AT THE TOP OF THE INTERNEURONAL PYRAMID – CALRETININ EXPRESSING CORTICAL INTERNEURONS

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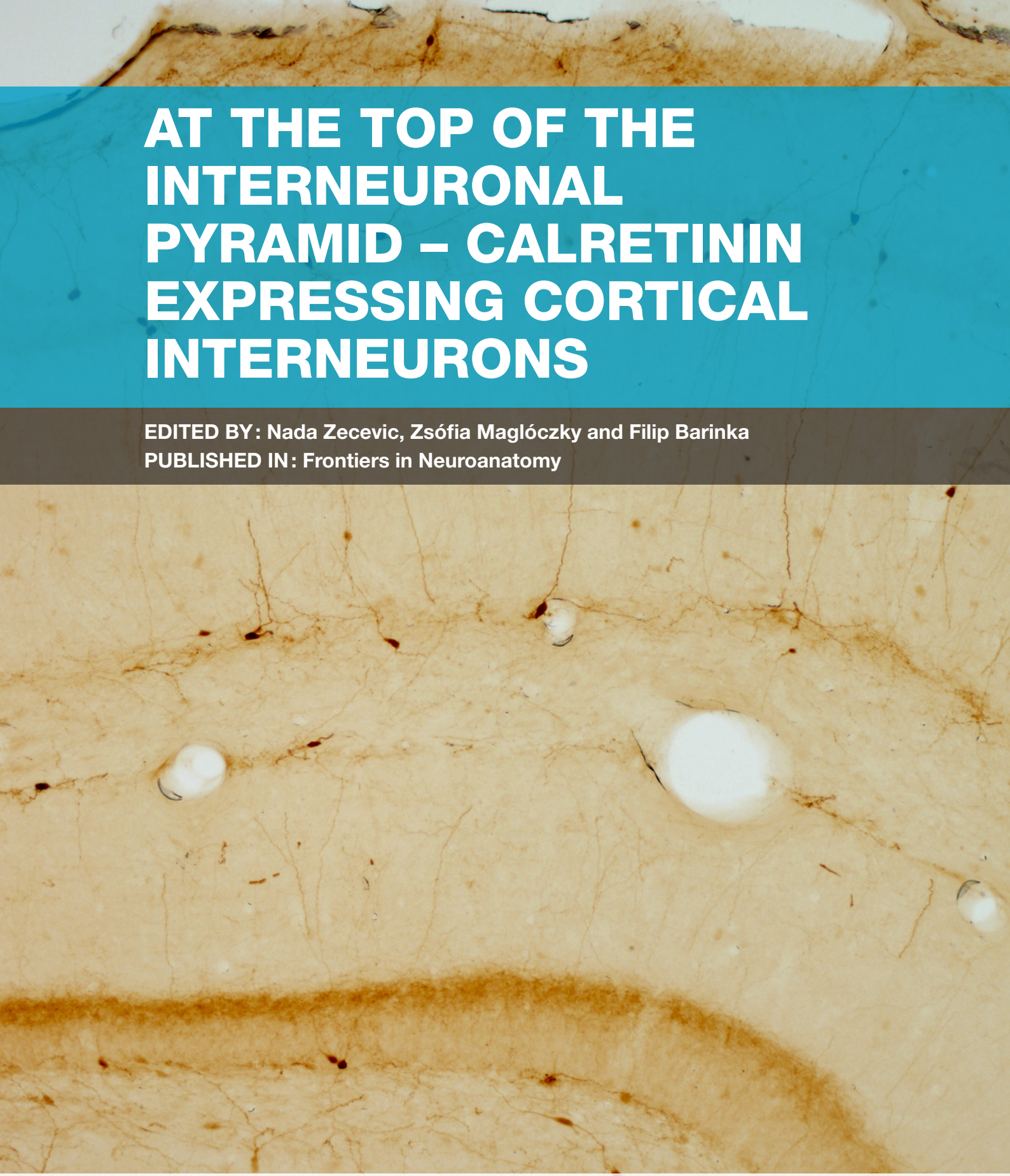

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AT THE TOP OF THE INTERNEURONAL PYRAMID – CALRETININ EXPRESSING CORTICAL INTERNEURONS

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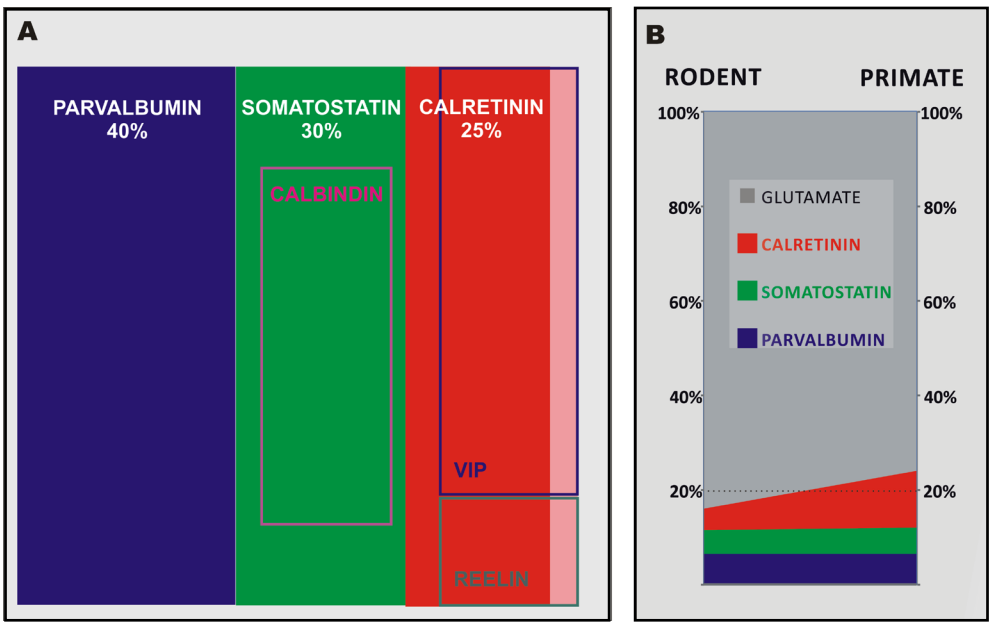

A contribution of main cortical GABAergic neuron subpopulations to total number of GABAergic neurons in rodents and their contribution to total number of neurons in rodents and primates. (A) Panel demonstrates contribution of different subpopulations of GABAergic neurons in rodents, showing that parvalbumin, somatostatin, and calretinin comprise more than 90% of all GABAergic neurons. (B) Panel demonstrates 50% increase of the contribution of cortical GABAergic neurons from rodents (≈16%) to primates (≈24%), which can be attributed to three fold increase in number of calretinin neurons (shown in red; from ≈4% in rodents to ≈12% of the total neuron number in primates). (Ana Hladnik, Domagoj Džaja, Sanja Darmopil, Nataša Jovanov-Milošević and Zdravko Petanjek doi: 10.3389/fnana.2014.00050)

COVER FIGURE:
Light micrograph of a rat hippocampus immunostained for calretinin (CR) shows numerous, morphologically heterogeneous interneurons present in all regions of the hippocampus. In the CA1 region, they are most frequent in the strata radiatum and pyramidale. Their dendrites are smooth or sparsely spiny, run radially in the strata pyramidale and radiatum, and horizontally in the stratum oriens. Numerous multipolar and fusiform CR- positive cells are present in every layer of the dentate gyrus. A dense CR-positive axon-bundle is visible at the top of the granule cell layer, originating from the supramammillary nucleus. The interneurons and their dense, gracile dendritic networks are vulnerable to various neurological disorders, including e.g. epilepsy, ischemia and Alzheimer Disease (Kinga Tóth and Zsófia Maglóczky, doi: 10.3389/fnana.2014.00100)

It is in general well appreciated that the cortical interneurons play various important roles in cortical neuronal networks both in normal and pathological states. Based on connectivity pattern, developmental, morphological and electrophysiological properties, distinct subgroups of GABAergic interneurons can be differentiated in the neocortex as well as in the hippocampal formation. In this E-Book, we are focusing our attention on inhibitory interneurons expressing calcium-binding protein calretinin (CR). The aim of the E-Book is to consolidate the knowledge about this interneuronal population and to inspire further research on the function and malfunction of these neurons, which – functionally – seem to stand "at the top of the pyramid" of cortical interneuronal types.

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EDITORIAL
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Editorial: At the top of the interneuronal pyramid—calretinin expressing cortical interneurons

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Keywords: calretinin, interneurons, cortical development, epilepsy, primate cortex, corticogenesis, calcium-binding proteins

Intracellular protein calretinin (CR) acquired its name based on structural similarity to calbindin D28k and the site of first detection—retina (Rogers, 1987). Soon after this discovery, a basic description of the distribution of CR in rodent brain emerged from the work of several authors (reviewed by Baimbridge et al., 1992; Andressen et al., 1993). In the upcoming years, calcium-binding proteins (CaBP) calretinin, parvalbumin (PV), and calbindin (CB) could be shown to be expressed in largely non-overlapping interneuronal populations. The usefulness of utilizing PV, CB, and CR for categorization of interneuronal subpopulations was validated by gene cluster analysis (Toledo-Rodriguez et al., 2004).

While extensive literature on PV expressing (PV+) interneurons' physiological functions and changes in various pathological conditions have been published, we still have a limited view about the CR+ (and CB+) interneurons. This is quite disappointing, considering their increasing number in primates and various unique features they possess. In the Research Topic "At the top of the interneuronal pyramid—calretinin expressing cortical interneurons," we intended to provide a summary of current knowledge on CR+ cortical neurons and especially to emphasize and discuss those "unique features" of CR+ neurons mentioned above.

In the first article of this compilation, the story begins with a mini-review on protein calretinin from Beat Schwaller (Schwaller, 2014). The Author not only offers a summary of what is known about the expression and physiological role of calretinin in cellular homeostasis, but, to mention one of many interesting aspects, the reader also learns why calretinin "may be a potential therapeutic target for treatment of Huntington disease" (see Dong et al., 2012 for original research report).

Further two original research papers focus on the development of CR+ neurons during the ontogenesis of human cerebral cortex. Miriam Gonzáles-Gómez and Gundela Meyer (González-Gómez and Meyer, 2014) put emphasis on the expression of CR in neuronal progenitors during the early period (between 6 and 14 gestational weeks, gw) of human corticogenesis. At the early embryonic time, the expression of CR is unrelated to the GABAergic neurons and could be seen also in glutamatergic neurons of the preplate and "pioneer" cortical plate. Complementary to this paper, Nevena V. Radonjić with colleagues (Radonjic et al., 2014) describes the pattern of expression of a transcription factor (Gsx2) and its two downstream effectors, (Ascl1 and Sp8) proposed to be involved in the CR cell lineage, in the human fetal cortex at later stadium of corticogenesis, at midgestation (20 gw). The authors reported the co-expression of these factors with CR+ cells that were dividing in the cortical ventricular and subventricular zones (VZ/SVZ). Their results support the hypothesis that the VZ/SVZ, besides the ganglionic eminences in subpallium, is an additional source of interneurons in the primate developing cortex. However, alternative hypothesis have been suggested (Hansen et al., 2013; Ma et al., 2013). In the next—4th article of

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our compilation, review paper from Ana Hladnik and colleagues (Hladnik et al., 2014), authors broadly discuss this controversial topic. They propose that contradictory results about the origin of cortical CR+ interneurons in primates might be partially explained by regional variations in the site of CR+ neurons origin. According to their hypothesis, the main source of CR+ interneurons for the frontal lobe might be the local pallial VZ/SVZ, while subpallial ganglionic eminences supply CR+ interneurons for posterior and lateral regions of primate cerebral cortex.

One fact, which indirectly supports the concept of an additional source of CR+ interneurons in primate cortex, is the significantly higher proportion of CR+ cells compared to non-primate mammals. Another two main classes of interneurons, those containing PV and CB (or somatostatin) do not show such a robust increase in proportion during evolution. In the 5th article of this Research Topic, mini review from Domagoj Džaja and colleagues (Džaja et al., 2014), authors address this topic and the significance of the 5-fold increase of CR+ neurons in the layers II/III of primate cortex in relation to rodents, for the function of neocortical neuronal circuits in primate brain. Importantly, they open and discuss the question of the role of those additional CR+ interneurons in formation of neuronal assemblies.

In the 6th article, Daniela Camillo with colleagues (Camillo et al., 2014) studies the visual response properties of neurons that express or have transiently expressed CR during their development in mouse primary visual cortex. Besides other interesting findings, their results again accentuate the (functionally not well understood) high extent of transient CR expression in pyramidal neuron precursors during corticogenesis.

Although the terms "interneurons" and "local circuits neurons" are often used synonymously, the CR+ interneurons were also shown to participate on long range cortical connectivity: by being targeted via connections from remote cortical areas (Gonchar and Burkhalter, 2003) as well as by sending long range cortico-cortical axons (Tomioka and Rockland, 2007). In the 7th article of our Research Topic, Rastislav Druga with colleagues (Druga et al., 2014) describes the distribution of CR+ neurons in the claustrum of the rat. Interestingly, a small portion of those neurons could be found to send dendrites in the neighboring telencephalic structures (striatum) or the insular cortex. This pattern of interconnectivity is consistent with the concept of claustrum as a global processing and synchrony detection system (Smythies et al., 2014), and implies that CR+ neurons could be directly involved in this function.

In the 8th article of our Research Topic, Bruno Cauli with colleagues (Cauli et al., 2014) provides a closing comprehensive summary of what is known about the anatomy and physiology of bipolar cortical CR+ interneurons. Besides the themes elaborated also in the individual articles of this compilation, additional exciting topics as for example the involvement of CR+ neurons in neurovascular coupling, are reviewed here. Summarizing the available evidence, authors state that probably the bipolar CR+ interneurons are amongst the most complex cells in the cerebral cortex because they seem to be involved in every major mechanism that supports successful neuronal computation. Hence, from this point of view, the heterogeneous population of CR expressing neurons really seems to stand "at the top of the interneuronal pyramid."

