonstrate any electrophysiological or morphological difference between VP- and septum-derived CRs compared to the remaining subtypes (Sava et al., 2010). In addition, the RELN-dependent control of cortical lamination is not severely affected upon ablation of specific CR subtypes (Bielle et al., 2005; Griveau et al., 2010; Yoshida et al., 2006), consistent with the fact that all CRs secrete RELN. However, mouse mutants in which septum-derived CRs are ablated display a redistribution of other subtypes, regional changes in cortical neurogenesis, and ultimately an increase in the size of the motor cortex associated with a displacement of somatosensory areas (Griveau et al., 2010). Furthermore, modulation of CR subtypes distribution without affecting their total numbers was shown to affect the size and positioning of higher-order cortical areas (Barber et al., 2015). These data demonstrate that CR diversity is relevant to cortical organization in the tangential dimension and support the hypothesis that CRs send signals to ventricular zone progenitors, either through contact with the glial endfeet in the MZ, or via secreted factors. Although the nature of such signals remains to be established, transcriptomic analyses indicated that it possibly corresponds to the release of subtype-specific morphogens and growth factors (Griveau et al., 2010). It is worth noting that the link between CR subpopulations and cortical regionalization is most likely bidirectional as the patterning genes *Emx1/2* and *Pax6* were shown to be important for CR specification (reviewed in Barber and Pierani, 2016) and the analysis of *Fgf8*, *Emx2*, *Gli3* and *Pax6* mutants revealed differential defects between CR subtypes (Zimmer et al., 2010). These observations led to the proposal that CRs behave as “transient signaling units”, secreting a subtype-specific combination of factors throughout their migration, thus conveying positional information over long distances in the growing cortex and acting as a complement to the well described signaling centers releasing morphogens diffusing over shorter distances (Borello and Pierani, 2010; Griveau et al., 2010). The respective and relative contribution of CR-derived and signaling centers-derived morphogens to cortical regionalization remains to be determined.

In the adult hippocampus, the physiological role of persistent Cajal-Retzius is not yet understood. The RELN/Dab1 signaling pathway is required for synaptic plasticity and memory (Herz and Chen, 2006). However, the secretion of RELN being taken over by inhibitory neurons throughout development, it is

reasonable to hypothesize that the role of adult hippocampal CRs is not restricted to the secretion of RELN. Anstötz *et al* observed that mice raised in enriched cages, an environment associated with enhanced hippocampal neurogenesis, display a higher density of surviving hippocampal CRs than animals maintained in classical cages (Anstötz et al., 2018a). The authors propose that hippocampal CRs might play a role in the regulation of postnatal neurogenesis. The role of adult CRs remains to be elucidated, but the observation that modifying the environment promotes the survival of hippocampal CRs without affecting the survival of neocortical CRs underlines the existence of subtype-specific functions.

Given the accumulating evidence for specific roles for different subtypes, understanding the heterogeneity of CRs is clearly important. As discussed in the next section, deep transcriptomic characterization of CRs is likely to invigorate our understanding of their production, migration, function and disappearance.

Molecular profiling redefines CRs

While lineage tracing in mice has proved very useful to study the origins, functions and properties of CRs during development, only a few studies have undertaken detailed characterization of CR identities or the mechanisms involved in their production. In addition, a comprehensive molecular definition of CR subtype diversity is still lacking. Indeed, during the last two decades, the molecular toolkit available to identify CRs has been limited to a handful of marker genes and mouse lines. A first attempt to perform unbiased molecular profiling of CRs was reported by the group of Nakanishi using cDNA micro-arrays on FACS sorted cells expressing GFP under the regulation of the hem-derived CRs specific metabotropic glutamate receptor subtype 2 (*Grm2*) promoter (Yamazaki et al., 2004) (see Table 1). While their experimental design successfully led to the identification of CR-specific genes at E13.5 and P2 stages, these bulk data could not reveal subtype-specific facets of CR identity. Transcriptional diversity among CR subtypes was first suggested by performing microarray experiments on FACS-sorted Dbx1-derived neurons isolated from anterior medial and lateral regions of the developing brain at E12.5 (Griveau et al., 2010). Despite the bulk approach, the differential enrichment in CR subtypes in the two fractions analyzed led to the proposal that each subset express its own repertoire of signaling molecules.

