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► **To cite this version:**

Frédéric Causeret, Matthieu X Moreau, Alessandra Pierani, Oriane Blanquie. The multiple facets of Cajal-Retzius neurons. *Development* (Cambridge, England), 2021, 148 (11), pp.dev199409. 10.1242/dev.199409 . inserm-03242189

HAL Id: inserm-03242189

<https://inserm.hal.science/inserm-03242189v1>

Submitted on 30 May 2021

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1 The multiple facets of Cajal-Retzius neurons

2

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17 Abstract

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

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

33

34 **Introduction**

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

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

56

57 **Morphology and physiology of Cajal-Retzius neurons**

58 *Location, birth and death of CR neurons*

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

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

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

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

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

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

103 *Neuronal properties*

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

124 CRs are glutamatergic neurons (Del Río et al., 1995; Hevner et al., 2003; Quattrococo and Maccaferri,
125 2014), express a variety of receptors including GABA_A, NMDA, AMPA/Kainate, mGlu and
126 serotonergic receptors (de Frutos et al., 2016; López-Bendito et al., 2002; Martínez-Galán et al., 2001;
127 Schwartz et al., 1998) and receive synaptic contacts from both GABAergic and non-GABAergic neurons
128 onto their somata and dendrites, as demonstrated by electron microscopy and immunohistology (Anstötz
129 et al., 2014; Radnikow et al., 2002). While CRs undeniably exhibit GABAergic post-synaptic currents
130 (PSCs), the existence of functional glutamatergic synapses remains unclear. Several groups performing
131 whole cell recordings in the mouse postnatal neocortex have reported that evoked excitatory PSCs are
132 insensitive to the NMDA and AMPA/Kainate receptor antagonists but fully abolished by GABA_A

133 receptor blockade, indicating that evoked PSCs are strictly GABAergic (Kirmse et al., 2007; Soda et al.,
134 2003; Sun et al., 2019). In terms of spontaneous PSCs, hippocampal and neocortical CRs have only been
135 reported to display spontaneous GABAergic PSCs, confirming that glutamatergic synapses are most
136 likely physiologically silent (Kilb and Luhmann, 2001; Kirmse et al., 2007; Marchionni et al., 2010;
137 Quattrocolo and Maccaferri, 2013; Soda et al., 2003; Sun et al., 2019). However, it should be noted that
138 some studies performed in the mouse, rat or human neocortex reported the existence of weak
139 glutamatergic inputs (Ceranik et al., 2000; Lu et al., 2001; Radnikow et al., 2002; Sun et al., 2019). In
140 these studies, discrepancies further appear as to whether these excitatory PSCs are mediated via NMDA-
141 or both NMDA- and AMPA/Kainate-receptors. It seems that these differences are due to the fact that
142 the expression of functional NMDA and AMPA/Kainate receptors is highly dependent on the species or
143 the genetic background, as demonstrated by comparative studies between rat and humans or between
144 C57BL/6J and ICR mouse strains (Chan and Yeh, 2003; Lu et al., 2001).

145

146 ***Connectivity of CRs***

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

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

163 In the hippocampus, optogenetics and electrophysiological experiments have established SLM
164 neurogliaform and oriens-lacunosum-moleculare interneurons as functional presynaptic inputs for CRs
165 (Quattrocolo and Maccaferri, 2013) and SLM interneurons as post-synaptic targets (Anstötz et al.,
166 2018a; Anstötz et al., 2018b; Quattrocolo and Maccaferri, 2014). Nevertheless, despite recent advances
167 in understanding the hippocampal and neocortical circuits involving CRs, the synaptic function of these
168 cells remains poorly understood and will require further investigation.