And finally, in the last, 9th article of this research topic, a "window" to an important research field—function and malfunction of CR+ interneurons in various neuropsychiatric disorders—is being opened. In this review paper, Kinga Tóth and Zsófia Maglóczky (Tóth and Maglóczky, 2014) discuss the vulnerability of CR+ hippocampal interneurons to epilepsy. They summarize that the sensitivity of CR+ interneurons to epileptic activity plays an important role in the impairment of dendritic inhibition in epilepsy, with potential significant involvement in epileptogenesis. A different situation, with preservation of CR+ interneurons and loss of PV+ and/or CB+ interneurons could be found in other conditions as schizophrenia, bipolar disorder, and major depression (Beasley et al., 2002; Cotter et al., 2002; Sakai et al., 2008). It remains an exciting task for the future to establish a more detailed view of the role of CR+ interneurons in various pathological states.

All the aforementioned contributions are collected also in a single e-book. We hope this may be useful for researchers approaching this research field. More than anything else, we hope to inspire further research on this intriguing neuronal population and on its function and malfunction in neural circuits.

Acknowledgments

At this point, we would like to kindly thank all authors who submitted their articles to Research Topic on CR+ neurons and to all reviewers for their great work. Further, we want to thank the Chief Editor of Frontiers of Neuroanatomy Professor Javier DeFelipe for the unique possibility to publish the collection in this superb journal. And finally, we also would like to acknowledge the excellent support we always got from the Frontiers Editorial Office Team.

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Calretinin: from a "simple" Ca^{2+} buffer to a multifunctional protein implicated in many biological processes

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The hexa-EF-hand Ca^{2+}-binding protein calretinin (CR) is predominantly expressed in specific neurons of the central and peripheral nervous system. However, CR expression is also observed in non-neuronal cells, e.g., during embryonic development and in mesothelioma cells. Of the 6 EF-hand domains, 5 are functional; the first 4 domains form 2 pairs showing high cooperativity within a pair that results in non-linear modulation of intracellular Ca^{2+} signals by CR. EF-hand domain 5 has a low affinity and represents the identified interaction site with CR-binding partners present in mouse cerebellar granule cells. CR binding to other targets including the pore-forming α_1 subunit of the Ca^{2+} channel $Ca_V2.1$, as well as to huntingtin indicates additional Ca^{2+} sensor functions besides the well-known Ca^{2+}-buffering functions. The absence of CR in cerebellar granule cells of $CR^{-/-}$ mice results in increased excitability and altered firing of Purkinje cells and promotes cerebellar 160-Hz oscillations impairing motor coordination. The putative role of CR in neuroprotection is still highly discussed. Altogether, CR emerges as a multi-functional protein also associated with development, i.e., cell proliferation, differentiation, and cell death.

Keywords: calretinin, calcium signaling, calcium sensor, calcium buffer, neuron excitability

BASIC FACTS ABOUT CALRETININ (CR)

Calretinin (CR; human gene symbol: *CALB2*), calbindin D-28k (CB; *CALB1*) and secretagogin (SCGN; *SCGN*) represent the 3 members of the hexa-EF-hand protein family, also named the calbindin sub-family [see recent reviews on CR (Camp et al., 2009), CB (Schmidt, 2012) and SCGN (Alpar et al., 2012)]. They all contain 6 structural motifs named EF-hand Ca^{2+}-binding domains. Each domain consists of an alpha-helix of approximately 10 amino acids, a Ca^{2+}-chelating loop of 12 amino acids and a second alpha-helix that is oriented perpendicular to the first one (for more details on the EF-hand structure, see (Schwaller, 2010). CR (M_r 31 kDa) initially discovered in the retina, thus the name: calcium + retina = CR consists of 271 amino acids in many species and is highly conserved; the number of amino acids varies from 269 (e.g., *Gallus gallus*; Chicken) to 273 (e.g., *Monodelphis domestica*; Gray short-tailed opossum). CR is also expressed in zebrafish (*Danio rerio)* and an invertebrate ortholog named calbindin 53E (*Cbp53E*; previously *calbindin-32*) exists in *Drosophila melanogaster* that shares the highest sequence identity with CR (Reifegerste et al., 1993). In CR, the first 5 EF-hand domains are capable of binding Ca^{2+} ions, while the sixth one is inactive (Stevens and Rogers, 1997; Schwaller et al., 1997). Moreover the Ca^{2+}-binding affinity for site 5 is very low (K_D: 36 μM) (Faas et al., 2007) indicating that this site is expected to be rarely in the Ca^{2+}-bound form in the cytoplasmic compartment except in microdomains close to Ca^{2+} channels. The other 4 functional Ca^{2+}-binding sites form 2 similar pairs likely consisting of domains 1 and 2, as well as 3 and 4 showing strong cooperativity within a pair (Faas et al., 2007). The apparent K_D ($K_{D,\ app}$) for the 4 sites is 1.4–1.5 μM with high cooperativity (n_H of 1.9; for more details on CR's properties, see **Table 1**). This property results in non-linear Ca^{2+} regulation in cells due to the presence of CR. In a situation when the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ is at resting (basal) levels of 50–100 nM, then upon a brief and limited increase in $[Ca^{2+}]_i$, CR behaves like a typical slow-onset buffer (EGTA). However, if the same increase occurs at elevated $[Ca^{2+}]_i$, in the order of 1 μM, when the first site of a pair is in the Ca^{2+}-bound form, cooperativity sets in and CR functions almost like the fast buffer BAPTA (for more details on this behavior, e.g., on the spatiotemporal patterns of IP_3-evoked Ca^{2+} signals, see Dargan et al. (2004) or on CR's role modeled for a train of intracellular Ca^{2+} signals, see Figure 3 in (Schwaller, 2009). Thus, the Ca^{2+}-binding kinetics of CR strongly depends on $[Ca^{2+}]_i$ levels at the time when another increase in $[Ca^{2+}]_i$ occurs. Besides these novel properties of Ca^{2+} binding in a protein, first described for CR, several studies in the 90's reported CR to undergo considerable Ca^{2+}-dependent conformational changes, which indicated that CR might also have "Ca^{2+} sensor" functions like the prototypical sensor calmodulin (CaM). Results in support of CR acting as a Ca^{2+} sensor are presented in Section III.

Up to date, no structural data of full-length CR have been reported. However, the NMR structure of the N-terminal 100 amino acids of rat CR (Palczewska et al., 2001) embracing EF-hand domains 1 and 2 are very similar to the NMR solution structure of the corresponding domains in rat CB (Kojetin et al., 2006). Together with the similar results from limited proteolysis experiments obtained with CR and CB, this suggests that

Table 1 | Properties of calretinin (modified from Schwaller, 2009, 2010, 2012).

General parameters	Value (range)	References/comments
Amino acids	269–273; 271 in most mammals; 310[a]	Reifegerste et al., 1993; Zimmermann and Schwaller, 2002
Molecular mass (M_r)	30–31 kDa	Rogers, 1987
EF-hand domains	6	Rogers, 1987
Functional Ca^{2+}-specific sites	5	Stevens and Rogers, 1997; Schwaller et al., 1997
Identified CR binding partners	α_1 subunit of $Ca_V2.1$, huntingtin	Christel et al., 2012; Dong et al., 2012
METAL BINDING PROPERTIES AND MOBILITY		
$K_{D,\ Ca}$	$K_{D(T)}$ 28 μM $K_{D(R)}$ 68 nM $K_{D(app)}$ 1.4 μM EF5: 36 μM[b]	CR has 2 cooperative pairs (sites EF1-4) with indistinguishable binding properties. In the absence of Ca^{2+}, the first site within a pair is in the tensed (T) state and changes upon Ca^{2+} binding of the first site within a pair to the relaxed (R) state. These results were obtained by flash photolysis experiments Faas et al., 2007
$K_{D,\ Mg}$	4.5 mM	Stevens and Rogers, 1997
$k_{on,\ Ca}$ ($\mu M^{-1}s^{-1}$)	Tensed (T) sites: 1.8 Relaxed (R) sites: 310 EF-hand domain 5: 7.3	Faas et al., 2007
Cooperativity	Yes $n_H \approx 1.3$–1.9	Stevens and Rogers, 1997; Faas et al., 2007
Mobility $D_{Cabuffer}$ ($\mu m^2 s^{-1}$) in H_2O	120 ± 1 (means ± s.e.m.)	Arendt et al., 2013
$D_{10\,ms}$ ($\mu m^2 s^{-1}$)[c]	3.2 (IQR 1.6–5.9)	
INTRACELLULAR CONCENTRATION IN		
Frog saccular hair cells	1.2 mM	Edmonds et al., 2000
Rat outer hair cells	35 μM	Hackney et al., 2005
Rat inner hair cells	20 μM	Hackney et al., 2005
Mouse cerebellar granule cells	30–40 μM[d]; 0.7–1.2 mM[e]	Gall et al., 2003

[a] *Drosophila melanogaster calbindin 53E (previously calbindin-32) shows the highest sequence homology to calretinins of different species.*

[b] *A lower affinity for EF5 (K_D: 0.5 mM) was determined with the flow dialysis method (Schwaller et al., 1997).*

[c] *Diffusion determined in cerebellar granule cell dendrites by an anomalous subdiffusion model.*

[d] *Based on BAPTA concentration (150 μM) needed to restore granule cell excitability in $CR^{-/-}$ cells.*

[e] *Based on numerical simulations of buffered Ca^{2+} diffusion near a single Ca^{2+} channel or a large cluster of Ca^{2+} channels (Saftenku, 2012).*

the overall structure of hexa-EF-hand proteins might be rather similar.

REGULATION OF CALRETININ EXPRESSION

Still relatively little is known on the mechanisms of regulation of CR expression in various tissues; altered CR expression levels have been reported as the consequence of experimental manipulations or are associated with certain diseases in humans and/or animal models of these diseases [for more details, see Schwaller, 2009, 2010, 2012]. Based on the substantial sequence homology in the promoter region including the TATA and CAAT boxes of the human *CALB2* and mouse *Calb2* gene (Strauss et al., 1997), it is reasonable to assume that CR expression is regulated in a similar manner in the two species, although species differences in CR expression have been reported before. Neuron-specific "CR-like" expression of a luciferase reporter gene in cortical cultures is achieved in the presence of the mouse *Calb2* promoter region from −115/+54. The 5′ region of this promoter fragment (−115/−71) selectively binds a nuclear protein present in cerebellar granule cells and contains an "AP2-like" element (−90/−80 bp; **Figure 1**). This element is essential for the neuron-specific reporter expression (Billing-Marczak et al., 2002). The same "AP2-like" element doesn't affect transcriptional activity in human colon carcinoma and mesothelioma cells indicating that CR expression in neurons and non-neuronal cell types is differently regulated (Billing-Marczak et al., 2004). In human colon cancer cells, CR expression is downregulated by butyrate (Marilley et al., 2001), a substance derived from intestinal fermentation of dietary fibers by bacteria. Butyrate, a known modulator of histone acetylation, inhibits the cell cycle and leads to enterocyte differentiation. Of the several putative butyrate-responsive elements (BREs) present in the human *CALB2* promoter, two elements embracing the TATA box act as butyrate-sensitive repressor elements in colon cancer cells, but not in cells of mesothelial origin (**Figure 1**; Haner et al., 2010). This supports the notion of cell type-specific *CALB2* regulation. The rat *Calb2* promoter region contains 3 binding motifs for the transcription factor LEF1/TCF that binds to β-catenin via its N-terminal region (**Figure 1**); β-catenin, not directly binding to DNA, contains a strong transactivation domain and is highly expressed in thalamic neurons. Down-regulation of β-catenin by its negative regulator Axin2 significantly reduces CR expression in cultured rat thalamic neurons indicating that β-catenin is a positive regulator of the *Calb2* gene (Wisniewska et al., 2012). In addition, several transcripts exist from the human *CALB2* gene (Schwaller et al., 1995), which are present in several colon cancer cell lines (Gander et al., 1996) and in tumor tissue from primary colon tumors (Schwaller et al., 1998). One splice variant with deletion of exons 8 and 9 results

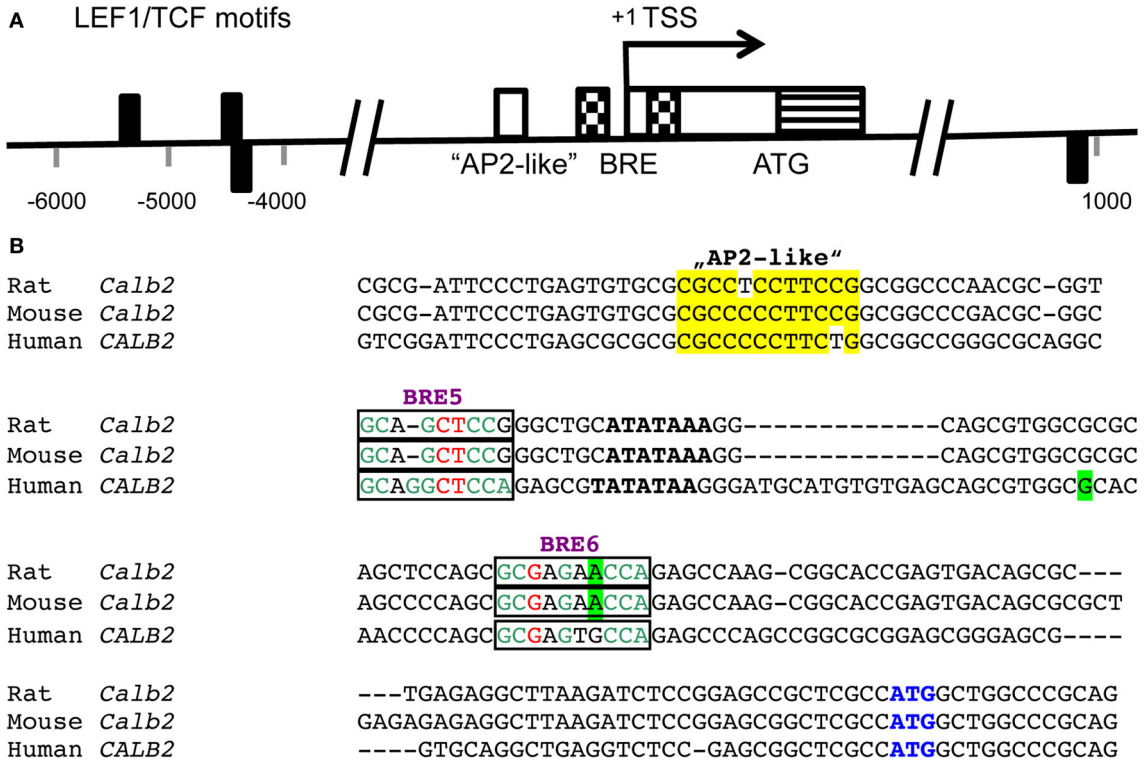

FIGURE 1 | (A) Positions of LEF1/TCF motifs (black rectangles) are present within conserved (human/rat) non-coding regions flanking the transcription start site (TSS; pos. +1) of the CALB2/Calb2 genes and are depicted either above or below the axis depending on the strand (modified from Wisniewska et al., 2012). The consensus sequence recognized by the DNA-binding domain of LEF1/TCFs is WWCAAAG (W is either A or T). Nucleotide numbering is shown with respect to the TSS. In the region −90/−80 of the mouse Calb2 gene, an "AP2-like" element (white box) is responsible for neuron-specific expression of the transcript (Billing-Marczak et al., 2002). A bipartite butyrate-responsive element (BRE; checkered boxes) surrounds the TATA box and the TSS (Haner et al., 2010). The non-coding part of exon 1 is shown as a white box and the coding region including the ATG start codon as a striped box. **(B)** Sequence comparison of the rat, mouse and human Calb2 gene around the TSS. The "AP2-like" region is boxed in yellow. The TATA box is marked in bold and the BREs 5 and 6 acting as butyrate-responsive repressors in colon cancer cells are boxed. The most highly conserved nucleotides in the BREs (consensus sequence: GCGGGCTCCA) are shown in green, the less conserved ones in red and the nucleotides not conforming to the consensus sequence are shown in black. The TSS (+1) is boxed in green. The start codon ATG is marked in blue.

in a truncated protein named CR-22k of 192 amino acids, which is expressed in certain tumors (Schwaller et al., 1998); the other transcript (deletion of exon 8) is currently known to exist only at the level of mRNA (Schwaller et al., 1995) and might have a function as an RNA molecule, possibly as a target for micro (mi)RNA or acting as a long non-coding (lnc) RNA.