The recent advances in single cell RNA sequencing (scRNAseq) technologies offer new opportunities to better characterize CRs among the diversity of cortical neuron types. Using this approach, Tasic and collaborators (Tasic et al., 2018) reported that the few CRs surviving in adult mice classify outside of the major glutamatergic branch, highlighting the very "special" nature of CRs in the cortex, as foreseen by Ramón y Cajal (Gil et al., 2014). The increased availability of published and public scRNAseq

datasets allows profiling of CRs from distinct regions or stages. A first attempt was made by Iacono and colleagues (Iacono et al., 2018) using the "1.3 million brain cells" dataset from10X Genomics (https://support.10xgenomics.com/single-cell-gene-expression/datasets/1.3.0/1M_neurons) that contains several thousand E18.5 CRs but their classification of neocortical CRs into 8 clusters awaits validation *in vivo* to understand how these clusters relate to the distinct CR subtypes that can be identified on biological grounds. Interestingly some of the clusters display an apoptotic signature, suggesting that the CR demise program is already initiated by late embryonic stages. However, the absence of *p73*-negative clusters indicate that not all CR diversity was sampled, probably due to the cortical region dissected. More recently, scRNAseq experiments performed at E12.5 allowed profiling of *p73*-positive and negative CRs, and indicated that the main distinction among subtypes is actually related to their VP vs medial (hem/septum/TE) origin (Moreau et al., 2020). Interestingly, VP-derived cells appear to lack a strong specific signature and differ from other CR subtypes by the absence of a complete module of genes usually considered as CR markers such as *Lhx1, Ebf3* and *Cdkn1a* (p21) in addition to *p73*.

An issue scientists are currently facing is the multiplicity of studies: choosing the relevant datasets and exploring them requires analytic skills and is time consuming. An additional challenge regarding CRs is related to their low density, which in the absence of enrichment methods may lead to very low sampling. Here, we give examples of two mouse datasets – in addition to the aforementioned "1.3 million brain cells" dataset - we believe are suited for the study of CRs as they contain a sufficient number of cells sequenced at a sufficient depth: one from E14.5 cortex (Loo et al., 2019, Figure 3A) and the "9k brain cells" dataset from 10X genomics (Figure 3B and https://support.10xgenomics.com/single-cell-gene-expression/datasets/2.1.0/neuron_9k). We also draw the attention of the reader to a very recent preprint containing nearly 300,000 cells collected from E7 to E18 brains that will certainly prove itself helpful in the deep characterization of CRs (La Manno et al., 2020). As a proof-of-concept that valuable information can be retrieved from such datasets, we present here some analysis focusing on CRs and their heterogeneity (see Supplementary Information). We illustrate in Figure 3A-C the very peculiar clustering of CRs, apart from all other neurons. Lists of genes enriched in CRs can be extracted to characterize in a comprehensive manner what makes CR distinct from other neuronal types (Figure 3D). The high sampling of CRs in the "1.3 million brain cells" dataset (more than 14,000 cells) additionally allows CR diversity to be investigated, although it only contains cells from the neocortex and hippocampus (Figure 3E).

A notable and defining feature of CRs is that they lack expression of the forebrain specifier *Foxg1*. The observation that *Foxg1* KO mice generate an excess of CRs prompted the hypothesis that CRs could represent a default fate (Hanashima, 2004; Muzio and Mallamaci, 2005). Accordingly, CRs would be produced by default in the cortex unless *Foxg1* is expressed and redirects cells towards a pyramidal fate,

reminiscent of the repression of alternative fates observed in the spinal cord (Kutejova et al., 2016). The underlying mechanisms were partially unraveled as *Foxg1* inhibition was shown to directly repress key transcription factors required for the acquisition and/or maintenance of CR traits, and especially their migratory behaviour, including Ebf and Dmrt family members (Chiara et al., 2012; Chuang et al., 2011; de Frutos et al., 2016; Kikkawa et al., 2020; Kumamoto et al., 2013). Furthermore, conditional *Foxg1* deletion at E13.5, after the end of normal CR production, leads to ectopic production of *p73*-negative VP-derived but not Hem-derived CRs (Hanashima et al., 2007), consistent with the idea that VP-derived CRs are quite distinct from other subtypes.