169

170 ***Molecular characteristics of CRs***

171 CRs gained significant attention after the discovery that they express high levels of Reelin (RELN), a
172 secreted extracellular matrix protein necessary for the lamination of the neocortex (D’Arcangelo et al.,
173 1995; Ogawa et al., 1995). This discovery shed light on an important developmental role of CRs and
174 provided scientists with a sensitive immunological marker. RELN has since then been widely used to
175 immunolabel CRs. At embryonic stages, the onset of expression appears slightly later in certain
176 subpopulations of CRs than others (Griveau et al., 2010). In addition, although all CRs express *RELN*,
177 it is important to underline that RELN expression is not specific to CRs (Anstötz et al., 2016; Chowdhury
178 et al., 2010; Frade-Pérez et al., 2017; Moreau et al., 2020). For example, *Reln* mRNA can be detected
179 in the cortical plate below the MZ from E12.5 onwards (Yoshida et al., 2006). In addition, from late
180 embryonic stages, the number of interneurons positive for *Reln* within the MZ itself progressively
181 increases (Alcántara et al., 1998; Anstötz and Maccaferri, 2020), which makes it inappropriate to rely
182 on RELN positivity and localization in the MZ to ascertain CRs identity (see Figure 1C).

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

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

198

199 **CR subtypes differ in their origins, migration routes and distribution**

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

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

217 It is the use of genetic fate-mapping in mice in combination with *in utero* electroporation that has
218 allowed the precise sites of CRs production to be pinpointed. In Table 1 we provide a list of all the
219 transgenic mouse lines currently available which can be used to follow CRs through the expression of
220 Cre recombinase, GFP or β -galactosidase. To date, four sources at the borders of the pallium have been
221 precisely described (Figure 2): the cortical hem (Takiguchi-Hayashi, 2004), the ventral pallium (VP,
222 also referred to as PSB, pallial-subpallial boundary) (Bielle et al., 2005), the pallial septum (Bielle et
223 al., 2005), and the thalamic eminence (TE) (Meyer et al., 2002; Ruiz-Reig et al., 2017; Tissir et al.,
224 2009). It is generally accepted that the hem provides the majority of CRs, especially in the dorsal
225 pallium. From these focal sites at the border, but within the pallial anlage, CRs reach the neocortical
226 surface by tangential not radial migration, at least in rodents.

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

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

242 Once generated, CRs migrate tangentially following a complex choreography (Figure 2): VP and
243 septum-derived CRs (identified by the *Dbx1-Cre* mouse line) follow both dorsal and ventral flows
244 (Bielle et al., 2005). Hem-derived CRs (identified by the *Wnt3a-Cre* mouse line) migrate anteriorly and
245 laterally but seem to encounter a semi-permeable lateral boundary, possibly corresponding to the lateral
246 olfactory tract (LOT), resulting in most of them being eventually distributed above hippocampal and
247 neocortical territories (Yoshida et al., 2006). TE-derived CRs (identified by the *$\Delta Np73$ -Cre* mouse line
248 together with septum- and hem-derived CRs) follow an anterior stream along the LOT together with
249 other neurons migrating towards the olfactory bulb (Ruiz-Reig et al., 2017).

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

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

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

274 **CR death: sub-type specific mechanisms and timing**

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

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

299 Within the hippocampal formation, the time course of cell death of CRs appears delayed in the
300 hippocampal fissure and dentate gyrus compared to the subiculum and entorhinal cortex, which display
301 neocortical-like kinetics (Anstötz et al., 2016). The differences go further than a simple variation
302 between the neocortex and hippocampal formation. Comparing the density of Δ Np73-derived
303 (hem/septum) versus Dbx1-derived (VP/septum) CRs along the rostrocaudal axis of the neocortex
304 reveals that the extent of cell death depends on the cortical territories but also on the subtype of CRs:
305 Dbx1-derived CRs decline in numbers as early as P4, especially in rostral regions, whereas Δ Np73-
306 derived CRs mostly decline between P4 and P10 (Ledonne et al., 2016). Moreover, CRs in the medio-
307 rostral cortex undergo death with delayed kinetics with respect to their ontogenetically related
308 equivalents in the dorso-lateral neocortex. These heterogeneities raise the question of the mechanisms

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

312 The time course and rate of apoptosis of hem-derived CRs depends on their final location (hippocampus
313 vs cortex) (Anstötz and Maccaferri, 2020), indicating that the environment represents an important
314 factor controlling their cell death. Although the mechanisms are not yet fully understood, experimental
315 data indicate that electrical activity is a major factor. In the 1990s, *in vitro* pharmacological studies
316 revealed the pro-apoptotic role of electrical activity on CRs (Del Río et al., 1996; Mienville and Pesold,
317 1999), an effect that contrasts with its pro-survival role on other cortical neurons during the same period.
318 Another line of evidence is the observation that CRs survive when cultured in pure hippocampal explants
319 but degenerate in entorhino-hippocampal cocultures, a model that preserves the afferent inputs into CRs
320 (Del Río et al., 1996). These data led to the hypothesis that CR death results from an overload of
321 intracellular calcium via the direct stimulation of their glutamatergic receptors or a global increase in
322 neuronal network activity.