THE IDENTIFICATION OF CALRETININ-INTERACTING TARGETS SUPPORTS CR'S ROLE AS A CA^{2+} SENSOR

The finding that purified CR undergoes significant Ca^{2+}-dependent conformational changes *in vitro* (Kuznicki et al., 1995; Schwaller et al., 1997), together with the observation that CR immunoreactivity in chick brainstem auditory neurons changes from diffuse cytosolic staining to intense localized staining beneath the plasma membrane, which occurs together with the onset of spontaneous activity (Hack et al., 2000), suggested already in the late 90's that CR might have additional Ca^{2+}-sensor functions (Billing-Marczak and Kuznicki, 1995). Furthermore, CR was shown to be present in membrane fractions of rat cerebellum (Winsky and Kuznicki, 1995) and to bind to cytoskeletal elements in WiDr colon cancer cells (Marilley and Schwaller, 2000). In support of the presence of CR targets, CR translocates from the cytosol to the nucleus in a vitamin D_3-dependant and/or butyrate-dependent way in colon cancer cells *in vitro*, also indicating that CR might have nuclear interaction partners (Schwaller and Herrmann, 1997). Recently, in two studies CR targets were identified (Christel et al., 2012; Dong et al., 2012) and moreover, the interacting domain of CR that leads to a decreased Ca^{2+}-dependent mobility of CR in cerebellar granule cells was identified (Arendt et al., 2013). A yeast-two-hybrid screen with CR as bait, identified a consensus, strongly basic peptide sequence H(R/K)HRRR(E/D) as a putative CR-binding (CRB) motif (Christel et al., 2012). This motif is present in multiple copies (CRB1-5) in the cytoplasmic linker region between domains II and III of the channel-forming alpha 1 subunit of the high-voltage activated Ca^{2+} channel $Ca_V2.1$ (P/Q type). This channel is regulated in a dual fashion by Ca^{2+} ions, showing both, Ca^{2+}-dependent inactivation (CDI) and Ca^{2+}-dependent facilitation (CDF); both mechanisms influence synaptic plasticity in the nervous system. In cells expressing $Ca_V2.1$ *in vitro*,

co-expression of CR inhibits CDI and enhances CDF via a direct interaction with the $\alpha_1$2.1 subunit. The channel subunit $\alpha_1$2.1 co-immunoprecipitates with CR antibodies using either extracts from HEK293T cells transfected with CR and Ca_V2.1 or mouse cerebellar extracts. In mouse cerebellum, both CR and Ca_V2.1 are strongly expressed in granule cells and the absence of CR in $CR^{-/-}$ mice causes impairment in motor control (Schiffmann et al., 1999). This impairment is essentially caused by CR's absence in granule cells, since the motor phenotype can be rescued by selective re-expression of CR in granule cells (Bearzatto et al., 2006). Thus, the direct modulation of Ca_V2.1 by CR affects intracellular Ca^{2+} signaling and probably also neuronal excitability via a mechanism that is different from CR's previously proven Ca^{2+} buffering function as discussed in Section IV. The interaction between CR and interacting partners likely including Ca_V2.1 was studied in granule cells by fluorescence recovery after photobleaching (FRAP). The diffusion of fluorescence-labeled CR molecules is much slower than the one of freely diffusible molecules (fluorescein dextrans) of comparable size (Arendt et al., 2013). Moreover, during a burst of action potentials (APs) that leads to an increase in dendritic $[Ca^{2+}]_i$, CR's mobility is further decreased, indicative of Ca^{2+}-dependent interactions. Addition of a peptide consisting of EF-hand 5 of CR to granule cells, considerably increases CR's mobility implicating that the CR interactions occur mainly via the region of EF-hand 5, the Ca^{2+}-binding site with very low affinity. Estimations on the density (concentration) of Ca_V2.1 channels and CR in granule cells indicate that channel numbers are too low to account for the strong effect on CR's mobility implicating additional, yet unidentified CR-binding partners in these neurons. A binding partner interacting with CR was found to be huntingtin (Htt), identified by tandem affinity purification (Dong et al., 2012). Binding to CR is even stronger with a mutant form of Htt characterized by a polyglutamine (polyQ) region that is typical for Huntington's disease (HD). In neuronal cultures, CR colocalizes with Htt and a CR/Htt complex can be isolated by co-immunoprecipitation. In CR-overexpressing HEK293 cells, levels of phosphorylated AKT (p-AKT) are increased. At the functional level, CR overexpression reduces mHtt-related H_2O_2 cytotoxicity in various HD *in vitro* models. This might be directly linked to CR's capacity to decrease $[Ca^{2+}]_i$ in these cells and/or to indirectly increase levels of p-AKT considered as a pro-survival signal. On the other hand, CR down-regulation by shRNA enhances mHtt-mediated neuronal cell death. Based on their findings, the authors conclude that "CR may be a potential therapeutic target for treatment of HD." A link between CR and p-AKT was reported before; increased expression levels of CR strongly correlate with increased resistance to asbestos cytotoxicity in immortalized Met-5A mesothelial cells (Henzi et al., 2009). This protective effect is abrogated in the presence of phosphatidylinositol 3-kinase (PI3K) inhibitors, in support of the above findings that increased PI3K/AKT signaling (increased p-AKT) caused by CR up-regulation favors cell survival. Thus, in the case of CR-expressing mesothelial and mesothelioma cells, CR or more precisely its down-regulation, might be viewed as a potential new target/strategy for malignant mesothelioma therapy (Blum and Schwaller, 2013).

THE EFFECT OF CALRETININ ON INTRACELLULAR CA^{2+} SIGNALING

The particular Ca^{2+}-binding properties of CR together with its mobility differently affect intracellular Ca^{2+} signals, however only to a measurable extent, if present at a sufficiently high concentration, typically in the range of tens of μM in neurons. Generally, lower CR concentrations ($\approx$1 μM) don't affect Ca^{2+} signals and e.g., don't protect PC12 cells against Ca^{2+} overload induced by ionophore treatment or trophic factor deprivation (Kuznicki et al., 1996). Effects of CR on Ca^{2+} signals are often deduced from comparing signals in neurons from WT and $CR^{-/-}$ mice (Schmidt et al., 2013) or when overexpressing or down-regulating CR in cell culture models (Billing-Marczak et al., 1999; D'Orlando et al., 2001; Pecze et al., 2013). CR's particular properties, i.e., its low Ca^{2+} occupancy at resting $[Ca^{2+}]_i$ together with the high cooperativity resulting in non-linear binding properties in a setting in which neurotransmitter release depends supralinearly on Ca^{2+} (e.g., in parallel-fiber (PF) terminals onto Purkinje cells) result in considerable nanodomain Ca^{2+}-buffering by CR. As a consequence, a minor increase in the amplitude of AP-evoked Ca^{2+} signals in $CR^{-/-}$ PF boutons results in a considerably higher release probability (Schmidt et al., 2013). CR-deficient cerebellar granule cells are characterized by faster APs and, when electrically stimulated to generate repetitive spike discharges, show enhanced frequency increase with injected currents, i.e., increased excitability (Gall et al., 2003). The excitability can be reverted to the situation seen in WT cells, by loading the cells with the fast buffer BAPTA (150 μM) strongly indicating that the "fast" Ca^{2+} buffering function of CR is most likely responsible for limiting granule cell excitability. From these experiments it was also deduced that the CR concentration in these neurons is in the order of 40 μM, based on CR's 4 high-affinity Ca^{2+}-binding sites; this estimation is in line with modeling studies on CR function (Roussel et al., 2006). However, other models taking into account CR's cooperativity of Ca^{2+} binding resulting in a delayed equilibration with Ca^{2+} predict the concentration of CR to be even higher, in the order of 0.7–1.2 mM (Saftenku, 2012), a value estimated to be present in frog saccular hair cells (Edmonds et al., 2000). However, in this model the modulation of the main voltage-activated Ca^{2+} channel in granule cells, Ca_V2.1, by CR (Christel et al., 2012) was not taken into account. Thus, the precise concentration of CR in granule cells has to be determined yet, possibly by a *in situ* calibration method as previously used for the determination of the concentration of CB ($\approx$40 μM) in hippocampal granule cells (Muller et al., 2005) or of PV in DG basket cells (11.9 $\pm$ 1.6 μM) or in cerebellar basket cells (563 $\pm$ 66 μM) (Eggermann and Jonas, 2012). The particular biophysical properties of CR also acting as a slow-onset Ca^{2+} buffer are best appreciated from studies in *Xenopus* oocytes (Dargan et al., 2004). Photo-release of inositol 1,4,5 triphosphate (IP_3) evokes Ca^{2+} signals that are differently modulated by endogenous or synthetic Ca^{2+} buffers (Dargan and Parker, 2003). In the presence of slow buffers such as PV or EGTA, global Ca^{2+} signals are fragmented into local Ca^{2+} puffs, resulting from Ca^{2+} release from discrete clusters of IP_3 receptors, while low concentrations of fast buffers (CB, BAPTA) decrease the amplitude of Ca^{2+} signals and favor

"globalization" of spatially uniform Ca^{2+} signals, in particular, at high $[IP_3]$. Interestingly, puffs are observed in the presence of CR at low stimulation intensities, i.e., at low $[IP_3]$, an effect never occurring in the presence of CB or BAPTA. Thus, under conditions of small elevations in $[Ca^{2+}]_i$ from resting Ca^{2+} levels, CR has properties of a slow Ca^{2+} buffer such as PV or EGTA.

CALRETININ EXPRESSION IS LINKED TO NEURONAL DEVELOPMENT

The detailed analyses of temporal and spatial expression of CR in the brain is the major focus of this Frontiers series, has been summarized in several papers and reviews Arai et al. (1991); Jacobowitz and Winsky (1991); Hof et al. (1999); Barinka and Druga (2010) and is thus not covered in this mini-review. Recent findings on CR expression (often transient) and neurogenesis are briefly summarized. Olfactory receptor neurons are generated throughout lifetime and are characterized by a short period of CR expression just before these neurons are fully mature (Wei et al., 2013), yet the functional significance is currently unknown. Also mouse adult hippocampal neurogenesis, more precisely, the early postmitotic stage of dentate gyrus (DG) granule cell development is characterized by transient CR expression (Brandt et al., 2003). This stage coincides with onset of differentiation and absence of CR in the immature early postmitotic granule cells of $CR^{-/-}$ mice (systematic name: $Calb2^{tm1Map}$) "causes an early loss in proliferative capacity of the subgranular zone that is maintained into adult age, when it has a further impact on the migration/survival of newborn granule cells" (Todkar et al., 2012). Interestingly, when in WT mice newborn cells are functionally integrated in the DG granule cell network, CR expression stops and is changed to CB, the typical marker for adult DG granule cells. The functional consequences of this swap from CR to CB expression for granule cell physiology are currently unknown, as well as the mechanisms that lead to such a change. Nonetheless, it indicates Ca^{2+}-binding protein-specific functions that cannot be shared and/or substituted even by apparently very similar proteins such as CB and CR.

WHAT IS THE PHYSIOLOGICAL ROLE OF CALRETININ?

As was previously reported for CB (Schmidt, 2012), CR certainly has more than one function, depending on various parameters including cell type (neurons vs. non-excitable cells including tumor cells), stages of development (adult vs. developmental stages) and probably also different neuronal subtypes. For some proposed roles of CR, e.g., a role in neuroprotection, the proportion of reports, mostly obtained in correlative studies, in favor or against such a role is almost 50:50 [for more details, see (Schwaller, 2009, 2010)], clearly necessitating more studies directly addressing this putative function of CR. Here, just the most important results obtained in $CR^{-/-}$ mice are summarized. The decreased LTP in the DG is thought to be the result of an increased excitatory drive from CR-depleted mossy cells onto hilar interneurons (Schurmans et al., 1997). Most findings on the function of CR are derived from studies in the cerebellum, where CR is expressed in cerebellar granule cells. Their increased excitability in the absence of CR (Gall et al., 2003) is linked to the altered firing properties (Cheron et al., 2000) and likely Ca^{2+} homeostasis of Purkinje cells and the emergence of cerebellar 160-Hz oscillations (Cheron et al., 2004) that result in impairment in motor coordination (Schiffmann et al., 1999), for more details, see Schwaller (2009). Thus, CR expression in granule cells appears necessary for correct computation that is crucial for the coding and storage of information in the cerebellum. Of note, there is currently no data available on CR's function in cortical interneurons, e.g., derived from $CR^{-/-}$ mice. I am also not aware that anybody has attempted to manipulate CR expression levels of cortical interneurons, e.g., by shRNA and to investigate the functional consequences of CR down-regulation.