One longstanding question regards the conservation of CRs during cortical evolution. Important differences were reported between rodents and humans (Meyer and González-Gómez, 2018a). At the molecular level, species-specific differences also appear. For instance, human CRs specifically express HAR1A (also known as HAR1F), a non-coding RNA subjected to accelerated evolution in hominoids (Pollard et al., 2006). Nevertheless, scRNAseq data of the developing human cortex from gestational weeks 22-23 (prior to transient CR demise, Fan et al., 2018) confirms that mouse and human CRs are indeed a homologous cell type (Figure 3F). Although human CRs have been well described, a thorough comparison between human and rodent CRs in terms of developmental profile and subtype cannot yet be drawn because of the lack of longitudinal studies at all stages in human. Because human embryonic or fetal tissue is difficult to obtain, organoids represent an interesting alternative to gain access to human CR signatures, especially when combined with scRNAseq (Birey et al., 2017). We predict that deepening our knowledge of the species-specific features of CR will soon allow the assessment of CR contribution to brain evolution, especially in primates.

In sauropsids, only a handful of studies have addressed the matter and the issue is not as clear. As noted above, *Reln* expression has been described in the telencephalic MZ of turtles, lizards and birds (Bar et al., 2000; Goffinet, 2017). By contrast, expression of *p73* was reported to be very limited in lizards (Cabrera-Socorro et al., 2007) whereas in crocodiles most subpial *Reln*$^{+}$ cells were also found to be *p73*$^{+}$ (Tissir et al., 2003). In chick, *Lhx1* and *Lhx5* (two genes enriched in mouse CR, see Figure 3) are expressed in the septum and thalamic eminence, while Lhx5 is also found in the hem, but neither gene seems to be expressed in MZ cells of the cortex (Abellan et al., 2010a; Abellan et al., 2010b). Whether these structures can generate CRs in avian embryos remains an open question. An unequivocal assessment of the degree of CR conservation in sauropsids will likely come from scRNAseq experiments, but the only data available so far come from adult specimens (Tosches et al., 2018) which, as in mice, appear largely devoid of CRs.

Overall, molecular profiling has revealed the extent to which CRs distinguish themselves from all other cortical neurons and has exposed their heterogeneity. These data have also opened new perspectives on their generation, elimination and contribution to brain development. As the number and quality of such

datasets improve, and as data from more species are added, we expect single cell profiling to further contribute to our understanding of CRs.

Future challenges and perspectives

Over the last 15 years, thanks to the development of genetic tracing tools and more recently single-cell technologies, we have made tremendous progress in exposing the multiple facets of CRs during cortical development. The nature of subtype- and species-specific features have begun to be unraveled. However, the molecular mechanisms underpinning CR diversity and their relevance for the construction of functional or dysfunctional cortical networks is still largely unclear. For example, the topology of the gene regulatory networks (GRNs) controlling the acquisition and the maintenance of CR identity remains a black box. Further efforts will be necessary to identify the terminal selector transcription factors (Hobert, 2016) for CRs. New attempts to map the GRN of CRs will likely take advantages of scATACseq approaches allowing the identification of regulatory regions of the genome which are specifically accessible in CRs compared to other cortical neurons. New technologies such as spatial transcriptomics could prove helpful, especially in humans, to precisely map CR subsets and pinpoint their origins.

Besides a better classification of CRs, the precise architecture of transient cortical networks in which CRs are embedded is still lacking. Trans-synaptic viral tracing of the different subpopulations of CRs will contribute to the better characterization of such networks. This will pave the way to understand how CRs contribute to shape mature cortical networks during development and whether CRs subtypes are assigned to specific functions. In terms of brain evolution, CRs are a highly relevant cell type as their numbers and diversity increases from sauropsids to mammals. They control key processes in brain morphogenesis and emerge as potential players in cortical gyrification. How CRs have evolved across species and their contribution to brain complexification will be one of the questions to solve for the next decade. Another future challenge is to determine the contribution of CRs to neurodevelopmental disorders in humans. Abnormal CR survival has been suggested to occur in patients with polymicrogyria, focal cortical dysplasia or temporal lobe epilepsy, pathologies associated with seizures (Blümcke et al., 1999; Eriksson, 2001; Garbelli et al., 2001). The study of mouse models with extended CR survival (Ledonne et al., 2016; Riva et al., 2019) will certainly prove useful in this perspective.