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

336 Δ Np73, the N-terminally truncated version of p73, is specifically expressed by CRs from the septum,
337 TE and cortical hem (Meyer et al., 2002; Tissir et al., 2009). Whereas full-length p73 expression is
338 associated with apoptosis, Δ Np73 is necessary for maintaining neuronal survival (Pozniak et al., 2000)
339 and seems to control the lifetime of CRs since its genetic inactivation leads to a strong reduction in CR
340 numbers at birth (Tissir et al., 2009). This could explain the apparent faster decline of VP-derived CRs
341 as these cells never express Δ Np73 (Ledonne et al., 2016). More recently, the observation that septum-
342 derived CRs - but not hem-derived CRs - survive upon genetic overexpression of the hyperpolarizing
343 potassium channel Kir2.1 demonstrates that a similar input can differentially affect the survival fate of

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

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

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

360

361 **CR functions during development**

362 The principal function attributed to CRs is the control of radial migration through secretion of RELN
363 (D’Arcangelo et al., 1995; Ogawa et al., 1995). RELN is a large glycoprotein of about 400 kDa. Upon
364 binding to one of its two cognate receptors, the very low density lipoprotein receptor (VLDLR) and the
365 apolipoprotein-E receptor type 2 (ApoER2), the disabled-1 (Dab1) adaptor is phosphorylated and
366 subsequently activates various downstream pathways (D’Arcangelo et al., 1999; Hiesberger et al.,
367 1999). Mouse mutants for RELN (*reeler*) or *Dab1* (*scrambler*), present an abnormal layering in the
368 hippocampus, neocortex and cerebellum (D’Arcangelo et al., 1995; Howell et al., 1997; Sheldon et al.,
369 1997). Consistent with this, CR depletion in the hippocampus by genetic manipulation of p73 leads to
370 severe morphological defects (Amelio et al., 2020; Meyer et al., 2004; Meyer et al., 2019).

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

378 2011), dendritogenesis of cortical pyramidal neurons (de Frutos et al., 2016). Furthermore, CR subtype-
379 specific presence and distribution at the surface of the developing cortex participates in early patterning
380 and in the control of the size of cortical areas (Barber et al., 2015; Griveau et al., 2010). This function
381 does not rely on a RELN-dependent mechanism but rather subtype-specific signaling (see below).
382 Importantly, decreased CR density at embryonic stages affects cortical circuits even after CRs have
383 disappeared, supporting a long-term function for CRs despite their transient lifespan (de Frutos et al.,
384 2016).

385 The diversity of CRs in terms of ontogenic origins, migration routes, cell death and survival fate raises
386 the question whether each subtype fulfills a specific function. Patch-clamp experiments followed by
387 biocytin labelling in *Dbx1^{Cre}* mice failed to demonstrate any electrophysiological or morphological
388 difference between VP- and septum-derived CRs compared to the remaining subtypes (Sava et al.,
389 2010). In addition, the RELN-dependent control of cortical lamination is not severely affected upon
390 ablation of specific CR subtypes (Bielle et al., 2005; Griveau et al., 2010; Yoshida et al., 2006),
391 consistent with the fact that all CRs secrete RELN. However, mouse mutants in which septum-derived
392 CRs are ablated display a redistribution of other subtypes, regional changes in cortical neurogenesis,
393 and ultimately an increase in the size of the motor cortex associated with a displacement of
394 somatosensory areas (Griveau et al., 2010). Furthermore, modulation of CR subtypes distribution
395 without affecting their total numbers was shown to affect the size and positioning of higher-order
396 cortical areas (Barber et al., 2015). These data demonstrate that CR diversity is relevant to cortical
397 organization in the tangential dimension and support the hypothesis that CRs send signals to ventricular
398 zone progenitors, either through contact with the glial endfeet in the MZ, or via secreted factors.
399 Although the nature of such signals remains to be established, transcriptomic analyses indicated that it
400 possibly corresponds to the release of subtype-specific morphogens and growth factors (Griveau et al.,
401 2010). It is worth noting that the link between CR subpopulations and cortical regionalization is most
402 likely bidirectional as the patterning genes *Emx1/2* and *Pax6* were shown to be important for CR
403 specification (reviewed in Barber and Pierani, 2016) and the analysis of *Fgf8*, *Emx2*, *Gli3* and *Pax6*
404 mutants revealed differential defects between CR subtypes (Zimmer et al., 2010). These observations
405 led to the proposal that CRs behave as “transient signaling units”, secreting a subtype-specific
406 combination of factors throughout their migration, thus conveying positional information over long
407 distances in the growing cortex and acting as a complement to the well described signaling centers
408 releasing morphogens diffusing over shorter distances (Borello and Pierani, 2010; Griveau et al., 2010).
409 The respective and relative contribution of CR-derived and signaling centers-derived morphogens to
410 cortical regionalization remains to be determined.