In CR-expressing mesothelial cells, CR down-regulation causes a G_1 block and in mesothelioma-derived cells leads to apoptosis via strong activation of the intrinsic caspase 9-dependent pathway (Blum and Schwaller, 2013). A rather similar effect is also seen in CR-expressing colon cancer cells (Gander et al., 1996) indicating a role for CR in cell cycle regulation, proliferation, possibly differentiation and cell death. These findings are in line with transient CR expression during development, whether in the nervous system or in other tissues including mesenchymal tissue (Gangji et al., 1994). In summary, we have just started to unravel the likely many functions of CR in different settings and there are still plenty of interesting aspects on CR's function(s) to be discovered.

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Dynamic expression of calretinin in embryonic and early fetal human cortex

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Calretinin (CR) is one of the earliest neurochemical markers in human corticogenesis. In embryos from Carnegie stages (CS) 17 to 23, calbindin (CB) and CR stain opposite poles of the incipient cortex suggesting early regionalization: CB marks the neuroepithelium of the medial boundary of the cortex with the choroid plexus (cortical hem). By contrast, CR is confined to the subventricular zone (SVZ) of the lateral and caudal ganglionic eminences at the pallial-subpallial boundary (PSB, or antihem), from where CR+/Tbr1- neurons migrate toward piriform cortex and amygdala as a component of the lateral cortical stream. At CS 19, columns of CR+ cells arise in the rostral cortex, and contribute at CS 20 to the "monolayer" of horizontal Tbr1+/CR+ and GAD+ cells in the preplate. At CS 21, the "pioneer cortical plate" appears as a radial aggregation of CR+/Tbr1+ neurons, which cover the entire future neocortex and extend the first corticofugal axons. CR expression in early human corticogenesis is thus not restricted to interneurons, but is also present in the first excitatory projection neurons of the cortex. At CS 21/22, the cortical plate is established following a lateral to medial gradient, when Tbr1+/CR- neurons settle *within* the pioneer cortical plate, and thus separate superficial and deep pioneer neurons. CR+ pioneer neurons disappear shortly after the formation of the cortical plate. Reelin+ Cajal-Retzius cells begin to express CR around CS21 (7/8 PCW). At CS 21–23, the CR+ SVZ at the PSB is the source of CR+ interneurons migrating into the cortical SVZ. In turn, CB+ interneurons migrate from the subpallium into the intermediate zone following the fibers of the internal capsule. Early CR+ and CB+ interneurons thus have different origins and migratory routes. CR+ cell populations in the embryonic telencephalon take part in a complex sequence of events not analyzed so far in other mammalian species, which may represent a distinctive trait of the initial steps of human corticogenesis.

Keywords: preplate, pioneer cells, cortical plate, regionalization, lateral migratory stream, ganglionic eminence

INTRODUCTION

The calcium-binding protein calretinin (CR) is a multifunctional protein involved in a variety of activities in the developing and adult brain (Schwaller, 2014). In the adult cortex, CR is mainly expressed in GABAergic interneurons. In most mammalian species, CR+ neurons are concentrated in layers II and III, where they display a bipolar or bitufted, vertically aligned morphology, while multipolar morphologies are observed in deeper layers (Gabbott et al., 1997; Hof et al., 1999; Barinka and Druga, 2010). The double-bouquet cells, an interneuron type characteristic of primate cortex, co-express CR and calbindin (CB; Del Rio and DeFelipe, 1997). In adult human cortex, CR is also present in specific subsets of pyramidal cells, such as the pyramidal-shaped neurons in layers V and VI of entorhinal cortex (Mikkonen et al., 1997), or pyramidal cells in layer V of the anterior cingulate cortex (Hof et al., 2001).

In the rodent, virtually all interneurons have a subpallial origin (reviewed in Welagen and Anderson, 2011). In mice, the majority of CR+ interneurons derive from the caudal ganglionic eminence (CGE; Nery et al., 2002; Xu et al., 2004). These CGE-derived interneurons display bipolar morphologies and may colocalize CR and vasoactive intestinal peptide (VIP; Miyoshi et al., 2010; Lodato et al., 2011). A less common CR+ interneuron subtype with a multipolar morphology co-expresses CR and somatostatin, and may have its origins in the medial ganglionic eminence (MGE; Sousa et al., 2009). The lateral ganglionic eminence (LGE) seems to play a minor role in the generation of interneurons compared to MGE and CGE (see Welagen and Anderson, 2011).

The developmental origins of human interneurons are controversial. For many authors, human and monkey GABAergic interneurons have a double origin, with large proportions deriving from neocortical ventricular and subventricular zones (SVZ), in addition to a lineage originating from ganglionic eminences (GE; Letinic et al., 2002; Meyer et al., 2002b; Rakic and Zecevic, 2003; Petanjek et al., 2009a,b; Jakovcevski et al., 2011; Zecevic et al., 2011; Reinchisi et al., 2012; Al-Jaberi et al., 2013). Recent papers, however, reported that the vast majority of human cortical interneurons are produced in the GE, including a large contribution from non-epithelial SVZ stem cells of the CGE (Hansen et al., 2013; Ma et al., 2013).

The present report is focused on the early period of human corticogenesis, which precedes generation and migration of the large varieties and numbers of interneurons. The human cortex is unique insofar as it is the substrate of our cognitive faculties,

and displays an extraordinary structural and functional complexity. It is becoming evident that the mouse, the currently prevalent brain model, is evolutionary too divergent from human to serve as a paradigm for human cortex development. Recent reconstructions of the phenotype of the hypothetical placental ancestor suggested that its brain was gyrencephalic (O'Leary et al., 2013), to the point that the smooth mouse brain may represent a secondary lissencephaly as the result of a phenotypic reversal and a shift toward miniaturization (Kelava et al., 2013). The embryonic and early fetal period sets the framework and pace for the subsequent patterning, proliferation and differentiation events of the cortex. The description and neurochemical definition of its first cellular components are thus crucial for understanding how the human brain is built. Since CR marks early appearing neurons in the human telencephalon (Meyer et al., 2000; Meyer, 2007), co-expression of CR with other proteins may define their possible activities and origins in the initial steps of corticogenesis. In particular, we analyze co-expression of CR and Tbr1, a T-box transcription factor expressed in postmitotic glutamatergic neurons with a pallial origin (Bulfone et al., 1999; Puelles et al., 2000; Hevner et al., 2001). Other proteins co-expressed with CR in the embryonic cortex are Reelin, the extracellular matrix protein secreted by Cajal-Retzius cells (Meyer et al., 1999; reviewed by Tissir and Goffinet, 2003), and GAD (glutamate decarboxylase, the enzyme that catalyzes the decarboxylation of glutamate to GABA). The aim of our analysis is to reconstruct the chain of events that lead to the formation of the cortical plate (CP), the precursor of the adult cortex, in the embryonic human brain.

MATERIALS AND METHODS

The embryonic and early fetal brains, between 6 and 14 postconceptional weeks (PCW), were the same used in previous studies (Meyer et al., 2000, 2002a,b, 2003). They were obtained after legal abortions following national guidelines in our respective countries, under the supervision of the Ethical Committee of the University La Laguna. The embryos (6, 6.3, 6.5, 7, 7.3, 7.5, 8, 8.5 PCW), were staged according to Carnegie stages (CS) defined by O'Rahilly and Müller (1994). The embryonic heads and fetal brains were fixed in Bouin or 4% paraformaldehyde, embedded in paraffin, and cut in a mostly coronal plane into 7 or 10 μ-thick serial sections. The classification into CS was found more reliable for cortical development than the reported gestational or postconceptional age, where one or two days can result in significant changes of cortical structure. We re-examined previously stained material, in particular immunostaining for CR and CB, and processed unstained sections for Tbr1, Reelin, GAD and PCNA. Double-staining was performed by sequential two-color immunohistochemistry, and confocal microscopy.

IMMUNOHISTOCHEMISTRY

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

SEQUENTIAL TWO COLOR IMMUNOSTAINING

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

DOUBLE IMMUNOFLUORESCENCE

Mouse monoclonal anti- CR or anti-Reelin antibody 142 were mixed with rabbit polyclonal anti-Tbr1 or anti-GAD antibodies and sections were incubated overnight at room temperature. Then the secondary biotinylated anti-mouse IgG antibody (1:400, Amersham) and cyanine-3-coupled anti-rabbit IgG antibody (1:400, Amersham) were incubated for 1 h at room temperature in the dark, followed by cyanine-2 dye conjugated streptavidin (1:400, Amersham) for 1 h. Nuclei were stained with DAPI. Sections were washed in TBS and coverslipped with DABCO (1%) and glycerol-PBS (1:1). Negative controls omitted the primary antibodies. Fluorescence immunosignals were obtained using a Fluoview 1000 laser scanning confocal imaging system (Olympus Optical).

ANTIBODIES USED

Mouse monoclonal anti-reelin antibody 142 (IgG1, 1:500, de Bergeyck et al., 1998; gift of A. Goffinet),1/500; Mouse monoclonal anti-CR antibody Swant, 6B3, 1/200; Rabbit polyclonal anti-CR, Swant, 7699/4, 1/3000; Mouse monoclonal antibody anti-PCNA, Thermo Scientific, Ab-1 (clone PC10) 1/1000; Rabbit polyclonal anti-CB, Swant, CB-38a, 1/7000; Rabbit polyclonal anti-Tbr1, Abcam, ab31940, 1/300; Rabbit polyclonal antibody anti-GAD 65/67, Abcam, ab49832, 1/1000; Rabbit polyclonal B3 anti-Dab1 antibody, 1/100 (gift of B. Howell).

RESULTS

CS17: CR AND CB ARE EXPRESSED AT OPPOSITE BOUNDARIES OF THE CORTEX

In the earliest stage studied here, CS 17 (ca 6 PCW), CR (**Figure 1A**), and CB (**Figure 1B**) were expressed in distinct sectors of the telencephalon. High expression of CB was in the neuroepithelium at the medial edge of the cortical primordium, at the

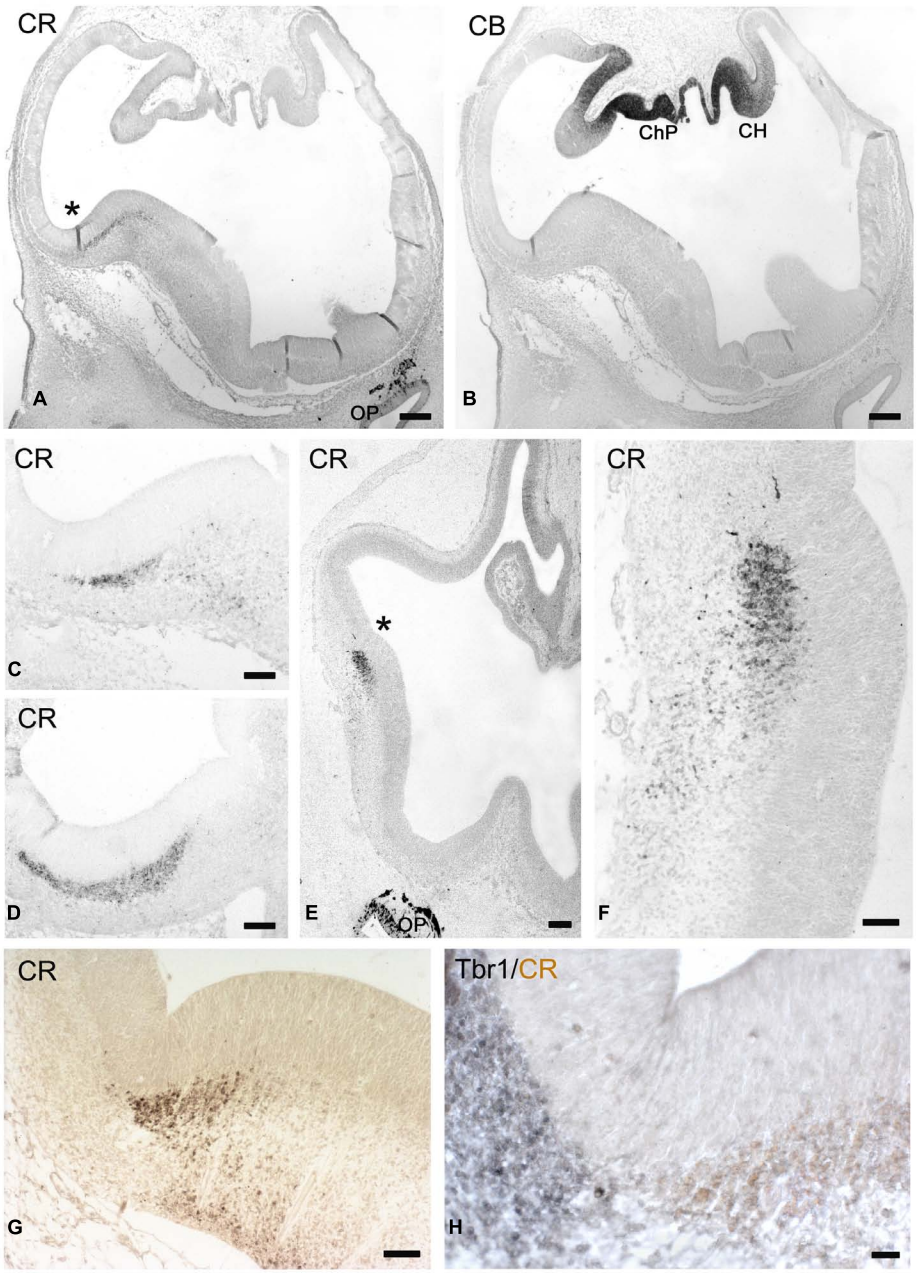

FIGURE 1 | (A,B) Low magnification views of human telencephalon at CS17 stained for CR **(A)** and CB **(B)**, which marks the cortical hem (CH). ChP: choroid plexus. **(B)** These coronal sections are slightly oblique, with the right side being more rostral than the left one. **(C)** Shows the CR+ patch in the lateral ganglionic eminence. The cortico-striatal angle is marked by an asterisk in **(A)**. **(D)** Shows the CR+ patch at the level of the caudal ganglionic eminence. **(E–H)** CS18. **(E)** Low magnification view, with the asterisk marking the rostral cortico-striatal angle. **(F)** The CR+ patch in the lateral GE and migration toward the paleocortex. **(G)** A more caudal view of the CR+ patch in the ganglionic eminence, and the ventral migration of the lateral cortical stream. **(H)** Two-color immunostaining of the pallial-subpallial boundary (PSB), with Tbr1 expression (gray) in its cortical aspect, and CR positivity (light brown) in its subpallial sector. Note the intense CR-positivity in the olfactory pit (OP) and its derivatives in **(A,E)**. Scale bars: 100 μm in **(A,B,E)**; 50 μm in **(C,D,F,G,H)**.

boundary with the choroid plexus anlage, known as the cortical hem, with the developing choroid plexus displaying the strongest CB-intensity (**Figure 1B**). By contrast, CR was present in the dorsal aspect of the LGE near the cortico-striatal angle, or PSB, while the remainder of the GE was CR-negative (we follow the terminology of Pauly et al., 2014). Here, CR-expression was detected in the SVZ along the entire rostro-caudal extent of the GE; few scattered CR+ cells with migratory morphologies were observed in the mantle zone lateral and ventral to the GE, which corresponds to the olfactory paleocortex at rostral levels (**Figure 1C**), and the

amygdala at the caudal end (**Figure 1D**). The patch of CR+ SVZ was larger in the CGE (**Figure 1D**). CR positivity was not yet observed in the incipient cortical preplate (at this moment rather a narrow marginal layer), which was occupied only by few small Reelin+/CR- cells representing early born Cajal-Retzius cells just outside the cortical neuroepithelium. Both the cortical hem and the PSB were proposed to be putative signaling centers (Grove et al., 1998; Assimacopoulos et al., 2003; Subramanian et al., 2009), and it is interesting that CB and CR are almost complementary in defining the opposite extremities, hem and antihem, respectively, of the cortical primordium.