The most fascinating aspect of CRs is certainly their almost purely developmental role. They are a unique example of a cell type that is produced very early during corticogenesis, whose migration and positioning all over the developing forebrain is tightly regulated, that perform essential functions in the establishment of brain architecture, and that is almost completely eliminated once development is over. The very few CRs surviving until adulthood might reveal themselves necessary for a physiological

551 cortical processing, but until then CRs remind us that there are important functions for neurons beyond
552 synaptic transmission.

Mouse line	Details	Targeted CRs	Also targeted	References	Remarks
Wnt3a-Cre	Insertion of IRES-Cre in the 3'UTR	Hem-derived CRs	Hem, choroid plexus	Wnt3a$^{tm1(cre)Eag}$ (Yoshida et al., 2006)	An equivalent line is available Wnt3a$^{tm1.1(cre)Mull}$ (Gil-Sanz et al., 2013)
ΔNp73-Cre	Knock-in of Cre-IRES-EGFP at the start codon of ΔNp73	Hem-, septum- and thalamic eminence-derived CRs	Choroid plexus, GnRH neurons, vomeronasal neurons, uncharacterized cells in the preoptic area and retrobulbar area	Trp73$^{tm1(cre)Agof}$ (Tissir et al., 2009)	
p73-LacZ	Insertion of IRES-LacZ downstream exon 4 of *p73*	Hem- and most likely septum- and thalamic eminence-derived CRs	Ependymal cells and choroid plexus	Trp73$^{tm1a(KOMP)Wtsi}$ (Fujitani et al., 2017; Skarnes et al., 2011)	
Dbx1-Cre	Insertion of IRES-Cre in the 3'UTR	VP- and septum-derived CRs	**Interneurons and oligodendrocyte precursor cells from the preoptic area, glutamatergic transient neurons from the PSB,** lateral area and Arcuate nucleus of the hypothalamus	Dbx1$^{tm2(cre)Apie}$ (Bielle et al., 2005)	Two similar lines also exist : Dbx1$^{tm1(cre/ERT2)Jcor}$ (Hirata et al., 2009) and Dbx1$^{tm1.1(cre)Mull}$ (Hua et al., 2014) but CR-targeting is unknown. A Dbx1-LacZ allele Dbx1^{tm1Tmj} (Pierani et al., 2001) also allows short-term labelling of VP- and septum-derived CRs
Fzd10-CreER^{TM}	Transgene containing 6.7kb upstream *Fzd10* followed by CreER^{TM}	Hem-derived CRs	Dorsal thalamus, cerebellum, dorsal spinal cord, dorsal root ganglia.	Tg(Fzd10-cre/Esr1*)1Chzh (Gu et al., 2009; Gu et al., 2011)	A Fzd10-LacZ transgenic line also labels CR cells (Zhao et al., 2006)
Ndnf-Cre	Insertion of IRES2-dgCre (destabilized EGFP/Cre fusion) in the 3'UTR	At least CRs persisting in adults. Unknown at embryonic or early postnatal stages	**Layer 1 interneurons.**	Ndnf$^{tm1.1(folA/cre)Hze}$ (Tasic et al., 2016; Tasic et al., 2018)	Two related lines exist: Ndnf$^{tm1.1(cre/ERT2)Ispgl}$ (Abs et al., 2018) and Ndnf$^{tm1.1(cre)Rudy}$ (Schuman et al., 2019) but CR targeting is unknown.
IG17	Transgene containing 18kb upstream *Grm2* (mGluR2) followed by GFP-fused *IL2RA* (human Interleukin-2 receptor alpha)	Not precisely documented. Numerous CRs in the neocortex and hippocampus.	Granule cells of the olfactory bulb, cerebellar Golgi cells and pontine neurons	Tg(Grm2-IL2RA/GFP)1Nak (Soda et al., 2003; Watanabe et al., 1998)	
Cxcr4-GFP	GENSAT BAC transgene of ~200kb (RP23-9A20) with EGFP inserted at the start codon	Not precisely documented. Numerous CRs in the postnatal neocortex and hippocampus.	Neural progenitor cells in the hippocampus. Unknown in the embryonic cortex.	Tg(Cxcr4-EGFP)CD73Gsat (Anstötz and Maccaferri, 2020; Gong et al., 2003; Marchionni et al., 2010)	
Pde1c-Cre	GENSAT BAC transgene of ~50kb (RP23-142L8) with Cre inserted at the start codon	Probably medial CRs but not precisely documented	**Preplate/Subplate neurons** at embryonic stages. Some hilar interneurons in the hippocampus.	Tg(Pde1c-Cre)IT146Gsat (Anstötz et al., 2018a; Gong et al., 2003; Osheroff and Hatten, 2009)	A GENSAT Pde1c-GFP transgenic line also exists Tg(Pde1c-EGFP)S45Gsat (Gong et al., 2003)
Ebf2-GFP	GENSAT BAC transgene of ~170kb (RP24-283N8) with EGFP inserted at the start codon	Possibly all CRs subtypes	**Preplate neurons.** Weak GFP expression in the cortical plate at E14.	Tg(Ebf2-EGFP)FB58Gsat (Chowdhury et al., 2010; Chuang et al., 2011; Gong et al., 2003)	
Ebf3-LacZ	Knock-in of LacZ at the start codon of Ebf3	Hem-, septum- and thalamic eminence-derived CRs		(de Frutos et al., 2016; Jin et al., 2014)	A similar line exists: Ebf3^{tm1Reed} (Wang, 2004) but CR targeting is unknown. A GENSAT Ebf3-GFP transgenic line also exists Tg(Ebf3-EGFP)LP183Gsat (Gong et al., 2003)