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

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

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

426

427 **Molecular profiling redefines CRs**

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

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

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

462 An issue scientists are currently facing is the multiplicity of studies: choosing the relevant datasets and
463 exploring them requires analytic skills and is time consuming. An additional challenge regarding CRs
464 is related to their low density, which in the absence of enrichment methods may lead to very low
465 sampling. Here, we give examples of two mouse datasets – in addition to the aforementioned “1.3
466 million brain cells” dataset - we believe are suited for the study of CRs as they contain a sufficient
467 number of cells sequenced at a sufficient depth: one from E14.5 cortex (Loo et al., 2019, Figure 3A) and
468 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
469 preprint containing nearly 300,000 cells collected from E7 to E18 brains that will certainly prove itself
470 helpful in the deep characterization of CRs (La Manno et al., 2020). As a proof-of-concept that valuable
471 information can be retrieved from such datasets, we present here some analysis focusing on CRs and
472 their heterogeneity (see Supplementary Information). We illustrate in Figure 3A-C the very peculiar
473 clustering of CRs, apart from all other neurons. Lists of genes enriched in CRs can be extracted to
474 characterize in a comprehensive manner what makes CR distinct from other neuronal types (Figure 3D).
475 The high sampling of CRs in the “1.3 million brain cells” dataset (more than 14,000 cells) additionally
476 allows CR diversity to be investigated, although it only contains cells from the neocortex and
477 hippocampus (Figure 3E).

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

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

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

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

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

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

520 **Future challenges and perspectives**

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

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

546 The most fascinating aspect of CRs is certainly their almost purely developmental role. They are a
547 unique example of a cell type that is produced very early during corticogenesis, whose migration and
548 positioning all over the developing forebrain is tightly regulated, that perform essential functions in the
549 establishment of brain architecture, and that is almost completely eliminated once development is over.
550 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)Apic} (Bielle et al., 2005)	Two similar lines also exist : Dbx1 ^{tm1(cre)ERT2Jcor} (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 <i>Fzd10</i> followed by CreER TM	Hem-derived CRs	Dorsal thalamus, cerebellum, dorsal spinal cord, dorsal root ganglia.	Tg(Fzd10-cre/Esrl*)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)ERT2Jspgl} (Abs et al., 2018) and Ndnf ^{tm1.1(cre)Rudy} (Schuman et al., 2019) but CR targeting is unknown.
IG17	Transgene containing 18kb upstream <i>Grm2</i> (mGluR2) followed by GFP-fused <i>IL2RA</i> (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)