CS 18: CR IN THE LATERAL CORTICAL STREAM

At CS 18 (ca 6.3 PCW; **Figures 1E–H**) the cortico-striatal angle was sharper and thus better defined than at previous stages, and the bulge of the GE was larger and more rounded (compare **Figures 1A,G**, from equivalent levels). Tbr1 marked the lateral, pallial side and CR the medial, subpallial side of the PSB, with a sharp border between them (**Figure 1H**). A SVZ defined by Tbr1+ expression was visible in the cortex lateral to the PBS. The CR+ SVZ of the LGE gave rise to a stream of CR+ neurons that descended toward the olfactory cortex rostrally (**Figures 1E,F**), and to the amygdala caudally, although apparently not dorsally into the cortex (**Figures 1F,G**), forming part of the lateral cortical stream (LCS) of Bayer and Altman (1991, 2006). At even more rostral levels (not shown), CR+ cells migrated ventrally to the retrobulbar area, close to the olfactory bulb anlage. At this moment, the neuroepithelium of the olfactory pit and the olfactory nerve expressed the highest levels of CR (**Figures 1A,E**). The only distinct postmitotic cell population in the early preplate/marginal layer were Reelin+ Cajal-Retzius cells (not shown) which did not yet express CR.

CS 19: CR AND Tbr1 IN THE INITIAL PREPLATE

Carnegie stages 19 (ca 6.5 PCW) was examined in a particularly well preserved case cut in a horizontal plane (**Figure 2**). This plane allowed us to directly address rostro-caudal differences and reconstruct the three-dimensional organization of the telencephalon at this stage. **Figures 2A,B** shows low magnification views of two dorsal levels of the cortex stained for Tbr1 (A) and CB (B). **Figure 2B** shows the intensely CB+ choroid plexus, and the adjacent hem regions rostrally and caudally to the choroid plexus, which were moderately CB+ and gave rise to a few CB+ cells in the marginal layer, possibly representing hem-derived Cajal-Retzius cells. **Figure 2C** is a more ventral section at the level of the lateral and caudal GEs, the SVZ of which was almost entirely CR+, but Tbr1- (not shown). Examination of sections at levels A and

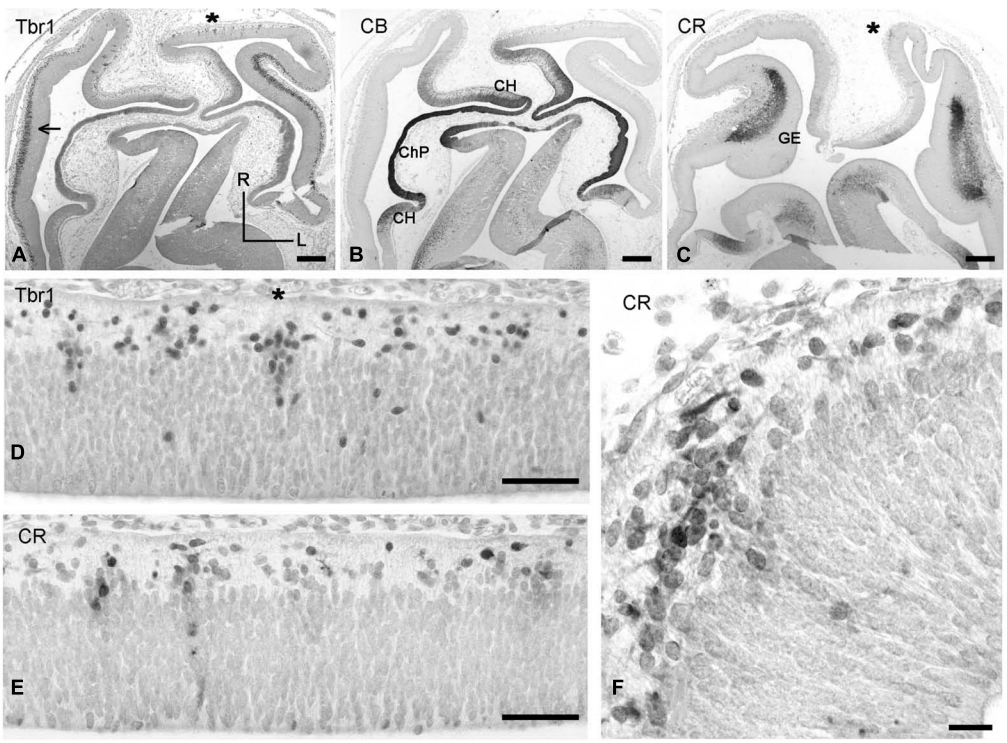

FIGURE 2 | (A–C) Low magnification views of horizontal sections through CS 19 brains, stained for Tbr-1 **(A)**, CB **(B),** and CR **(C)**. **(A,B)** Are at a level dorsal to the GE, while **(C)** is at the level of the GE. R, rostral, L, lateral. In **(A)**, the dorsolateral cortex displays a Tbr1+ SVZ (arrow). **(B)** CB defines the choroid plexus anlage (ChP) and adjacent cortical hem (CH). **(C)** CR stains the SVZ of the ganglionic eminences (GE). Fainter CR positivity is also observed in the cortical hem. **(D,E)** Two adjacent dorsal sections showing vertical columns of Tbr1+ **(D)** and CR+ **(E)** in the rostral cortical neuroepithelium. **(F)** Higher magnification of a CR+ column marked in **(C)** with an asterisk, and horizontal migration of CR+ neurons in the preplate. Scale bars: 200 μm in **(A–C)**; 50 μm in **(D,E)**; 25 μm in **(F)**.

C revealed that the cortical preplate was not homogenous but showed important regional differences. Importantly, a SVZ was observed in A in the lateral cortical sectors adjacent and dorsal to the GEs (which would be below this level, and not be visible in this horizontal plane of section). The cortical SVZ of the embryonic stages was Tbr1+ (**Figure 2A**; see also Bayatti et al., 2008b) and CR- (not shown). In turn, the cortical sectors rostral and caudal to the GE did not yet have a SVZ. However, the rostral sector (possibly representing the future prefrontal areas) displayed focal columns of Tbr1+ cells which exited the VZ and streamed into the marginal layer, or incipient preplate, where they adopted a horizontal orientation (**Figure 2D**). Similar columns of apparently postmitotic cells in the same location were CR+ (**Figures 2E,F**), suggesting colocalization of Tbr1 and CR in postmitotic cells in the rostral cortex.

The cortical sectors caudal to the GE (probably future temporal and occipital areas) did not show focal aggregations of Tbr1+ or CR+ cells, but only a homogeneous proliferative VZ and a narrow marginal layer containing few Reelin+ Cajal-Retzius cells.

CS 20: CR AND Tbr1 IN THE ADVANCED PREPLATE

At CS 20 (ca 7 PCW) further complexity was observed in the cell composition of the developing cortex, which presents a more advanced preplate (**Figure 3**). A Tbr1+ SVZ was now recognizable throughout almost the entire cortex, although it was thickest near the PSB adjacent to the LGE (**Figure 3A**). The PSB was clearly delimited by Tbr1-positivity on the pallial side, and CR-positivity on the subpallial side (**Figures 3A,B**). Double immunofluorescence for PCNA and CR (not shown) did not provide evidence for co-expression of both markers and suggested that the CR+ cells in the LGE were not proliferating. The prominence of the SVZ was in parallel with an increasing cellularity of the preplate; both followed a gradient decreasing from lateral to medial, as well as decreasing gradients toward the rostral and caudal poles of the cortex. The preplate overlying the SVZ was dominated by horizontal bipolar or monopolar CR+ cells (**Figures 3D,E**; the monolayer, Meyer et al., 2000). Even though the CR+ cells displayed a morphology usually attributed to Cajal-Retzius cells, they were Reelin-; the Reelin+ Cajal-Retzius cells at this stage were located closer to the pial surface and not yet CR+. We reported previously that some horizontal monolayer-cells expressed GAD (Meyer et al., 2000). We now analyzed the co-expression of CR and Tbr1 in the CS 20 preplate, and found that Tbr1+/CR+ cells predominated dorsally and medially (**Figure 3D**), whereas Tbr1-/CR+ cells were intermixed with Tbr1+/CR+ neurons in lateral regions (**Figure 3E**). The Tbr1- cells probably correspond to the GAD+/GABA+ cells previously described at this stage (Zecevic and Milosevic, 1997; Meyer et al., 2000; Rakic and Zecevic, 2003; Zecevic et al., 2011). Most of the CR+ cells migrating away from the LGE (**Figure 3C**) appeared to take a ventral and lateral route toward paleocortex and lateral neocortex following the LCS (Bayer and Altman, 1991) rather than traversing the PSB in a dorsal direction. An open question was the origin of the Tbr1+/CR+ cells in the advanced preplate, since cells in the cortical SVZ were Tbr1+ but did not express CR. We suggest that a possible origin of the Tbr1+/CR+ monolayer cells may be the Tbr1+/CR+ columns observed in the rostral cortical sector

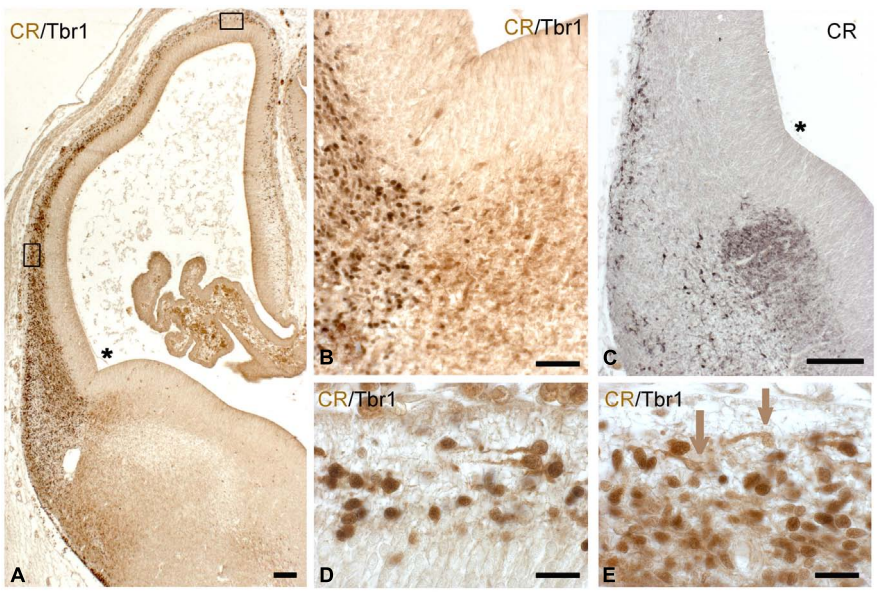

FIGURE 3 | CS 20. (A) Low magnification view of a coronal section doubly stained for Tbr-1 (dark brown or gray) and CR (light brown). **(B)** The PSB at higher magnification, showing a clear demarcation between the pallial, Tbr1+ side and the Tbr1-negative, CR+ subpallial side. **(C)** The CR+ patch in the LGE gives rise to a lateral and ventral migration stream; the monolayer of CR+ cells appears in the preplate. **(D,E)** Two-color immunohistochemistry of the dorsal **(D)** and lateral sector **(E)**, indicated by boxes in **(A)**. Dorsally, Tbr1+/CR+ cells predominate (dark nuclear staining with Tbr1), while more ventrally, both Tbr1-/CR+ (brown arrows) are side by side with Tbr1+/CR+ horizontal cells. The SVZ is lightly stained for Tbr1. Asterisks mark the cortico-striatal angle. Scale bars: 100 μm in **(A,C)**; 50 μm in **(B)**; 25 μm in **(D,E)**.

at CS 19, which would point to long-range tangential migrations within the preplate.

CS 21: THE EMERGENCE OF THE PIONEER CORTICAL PLATE

Carnegie stages 21 (ca 7.5 PCW) marks a turning point in corticogenesis: the condensation of the loosely arranged preplate into a compact cell "plate," the pioneer CP, or "pioneer plate" of Meyer et al. (2000). This aggregation of radially oriented neurons appeared first near the PSB (**Figure 4A**), while more dorsally we still observed a stage resembling the monolayer (**Figure 4D**), with a transitional, non-radially oriented compact cell condensation in a dorso-lateral position (**Figure 4C**). The lateral to medial progression of the pioneer CP varied among the fetuses of similar ages examined (7–8 PCW).