Table 1: Mouse lines allowing to follow CR cells.

Mouse lines reported to enable labelling or targeting of CRs. The precise cell types which are targeted (specifically or non-specifically) are not always very well described. The cell types indicated in bold are present in the cerebral cortex and could be mistaken for CRs depending on the experimental paradigm.

Figure 1. CRs in the postnatal brain.

(A) Sagittal (upper panel) and coronal (lower panel) sections of the postnatal mouse brain indicating the position of the neocortex, hippocampus and piriform cortex. **(B)** Expanded view of the delimited area shown in (A) picturing the position of CRs (pink dots) at the surface of the cortex and in the hippocampus. **(C)** Morphology of CRs in the cortex. In the upper panel, a drawing from Santiago Ramón y Cajal depicts the typical bipolar morphology of CRs (Ramón y Cajal, 1891). Axons and primary dendrites are oriented along the pial surface whereas secondary and tertiary dendrites extend perpendicularly, towards the pial surface. In the lower panel, a representative picture of the marginal zone of a P7 mouse pup shows the localization of CRs in the marginal zone of the neocortex. CRs are identified by Wnt3a-Cre genetic tracing (red) together with Reelin immunolabeling (green). DG: dentate gyrus, HF: hippocampal fissure, Hipp: hippocampus, MZ: marginal zone, NCx: neocortex, OML: outer molecular layer, Pir: piriform cortex, SLM: stratum lacunosum moleculare.

Figure 2. Origins and migratory routes of CR subtypes.

(**A**) 3D views of the embryonic brain depicting the surface views shown in B, C and the sections shown in D, E. (**B**) Lateral and (**C**) medial surface views of the developing forebrain indicating the migratory paths (arrows) of CRs from the ventral pallium (red), septum (green), hem (blue) and thalamic eminence (yellow). (**D**) and (**E**) represent CR migratory streams (arrows) on rostral and caudal coronal sections, respectively. CGE: caudal ganglionic eminence, ChP: choroid plexus, LGE: lateral ganglionic eminence, LOT: lateral olfactory tract, LV: lateral ventricle, MGE: medial ganglionic eminence, NCx: neocortex, Pir: piriform cortex, SP: subpallium.

Figure 3. scRNAseq for the study of CRs.