553 **Table 1: Mouse lines allowing to follow CR cells.**

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

557 **Figure 1. CRs in the postnatal brain.**

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

569

570 **Figure 2. Origins and migratory routes of CR subtypes.**

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

578

579 **Figure 3. scRNAseq for the study of CRs.**

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

594

595 **Acknowledgements**

596 We apologize to those authors whose work we have omitted due to space limitations. We thank our
597 coworkers in the Pierani lab and Luhmann lab for helpful discussions, especially Prof. Heiko Luhmann
598 and Dr Sergei Kirishuk for critical reading of the manuscript. We thank Giovanni Iacono from the Holger
599 Heyn lab for providing the subset of 14k CR from the 10X Genomics 1.3M neurons dataset. AP is a
600 CNRS investigator, FC is an Inserm researcher, MXM is the recipient of an Allocation Spécifique PhD
601 fellowship from the École Normale Supérieure. This work was supported by grants from Agence
602 Nationale de la Recherche (ANR-15-CE16-0003-01 and ANR-19-CE16-0017-03), Fondation pour la
603 recherche médicale (Équipe FRM DEQ20130326521 and EQU201903007836) to AP, state funding
604 from the Agence Nationale de la Recherche under “Investissements d’avenir” program (ANR-10-IAHU-
605 01) to the *Imagine* Institute and Deutsche Forschung Gemeinsam (BL 1633/1-1) to OB.

606

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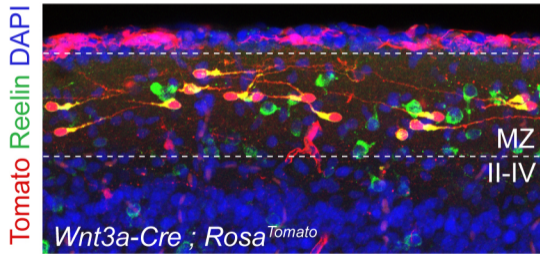
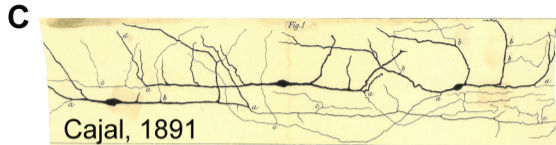
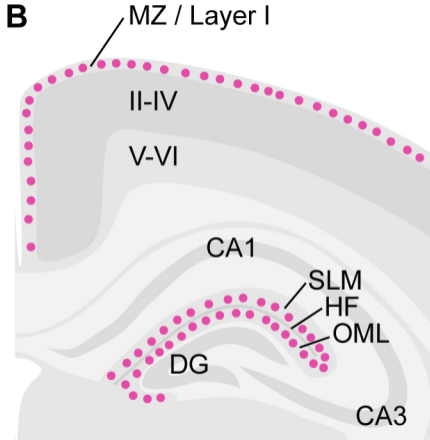
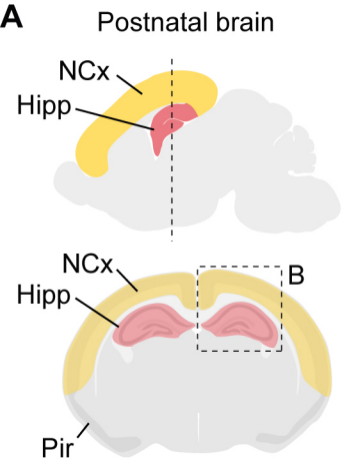
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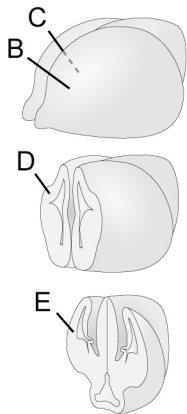
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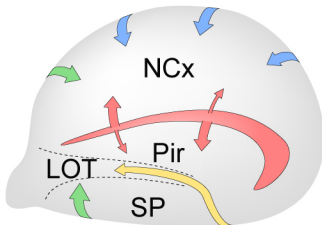
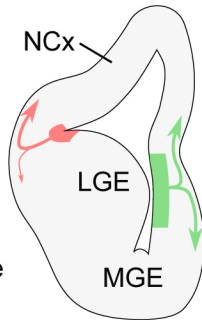
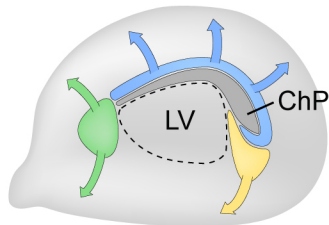
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- 1038



A Embryonic brain

■ Ventral Pallium
■ Hem
■ Septum
■ Thalamic Eminence

B Lateral surface view**D** Rostral section**C** Medial surface view**E** Caudal section