The most advanced pioneer CP with a thickness of several cell layers was found close to the future entrance of the internal capsule which at this stage was visible below the GE as a compact CB+ fiber bundle (not shown), but had not yet entered the cortex. Rostral and caudal cortical sectors lagged slightly behind. Virtually all cells of the pioneer CP were intensely CR+, and gave rise to a loose CR+ axonal plexus that followed a ventral and lateral course, representing the first corticofugal fibers (best visible in **Figure 4B**).

The apparently homogeneous CR+ pioneer CP of CS 21 turned out to be rather heterogeneous. Two-color immunohistochemistry (confocal microscopy using the monoclonal mouse CR antibody often failed in the early stages) revealed that both Tbr1+ and Tbr1- neurons formed part of the CR+ pioneer CP, as well as of the transitional dorsolateral cell condensation and the dorsomedial monolayer (**Figures 4A,C,D**). In the dorsolateral (**Figure 4C**) and dorsomedial (**Figure 4D**) regions,

Tbr1-/CR+ cells even seemed to outnumber Tbr1+/CR+ neurons. This finding may be interpreted as a massive and rather fast invasion of the preplate by Tbr1- cells, probably GABAergic neurons, compared to the rather slow emergence of the Tbr1+ SVZ which seemed to lag behind (compare the numbers of Tbr1+ cells in the dorsal cortex, compared to the CR+/Tbr1- cells). Furthermore, there was evidence of GAD+/CR+ and GAD+/CR- cells, as well as for Tbr1+/CR- cells. The GAD+/CR+ cells seemed to occupy a more superficial position, whereas the GAD+/CR- cells lay deeper (**Figure 4G**). The complexity of the advanced preplate cell populations points to an important role of GABAergic neurons in the earliest stages of cortex formation. Cajal-Retzius cells, characterized by Reelin-expression and a subpial location (**Figures 4E,F**) now also began to express CR, or CB when they were close to the cortical hem.

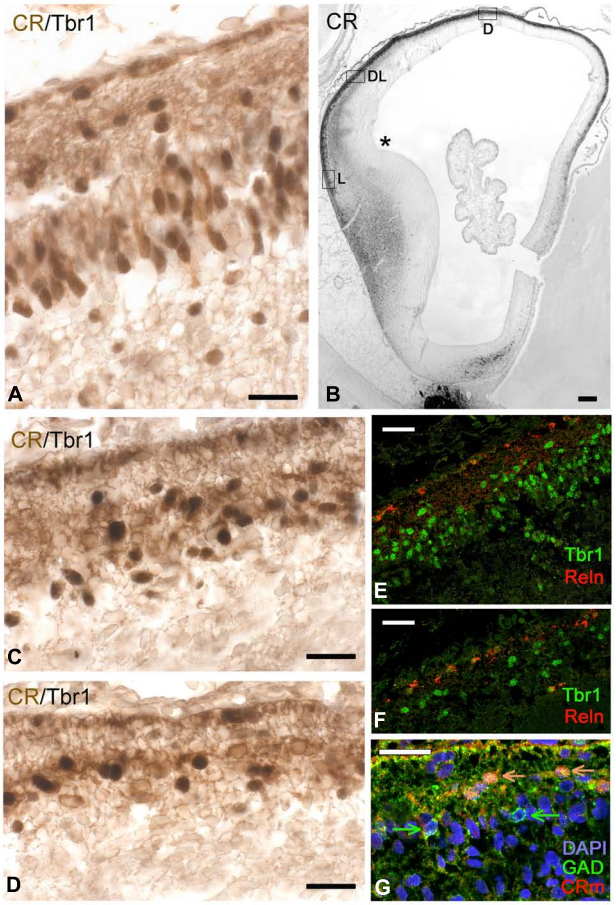

FIGURE 4 | CS 21. (A) Corresponds to a lateral (L), **(C)** to a dorsolateral (DL), and **(D)** to a dorsal (D) sector of the low magnification view in **(B)**, indicated by boxes. **(B)** Is immunostained for CR; **(A,C,D)** are doubly stained for Tbr1 (dark nuclei) and CR (light brown cytoplasm). Note that the lateral sector in **(A)** already shows a radially oriented pioneer cortical plate, whereas in **(C)**, Tbr1+/CR+ cells are intermixed with Tbr1-/CR+ cells, without any radial orientation. **(D)** Corresponds to a monolayer stage, with most cells showing a horizontal orientation, and Tbr1+/CR+ cells being outnumbered by Tbr1-/CR+ cells. **(E,F)** Confocal microscopy of Reelin+ Cajal-Retzius cells in a subpial location, segregated from pioneer plate **(E)** and monolayer cells **(F)**, marked with Tbr1. The level of **(E)** corresponds to that in **(A)**, the level of **(F)** to that of **(D)**. **(G)** Confocal microscopy of a dorsal sector of this case, showing colocalization of GAD and CR in superficial, horizontally oriented cells (orange arrows), while GAD alone was expressed in a deeper population (green arrows). Note that the mouse monoclonal CR-antibody was less sensitive than the rabbit CR antibody routinely used in the other panels. The asterisk in B marks the cortico-striatal angle. Scale bars: 100 μm in **(B)**; 50 μm in **(E,F)**; 25 μm in **(A,C,D,G)**.

CS 22/23: THE TRANSITION FROM PIONEER PLATE TO CORTICAL PLATE

The CP emerged at CS 22/23 (ca 8 PCW). There was actually a gradual transition from the radially organized pioneer CP, still visible in dorsal and medial areas (**Figures 5A–C**), and the CP in lateral, more advanced areas (**Figures 5D,E**). Pioneer cells were large neurons, particularly the deeper ones which had pyramidal-like shapes and may represent the future presubplate (Meyer et al., 2000; reviewed by Kostovic et al., 2011), while the superficial ones were more rounded (**Figure 5A**) and became increasingly sparse as the CP progressed. The axonal plexus had its origin in superficial and deep pioneer cells (see Meyer et al., 2000). The pioneer CP is an important step in cortex formation, because for the first time we observed a distinct expression of Dab1 in the apical tips of pioneer cells (**Figure 5B**), a staining pattern characteristic of the CP. Dab1 is a crucial component of the Reelin-Dab1 signaling pathway that responds to Reelin secreted by Cajal-Retzius cells (Meyer et al., 2003, reviewed by Tissir and Goffinet, 2003). Confocal microscopy revealed that pioneer neurons co-expressed Tbr1

and CR (**Figure 5C**), which is another feature of pyramidal neurons, demonstrating that they were indeed excitatory projection neurons. The CP was formed through the continuous arrival of new Tbr1+ cells and their insertion within the framework provided by the superficial and deep pioneer cells. CP-cells were lightly stained for CR in the dorso-lateral cortex (**Figure 5D**), and CR- in the lateral cortex (**Figure 5E**). In the latter, CR-positivity was still prominent in the presubplate and in the few remnants of the superficial pioneer cells. GAD-immunoreactivity was restricted to interneurons preferentially located along the upper and lower borders of the CP (**Figure 5F**).

Carnegie stages 22 and 23 were difficult to separate, because the five cases in our 8–10 PCW group were quite similar, and differed mainly in the size of the hemisphere, with all cases presenting a pioneer CP medially, and a multilayered CP laterally. Of note, this age represented another remarkable step in corticogenesis: the arrival of the internal capsule in the cortex, after traversing the PSB. The internal capsule was initially CB+ and accompanied by CB+ interneurons streaming into the intermediate zone (**Figure 5J**). The CR+ patch in the LGE adjacent to the PSB was now also the origin of a stream of CR+ interneurons which migrated into the SVZ, taking a deeper route than the CB+ interneurons (compare **Figures 5I,J**).

The pallial side of the PSB was clearly delineated by Tbr1 expression (**Figure 5H**); Tbr1 positivity was highest in the CP, but also visible in the SVZ and in neurons migrating

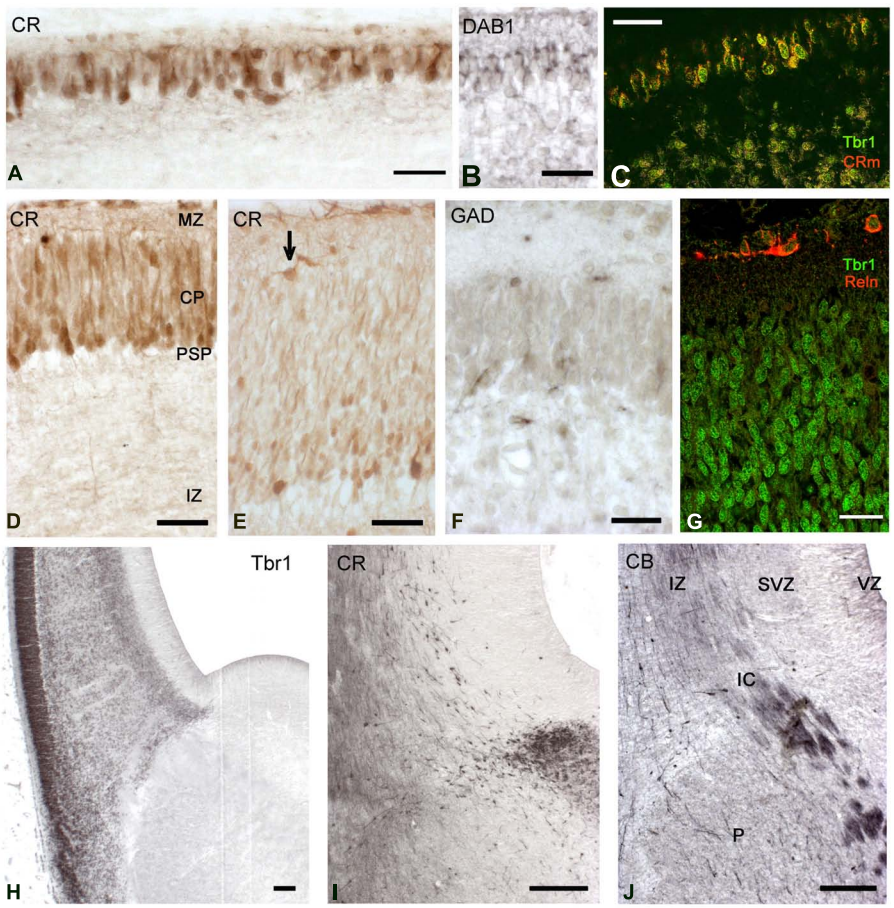

FIGURE 5 | CS22/23. Coronal sections from an 8.5 PCW-old case presenting pioneer cortical plate, cortical plate and intermediate stages. **(A–C)** The pioneer cortical plate, in the dorsal and medial sector of the cortex, is positive for CR **(A)**, Dab1 **(B)** and both Tbr1 and CR **(C)**, using confocal microscopy and the mouse monoclonal CR antibody (CRm). The pioneer cortical plate has a deep component of pyramidal-like neurons, and a superficial component of more rounded cells **(A)**. Both give rise to an axonal plexus beneath the pioneer plate **(A)**, and express Dab1 in the apical tips of their cytoplasm **(B)**. Pioneer cells co-express Tbr1 and CR **(C)**. **(D)** At an intermediate level, the cortical plate (CP) appears as lightly CR+ cells, while few superficial pioneer cells are still evident, and deep pioneer cells form the presubplate (PSP). **(E)** In the lateral sector, the cortical plate is widest, and CR-positivity has largely disappeared. Superficial pioneer cells are rare (arrow). **(F)** After the emergence of the CP, GAD+ cells are rare and mostly below and above the CP. **(G)** The CP is composed of Tbr1+ cells, and Reelin+ Cajal-Retzius cells appear now more differentiated. **(H)** Low magnification view of the PSB stained for Tbr1. Note the migration toward the ventral and lateral cortical areas, forming the lateral cortical stream, and the sharp delineation of the subpallium. **(I)** The CR+ patch in the LGE gives now rise to a dorsal migration stream into the SVZ of the cortex. Compare with **(J)** The first CB+ fibers of the internal capsule (IC) enter the cortex through the intermediate zone (IZ). CB+ interneurons migrate with the fibers, taking a more superficial route than the CR+ cells. P, Putamen. Scale bars: 100 μm in **(H–J)**; 50 μm in **(A–G)**.

radially through the IZ. Tbr1 also marked the LCS contributing pyramidal cells for paleocortex and insula. Cajal-Retzius cells, defined by Reelin-expression, had a more differentiated morphology (**Figure 5G**) and now clearly co-expressed CR (**Figures 5D,E**).

THE EARLY FETAL STAGE: DIFFERENTIATION OF INSULA AND TEMPORAL LOBE

The focus of this work is on the embryonic stages of corticogenesis rather than on the fetal cortex. However, as an indication of the ongoing complexity of human cortex development we show in **Figure 6** a coronal section of an 11-PCW old fetus immunostained for CR to point out a few important differences with rodent cortex that become evident after the embryonic period: first, the formation of a temporal lobe ventral to a largely increased insula; second, the ventral position of the CGE protruding into the temporal horn of the lateral ventricle; third, the mirror-like arrangement of the PSB in the fronto-parietal cortex dorsally, and the temporal cortex ventrally, with both displaying a CR+ patch in the lateral aspect of the GEs. It is clear from this figure that the medial temporal lobe does not show a CR+ pioneer CP, and thus apparently does not follow the sequence of events which we described for the fronto-parietal cortex, and which applies also to the prefrontal and occipital areas. Many aspects of the human cortex are as yet unknown, and will need a continued effort and much further work to understand its singularity.

DISCUSSION

Calretinin is expressed in early appearing neurons in the human embryonic and early fetal cortex, and analysis of CR immunostaining – along with that of other marker molecules – allowed us to obtain insight into early occurring telencephalic regionalization, and to distinguish a sequence of events that lead from the incipient preplate to the formation of the CP.