A, B: scRNAseq Spring plots show that CRs (highlighted in red) are always positioned apart from both glutamatergic and GABAergic neurons. Data analyzed from Loo et al., (2019) E14.5 mouse embryos (A) or 10X Genomics E18.5 mouse embryos (B). (**C**) Hierarchical clustering (here from the E18.5 10X Genomics dataset) shows that CRs branch apart all other neuronal types. (**D**) Identification of genes with particular functions/subcellular localizations that are specifically enriched in CRs. Such factors constitute candidates for CR markers and for key regulators of CR development and function. (**E**) Examples of CR-enriched genes differentially expressed within the CR cluster from the 10X Genomics E18.5 dataset. Whether these differences reflect CR origins, localization, progression towards apoptosis, or any other process, is currently unknown. **(F)** Comparison between human (Fan et al., 2018) and mouse (E18.5 10X Genomics) datasets demonstrate a significant (black dot) positive correlation between gene modules defining CRs in each species and a negative correlation when comparing CRs with other cortical neurons (inhibitory and excitatory). Such a strategy allows identification of species-specific features or conserved signatures, as pictured by the theoretical Venn diagram. Links to the datasets and details regarding the pipelines used for data analysis can be found in Supplementary Information.

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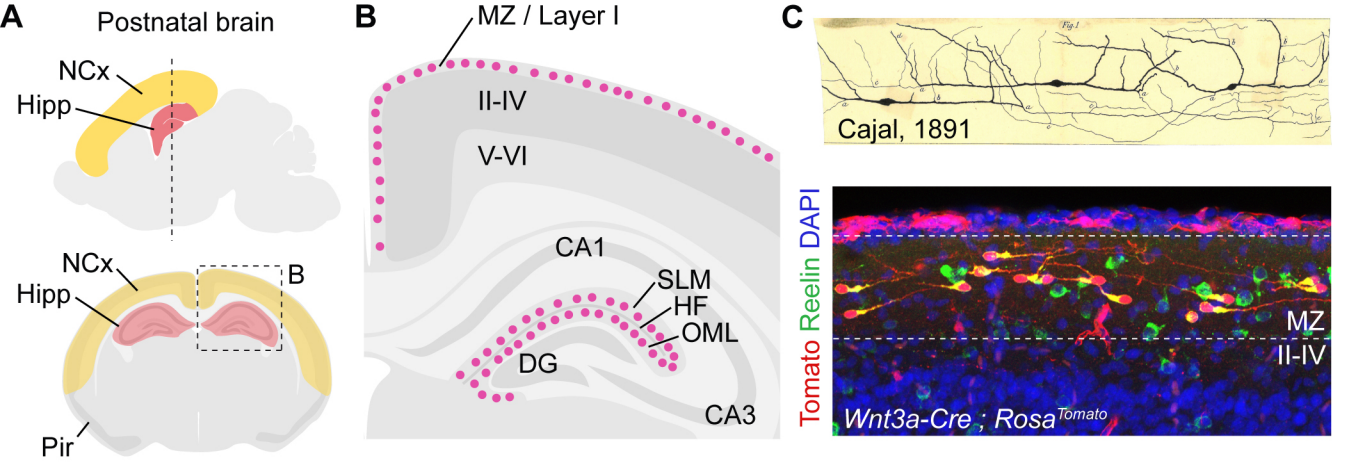
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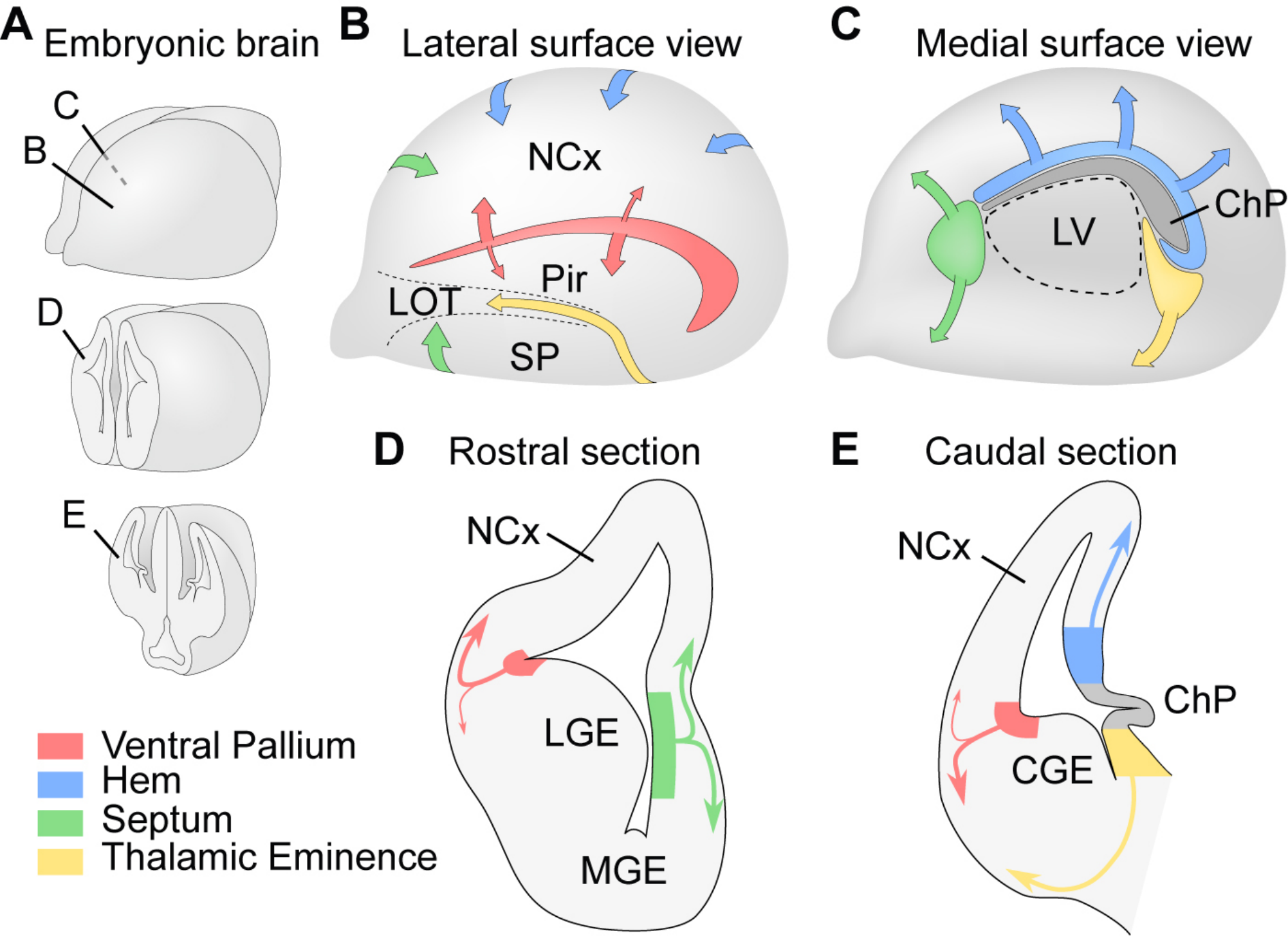
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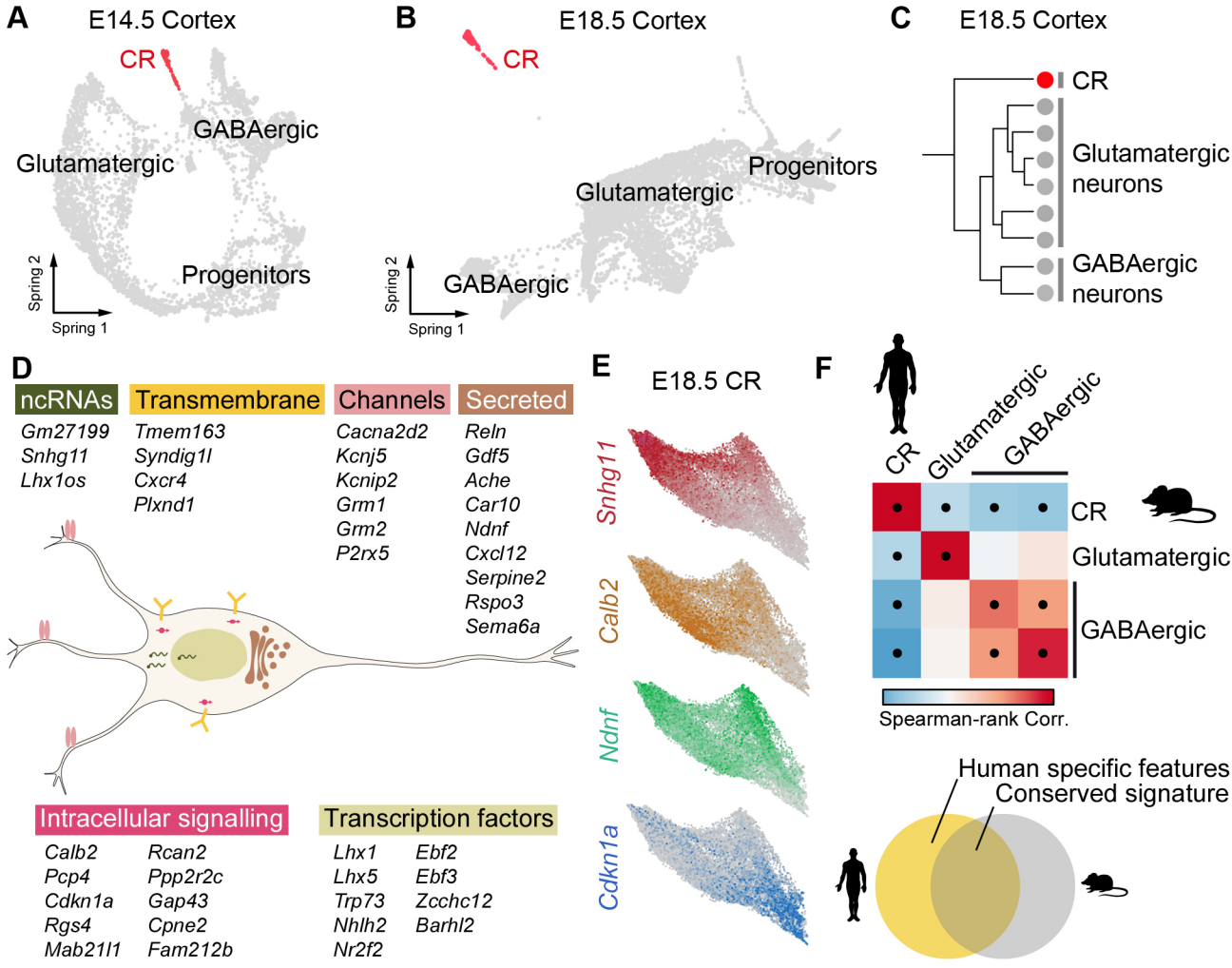
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A
Postnatal brain
NCx
Hipp
NCx
Hipp
B
Pir
B
MZ / Layer I
II-IV
V-VI
CA1
SLM
HF
OML
DG
CA3
C
Cajal, 1891
Tomato Reelin DAPI
MZ
II-IV
Wnt3a-Cre ; Rosa Tomato