EXPRESSION OF CR AND CB REFLECTS AN EARLY REGIONALIZATION OF THE HUMAN CORTEX

Already in the earliest stage examined, CS 17 (ca 6 PCW), CR is defining the lateral domain of the GE, which directly abuts the ventral border of the future cortex. The PSB is delineated by the expression of Tbr1, a marker of pallial glutamatergic cells (Bulfone et al., 1999; Puelles et al., 2000; Hevner et al., 2001), in

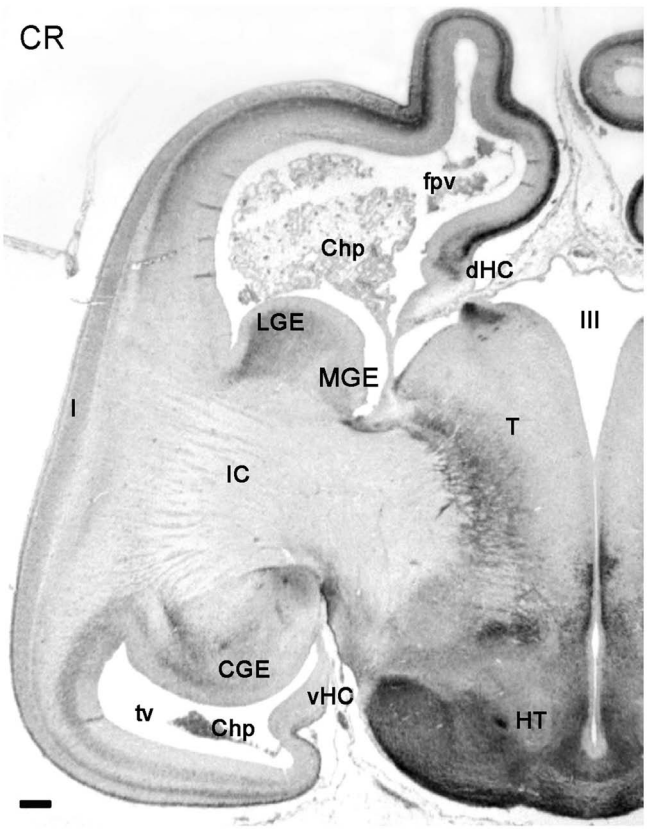

FIGURE 6 | Low magnification view of a hemisphere at 11PCW, stained for CR. The early fetal stages are characterized by growth and differentiation processes which, among many other features, allow the distinction of the temporal horn of the lateral ventricle (tv), the enormous growth increase of the internal capsule (IC) and the insula (I), the ventral position of temporal lobe and caudal ganglionic eminence (CGE), and the appearance of the ventral hippocampus (vHC). The intensely CR+ pioneer cortical plate is still recognizable in the frontal and parietal lobes, but not evident in the temporal lobe. CR is expressed in the LGE and the CGE. Other abbreviations: Chp, choroid plexus; fpv, fronto-parietal ventricle; dHC, dorsal hippocampus; HT, hypothalamus; MGE, medial ganglionic eminence; T, thalamus; III, third ventricle. Scale bar: 100 m.

the lateral aspect of the cortico-striatal angle, and the presence of CR in cells medial to this angle, with the absence of Tbr1 indicating their subpallial GABAergic identity. We report here that CR+ cells in the SVZ of the LGE are among the first postmitotic cells of the telencephalon, and differentiate earlier than cells in the pallium, with the exception of the first cohort of Cajal-Retzius cells (Meyer et al., 2000). What we find interesting is the positivity for CB in the medial aspect of the hemisphere, where cortical hem and choroid plexus are developing. CB is an early marker of the human hem system (Meyer, 2010; Roy et al., 2013), which includes the medial edge of the cortex as a putative signaling center involved in patterning of hippocampus and cortex (Grove et al., 1998), as well as the boundaries of prosencephalic structures such as septum and thalamic eminence with the prospective choroid plexus (Meyer, 2010; Roy et al., 2013). The structures belonging to the hem system express unique complements of signaling molecules, and require the transcription factor Lhx2 to delimit their extent (Roy et al., 2013). The PSB was proposed to represent an antihem, a putative signaling center at the opposite, lateral end of the pallium (Assimacopoulos et al., 2003). While the hem is characterized by the expression of Wnt and Bmp-genes (Grove et al., 1998), the antihem expresses the Wnt antagonist Sfrp2, members of the epidermal growth factor family, and Fgf7 (Assimacopoulos et al., 2003; Subramanian et al., 2009). It is tempting to propose that CR defines cell populations of the antihem, and CB of the hem, even though these calcium-binding proteins may not be directly involved in the signaling cascades. The first derivatives of the hem are CB+/p73+ Cajal-Retzius cells (Meyer et al., 2002a), whereas the first postmitotic neurons in the antihem form the CR+ cell cluster in the LGE that will be discussed below. In the mouse, neither CB nor CR are present in hem and antihem, which can be detected only through the expression of their characteristic morphogens and transcription factors. The coordinated activity of hem and antihem may be involved in delimiting the extent of the proliferative neuroepithelium of the future cortex.

In addition, and possibly independently from the hem/antihem signaling centers, the distinct and rather selective columnar expression pattern of CR/Tbr1 in the prospective frontal lobe at CS 19 suggests a role of CR/Tbr1 in cortical patterning, since Tbr1 is a transcription factor involved in promoting frontal identities in postmitotic neurons and in modulating the balance of cortical arealization (Bedogni et al., 2010). We want to point out the difference of CR expression in the subpallium, where it marks GABAergic interneurons, and CR expression in the pallium, where it can be associated with Tbr1, a marker of glutamatergic projection neurons. CR expression is thus *a priori* unrelated to the GABAergic or glutamatergic signature of a neuron.

CR MARKS AN EARLY MIGRATION FROM THE PSB VIA THE LATERAL CORTICAL STREAM

The CR+ domain in the SVZ of LGE and CGE appeared already at CS17, along with the onset of a migration of initially few CR+ cells toward the ventral areas, which were, from rostral to caudal, the retrobulbar area, future piriform cortex and amygdala. This migration became more prominent in the following stages when the morphology and architecture of the ventral telencephalon increased in complexity. The complementary expression of Tbr1 and CR in the PSB points to a parallel migration from both sides of the cortico-striatal border in a lateral and ventral direction and indicates that both form part of the LCS described by Bayer and Altman (1991) in the rat and identified also in human (Bayer and Altman, 2006). The LCS, also described as the lateral and ventral migratory streams (Medina et al., 2004), has been studied extensively in the rodent. According to Bayer and Altman (1991), the LCS contributes neurons to various ventral forebrain structures including piriform cortex and amygdala. Further destinations are the lateral neocortex, claustrum, endopiriform nucleus and olfactory tubercle, or, more generally, centers belonging to the lateral and ventral pallium of chick and mice (Puelles et al., 2000; Tamamaki et al., 2001; Medina et al., 2004; Bai et al., 2008). The LCS is formed by heterogeneous cell populations arising from distinct compartments of the PSB; transcription factors Tbr1, Pax6, and Emx2 are expressed in cells emerging from the pallial compartment, whereas cells arising in the subpallial compartment express Dlx2 or co-express Pax6 and Dlx2 (Puelles et al., 2000; Carney et al., 2006). In addition, a specific histogenetic zone at the PSB is defined by Dbx1, which provides cells for the ventral pallium (Puelles et al., 2000; Medina et al., 2004), and for an early born transient glutamatergic cell population migrating over the entire CP, plus a subset of Cajal-Retzius cells (Teissier et al., 2010) which we could not confirm in human.

The PSB described here in human embryos may have played a crucial role in the evolution of the mammalian forebrain through the LCS (Molnár and Butler, 2002). We want to point out some features that may be relevant for human cortical development. Importantly, the cell populations (Tbr1+ and CR+) on their respective sides of the PSB arise very early in embryonic life, at CS 17 and perhaps even earlier, marking the onset of cortical development. Tbr1+ glutamatergic neurons populate the olfactory paleocortex, amygdale, and future lateral neocortex concurrently with Tbr1-/CR+ putative GABAergic neurons via the LCS. However, as shown in **Figure 6**, shortly after the embryonic period, areal patterning and architecture of the lateral and ventral neocortical areas take a distinct course in human development, which is not comparable to that of rodents. This is to say that the PBS and LCS of the human embryo implicitly contain the progenitor areas and future migratory pathways necessary for the formation of the insular lobe in parallel with an expansion of the claustrum, and the establishment of the temporal lobe ventral to the insula. Interestingly, 3-D models of the telencephalon of CS19 human embryos indicated a relative size increase of the region corresponding to the ventral pallium of mice, which was interpreted as a sign of higher complexity of the human claustroamygdaloid structures (Lindsay et al., 2005). The term "ventral pallium" (Puelles et al., 2000) has different connotations in mouse and human, and much further work is needed to fill in the gap between human and rodent-related knowledge.

The origin of interneurons from transcription factor Dlx1/2 and Nkx2.1 domains in the GE is a well-established fact in rodents (reviewed by Welagen and Anderson, 2011). In primates, the situation is more complex and in part disputed. Our finding of an early

CR+ domain in the LGE and CGE that gives rise first to a latero-ventral migration, and later, at CS 22, to a dorsal migration into the cortex, does not imply that during fetal life all CR+ interneurons derive from LGE and CGE. Fetal interneuronogenesis is complicated by the fact that significant numbers of GABAergic cells emerge from the cortical VZ and SVZ (Letinic et al., 2002; Rakic and Zecevic, 2003; Petanjek et al., 2009a,b; Zecevic et al., 2011; Jakovcevski et al., 2011; Reinchisi et al., 2012; Al-Jaberi et al., 2013). The LGE and CGE may be the origins only of the first of successive CR+ interneuron migrations, most of which occur later in development and in different places.

CALRETININ AND Tbr1 DEFINE DISTINCT CELL POPULATIONS IN THE HUMAN PREPLATE

The original concept of the preplate, or primordial plexiform layer, postulated that its components – early born Cajal-Retzius cells and subplate cells – are split by the arrival of later born cortical-plate cells (Marin-Padilla, 1978). This concept, based on the Golgi method, was modified and adapted to the human (Meyer et al., 2000), primate (Smart et al., 2002) and rodent (Meyer et al., 1998) cortex with the advent of immunohistochemistry and the appearance of neurochemical markers for specific cell types.

In human embryos, the preplate is continuously changing because newly arriving cell components are added at different moments of development (Zecevic and Milosevic, 1997; Meyer et al., 2000, 2002b; Rakic and Zecevic, 2003; Zecevic et al., 2011). Initially, from 5.5 to 6.3 PCW (CS 16–18), the early marginal layer of the prospective neocortex contains only Reelin+ Cajal-Retzius cells that may have originated locally from Reelin+ radial cell columns in the neuroepithelium (Meyer et al., 2000, 2002b), shortly before the cortical hem produces additional Reelin+/p73+ Cajal-Retzius cells (Meyer et al., 2002a). In agreement with our previous work, we consider CS 19 (6.5 PCW) as the initial preplate stage, since for the first time non-Cajal-Retzius cells appear in the future neocortex. We show now that CR+ cells at CS 19 originate from focal columns embedded in the rostral cortical neuroepithelium, similar to Tbr1+ cells, and suggest that Tbr1+/CR+ cells migrate tangentially through the preplate and form part of the "monolayer" of horizontal cells characteristic of the advanced preplate at CS 20 (Meyer et al., 2000). The next step would be the aggregation of Tbr1+/CR+ cells in the compact pioneer CP at CS 21, and the appearance of the first pioneer axons of the neocortex.

The neurochemical heterogeneity of the advanced preplate complicates a straightforward interpretation of these early events. We show that at CS 20 (ca 7 PCW) and CS21(ca 7.5 PCW), Tbr1+/CR+ cells coexist with Tbr1-/CR+ cells, with the latter probably representing part of the GABAergic neurons which are a prominent part of the advanced preplate. We did not observe local aggregations or columns of GAD+ cells in cortical territory at earlier stages, and rather propose a subpallial origin for Tbr1- preplate cells. In fact, Rakic and Zecevic (2003) provided evidence that a subset of GABAergic cells in the CS 19 and 20 preplate co-expressed either Nkx2.1 or Dlx transcription factors, which is consistent with their origin in GE and subsequent tangential migration into the preplate (Zecevic et al., 2011). Alternatively, or in addition, GABAergic cells may also be born locally in distinct sectors of the cortical neuroepithelium (Rakic and Zecevic, 2003; Al-Jaberi et al., 2013). The prominence of GABAergic cells in the advanced preplate is supported by the work of Ben-Ari (reviewed by Ben-Ari et al., 2004) who described that GABAergic neurons act as the true pioneers in the developing cortical network, establishing functional synapses earlier than glutamatergic projection neurons. According to this author, the early established GABAergic synapses are depolarizing, and may modulate the maturation of immature, silent principal neurons. The time point of the massive presence of GABA in the monolayer is just a few days/hours before the emergence of the pioneer plate and the emission of the first projection fibers from pioneer neurons. In fact, the coexistence of pioneer CP and monolayer in the lateral and medial regions, respectively, in the same embryo suggests that in a very short time the cells of the monolayer aggregate to form the compact pioneer CP.

CR AND Tbr1 IN PROJECTION NEURONS OF PIONEER CORTICAL PLATE AND CORTICAL PLATE

The human pioneer CP is a short-lived and transient structure, which is important because it constitutes the first radially organized, Tbr1+ cell layer that gives rise to the efferent pioneer projections. The transitional stages between pioneer CP and CP, which follow the general maturation gradient from lateral to medial (Bayer and Altman, 1991), indicate that preplate splitting consists actually of a splitting of the pioneer CP, when CR-CPcells settle between the superficial and deep CR+ pioneer cells. CR in pioneer cells should not be interpreted as a marker of interneuron identity, since CR and Tbr1 colocalize in the pioneer CP. The putatively glutamatergic pioneer cells may derive from the CR+ radial cell columns observed at CS 19 in the rostral cortex, which may adopt a bipolar migratory morphology at CS 20, and assemble in the compact pioneer plate at CS 21. CR expression in these early cell populations may be related to specific functional roles of this calcium-binding protein which are as yet unknown, but probably independent of neurotransmitter identity.