A
Embryonic brain
C
B
D
E
B
Lateral surface view
NCx
Pir
LOT
SP
C
Medial surface view
ChP
LV
D
Rostral section
NCx
LGE
MGE
E
Caudal section
NCx
ChP
CGE
Ventral Pallium
Hem
Septum
Thalamic Eminence


A
E14.5 Cortex
CR
GABAergic
Glutamatergic
Progenitors
Spring 2
Spring 1
B
E18.5 Cortex
CR
Progenitors
Glutamatergic
GABAergic
Spring 2
Spring 1
C
E18.5 Cortex
CR
Glutamatergic neurons
GABAergic neurons
D
ncRNAs
Gm27199
Snhg11
Lhx1os
Transmembrane
Tmem163
Syndig1l
Cxcr4
Plxnd1
Channels
Cacna2d2
Kcnj5
Kcnip2
Grm1
Grm2
P2rx5
Secreted
Reln
Gdf5
Ache
Car10
Ndnf
Cxcl12
Serpine2
Rspo3
Sema6a
Intracellular signalling
Calb2
Pcp4
Cdkn1a
Rgs4
Mab21l1
Rcan2
Ppp2r2c
Gap43
Cpne2
Fam212b
Transcription factors
Lhx1
Lhx5
Trp73
Nhlh2
Nr2f2
Ebf2
Ebf3
Zcchc12
Barhl2
E
E18.5 CR
Snhg11
Calb2
Ndnf
Cdkn1a
F
CR
Glutamatergic
GABAergic
CR
Glutamatergic
GABAergic
Spearman-rank Corr.
Human specific features
Conserved signature