The pioneer CP is not restricted to human cortex. In the rat, pioneer cells at embryonic day 13 express CB and CR (Meyer et al., 1998), although they do not in the mouse (Espinosa et al., 2009). The rodent pioneer neurons emit the first fiber bundle of the cortex which directs toward the GE and the internal capsule but probably does not cross the telencephalic-diencephalic boundary (Meyer et al., 1998; Espinosa et al., 2009). The deep pioneer neurons are usually identified as subplate cells, while the presence of superficial pioneer cells in MZ and superficial CP is easily overlooked. In fact, human superficial pioneer cells are rare in the lateral CP at CS 23 (ca 8.5 PCW), which may be due to cell death or dilution in a rapidly growing cortex. We do not want to enter here the discussion on the human subplate, which has been the subject of many comprehensive studies and reviews demonstrating that the deep pioneer neurons rather represent a presubplate (Meyer et al., 2000; Smart et al., 2002; Meyer, 2007; Kostovic et al., 2011). The human subplate arises around 10 PCW, expands after 13 PCW (Bayatti et al., 2008a), attains highest differentiation in the second half of gestation, and may not be directly comparable to the rodent subplate.

We pointed out previously (Meyer et al., 2000) that Cajal-Retzius cells do not form part physically of the splitting of the preplate, because already at CS 19 they are confined to a subpial position, spatially segregated from the other cell populations in the preplate. However, the Reelin signal in Cajal-Retzius cells is crucial for preplate (or rather pioneer CP) splitting, because absence of Reelin prevents this process, leading in mice to the *reeler* phenotype characterized by a roughly inverted cortex (reviewed by Tissir and Goffinet, 2003). The Dab1+ pioneer plate may represent the first target population of Reelin-Dab1 signaling activity involved in radial migration.

As discussed here, the embryonic development of the human cortex leaves open many questions that require further clarification. In particular, the possible birthplace of early GABAergic neurons and Tbr1+ pioneer neurons in specific sectors of the cortical neuroepithelium, followed by tangential pathways through the preplate, requires further investigation. The difficulties and ethical limitations in obtaining well preserved human embryos make it imperative to explore other animal models, beyond the prevailing but insufficient mouse model.

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The complexity of the calretinin-expressing progenitors in the human cerebral cortex

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The complex structure and function of the cerebral cortex critically depend on the balance of excitation and inhibition provided by the pyramidal projection neurons and GABAergic interneurons, respectively. The calretinin-expressing (CalR$^+$) cell is a subtype of GABAergic cortical interneurons that is more prevalent in humans than in rodents. In rodents, CalR$^+$ interneurons originate in the caudal ganglionic eminence (CGE) from Gsx2$^+$ progenitors, but in humans it has been suggested that a subpopulation of CalR$^+$ cells can also be generated in the cortical ventricular/subventricular zone (VZ/SVZ). The progenitors for cortically generated CalR$^+$ subpopulation in primates are not yet characterized. Hence, the aim of this study was to identify patterns of expression of the transcription factors (TFs) that commit cortical stem cells to the CalR fate, with a focus on Gsx2. First, we studied the expression of Gsx2 and its downstream effectors, Ascl1 and Sp8 in the cortical regions of the fetal human forebrain at midgestation. Next, we established that a subpopulation of cells expressing these TFs are proliferating in the cortical SVZ, and can be co-labeled with CalR. The presence and proliferation of Gsx2$^+$ cells, not only in the ventral telencephalon (GE) as previously reported, but also in the cerebral cortex suggests cortical origin of a subpopulation of CalR$^+$ neurons in humans. *In vitro* treatment of human cortical progenitors with Sonic hedgehog (Shh), an important morphogen in the specification of interneurons, decreased levels of Ascl1 and Sp8 proteins, but did not affect Gsx2 levels. Taken together, our *ex-vivo* and *in vitro* results on human fetal brain suggest complex endogenous and exogenous regulation of TFs implied in the specification of different subtypes of CalR$^+$ cortical interneurons.

Keywords: cortical interneurons, cortical development, transcription factors, Gsx2, GABA

INTRODUCTION

The increased complexity of cortical progenitors is considered to be an evolutionary adaptation necessary for the development of higher brain functions in primates, and particularly in humans. Complex structure and function of the cerebral cortex critically depend on the balance of excitation and inhibition provided by pyramidal neurons and γ-aminobutyric acid-containing (GABAergic) interneurons, respectively. Interneurons comprise 20% of all cortical neurons in rodents and up to 35% in humans (Gabbott et al., 1997; DeFelipe et al., 2002; Jones, 2009). Importantly, their impairment has been described in various psychiatric and neurological disorders, including epilepsy, schizophrenia and autism (Yan et al., 1995; DeFelipe, 1999; Ulfig, 2001; Brandt et al., 2003; Baraban and Tallent, 2004; Lewis et al., 2005; Marin, 2012). In contrast to rodents, where the majority, if not all, cortical interneurons are generated in the ventral telencephalon (ganglionic eminence, GE; Anderson et al., 1997; Tamamaki et al., 1997; Parnavelas, 2000; Marin and Rubenstein, 2001), several groups have reported that cortical interneurons in primates originate both ventrally (in the GE) and dorsally, in the cortical subventricular zone (SVZ; Letinic et al., 2002; Rakic and Zecevic, 2003; Petanjek et al., 2009b; Jakovcevski et al., 2011; Al-Jaberi et al., 2013). This topic is still open for discussion since other groups reported that similar to rodents, the majority of cortical interneurons in primates originate in the GE and no proliferation of interneuron progenitors was demonstrated in the cortex (Hansen et al., 2013; Ma et al., 2013).

Cortical interneurons represent a heterogeneous group of cells that can be classified according to physiological, morphological or molecular criteria (Petilla Interneuron Nomenclature Group1 et al., 2008). In this study, we have focused on calretinin-expressing (CalR$^+$) interneurons which have variable morphology and are comprised of bipolar and double bouquet cells (DeFelipe, 1997). In humans, up to 50% of all CalR$^+$ cells are bipolar, localized predominantly in the upper cortical layers vs. approximately 15% in rodents (Condé et al., 1994; Yan et al., 1996; Gabbott et al., 1997; DeFelipe et al., 2006; Bayatti et al., 2008). By their electrophysiological output, they belong to accommodating or irregular-spiking interneurons (Porter et al., 1998) that target not only the distal dendrites of pyramidal cells, but importantly also other GABAergic interneurons. Their function in the primate cortex is complex, since they can disinhibit pyramidal cells by making synapses on

each other (DeFelipe et al., 1999; Schlösser et al., 1999; Lewis et al., 2005; Zaitsev et al., 2005). The specific distribution of CalR$^+$ cells in human upper cortical layers (II/III) suggests a role in cortical circuit formation necessary for higher brain functions specific to humans, such as abstract thinking and language (Hill and Walsh, 2005; Jones, 2009; Rakic, 2009).

Timing and specification of cortical interneuron development are guided by a variety of transcription factors (TFs). In mice, CalR$^+$ interneurons originate mainly from the caudal ganglionic eminence (CGE; Xu et al., 2004; Butt et al., 2005) from progenitor cells expressing the TF genomic screened homeobox 2 (Gsx2; Hsieh-Li et al., 1995). The TFs Achaete-scute homolog 1 (Ascl1, originally named Mash1) and specificity protein 8 (Sp8) are downstream effectors of Gsx2 (Waclaw et al., 2009; Wang et al., 2009). Gsx2 has a role in the specification of cortical interneurons (Xu et al., 2010), olfactory bulb interneurons and striatal projection neurons (Stenman et al., 2003). In the human fetal telencephalon Gsx2 has been reported exclusively in the GE (Ma et al., 2013). However, its role in the generation of CalR$^+$ interneurons in the developing human cerebral cortex still remains elusive.

It has been reported that CalR$^+$ cells during early human developmental stages (from 6 gestational weeks, gw) are generated in the GE and tangentially migrate into the cortex (Zecevic et al., 1999; Meyer et al., 2000; Rakic and Zecevic, 2003), whereas by midgestation (20 gw) an additional subgroup of proliferating cells in the outer subventricular zone (oSVZ) express CalR, suggesting their local origin at that stage of development (Jakovcevski et al., 2011; Zecevic et al., 2011). Our *in vitro* studies confirmed that CalR$^+$ cells originate from genetically labeled cortical human progenitors (Mo et al., 2007). Moreover, we reported that cortical radial glia cells (RGCs) *in vitro* generate CalR$^+$ cells in human but not in mice (Yu and Zecevic, 2011).

Here, we describe a specific pattern of expression in the human fetal cortex of the three TFs proposed to be involved in the CalR lineage: Gsx2, and its downstream targets Ascl1 and Sp8. In addition, cortical RGCs cultures treated with Sonic hedgehog (Shh), a morphogen known to play a role in interneuron specification, show a selective effect on these TFs that may influence specification of CalR$^+$ cells in human. These results indicate a complex endogenous and exogenous regulation of TFs implicated in the specification of the human CalR$^+$ cortical interneurons.

MATERIALS AND METHODS

HUMAN TISSUE PROCESSING AND IMMUNOSTAINING

Fetal brain tissues at midgestation (14–24 gw, $n = 22$; **Table 1**) were obtained from the Tissue Repository at The Albert Einstein College of Medicine, Bronx, NY, USA and StemExpress, Diamond Springs, CA, USA. Handling of the human material was done with special care following all necessary requirements and regulations set by the Institutional Ethics Committee. Ultrasonography and gross neuropathological examination confirmed that the brain tissue was normal. Embryonic brains were fixed overnight in 4% PFA/0.1 M PBS (pH 7.4), then cryoprotected in 30% sucrose/PBS, frozen in TissueTek OCT and sectioned on a cryostat (15 μm). Slices were incubated for 1 h at room temperature (RT) in blocking solution (10% NGS and 0.5% Tween-20 in PBS). Primary antibodies (**Table 2**) were applied overnight at 4°C in blocking solution followed by corresponding secondary antibodies (Jackson Immuno-Research Lab, West Grove, PA, USA) for 1 h at RT, and a short incubation in a nuclear stain bis-benzimide (Sigma).

Table 1 | Fetal human brain tissues analyzed in the study.

Cases	Gestational week (gw)	Gender	Direct tissue application	Cell culture application
1	14	NP	–	WB
2	15	♀	IHC	–
3	16	♂	RT-PCR	–
4	16	♀	IHC	–
5	17	♂	–	RT-PCR, WB
6	17	NP	IHC	–
7	17	♂	IHC	–
8	17	♂	–	WB
9	18	NP	RT-PCR	RT-PCR, WB
10	18	♂	IHC/ISH	–
11	18	NP	IHC	–
12	19	NP	–	WB
13	19	♀	IHC	–
14	20	♀	IHC/ISH	–
15	20	NP	IHC/ISH	–
16	21	NP	IHC/ISH	–
17	21	NP	IHC/ISH	–
18	22	NP	IHC/ISH	–
19	22	♂	IHC/ISH	–
20	23	♀	IHC/ISH	–
21	24	♂	IHC/ISH	–
22	24	♀	IHC/ISH	–

♀, *female;* ♂, *male; ICC, Immunocytochemistry; IHC, Immunohistochemistry; ISH, In situ hybridization; NP, Not provided; RT-PCR, real-time PCR; WB, Western blot*

IN SITU HYBRIDIZATION

The human *Gsx2* full coding sequence plasmid was obtained from OpenBiosystems (IMAGE:30915601). Riboprobe was generated from the linearized vector construct by *in vitro* transcription using digoxigenin (DIG)-UTP (Roche) as the label. *In situ* hybridization (ISH) was performed on cryosections (15 μm) described above. Sections were dried at RT for 2 h and subsequently fixed for 10 min with 4% PFA, before overnight incubation at 68°C in hybridization buffer 1× DEPC-treated "salts" (200 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5, 5 mM $NaH_2PO_4{\cdot}2H_2O$, 5 mM Na_2HPO_4; Sigma–Aldrich), 50% deionized formamide (Roche), 0.1 mg/ml RNase-free yeast tRNA (Invitrogen), 1× Denhardts (RNase/DNase free; Invitrogen), 10% dextran sulfate (Sigma-Aldrich) containing 100–500 ng/ml DIG-labeled RNA probe. After hybridization, sections were washed three times in a solution containing 50% formamide 1× SSC (Invitrogen) and 0.1% Tween 20 (Sigma–Aldrich) at 65°C, and two times at RT

Table 2 | Primary antibodies used in this study (in alphabetical order).

Antigen	Host	Clone	Dilution	Manufacturer	Catalog no.
Ascl1	Mouse IgG1	24B72D11.1	1:500	BD Pharmingen	556604
β-Actin	Mouse IgG		1:2000	Thermo Scientific	MA5-15739
CalR	Mouse IgG	6B8.2	1:500	Millipore Chemicon	MAB1568
	Rabbit IgG		1:500	Sigma	PA007306
GABA	Rabbit IgG		1:2000	Sigma	A2052
Gsx2	Rabbit IgG		1:250	Abcam	AB26255
	Mouse IgG		1:500	Millipore Chemicon	ABN162
Ki67	Mouse	MIB1	1:50	DAKO	M7240
Nkx2.1	Rabbit IgG	EP1584Y	1:500	Abcam	AB76013
Sp8 (C-18)	Goat IgG		1:500	Santa Cruz	Sc-104661
Sox2	Goat IgG		1:500	Santa Cruz	Sc-17320
Tbr1	Rabbit IgG		1:500	Proteintech	20932-1-AP
Tbr2	Rabbit IgG		1:500	Gift from R. Hevner	

in 1× MABT (20 mM maleic acid, 30 mM NaCl, 0.1% Tween 20; Sigma-Aldrich) before incubating in a solution containing 2% blocking reagent (Roche) and 10% heat inactivated sheep serum (HISS) in MABT, followed by overnight incubation in alkaline phosphatase (AP)-conjugated anti-DIG antibody (1:1500; Roche Applied Science). Fast Red (Roche) was used for fluorescent color detection of probe (FISH) by incubation in 100 mM Tris, pH 8.2, 400 mM NaCl containing Fast Red for 1–2 h at 37°C. Sections were counterstained with bis-benzimide and coverslipped using Fluoromount G mounting medium. Specificity of the procedure was assessed with a probe corresponding to the sense strand of *Gsx2*.

IMMUNOHISTOCHEMISTRY AFTER *IN SITU*

Following overnight incubation with mouse anti-Ascl1, rabbit anti-calretinin and goat anti-Sp8 antibodies, sections were thoroughly washed in PBST (0.2% Triton) and incubated with Alexa 488 secondary antibody to detect immunoreactivity. Nuclei were counterstained with bis-benzimide.

DISSOCIATED MIXED CELL CULTURE AND ENRICHMENT OF RGCs

Human fetal brain tissue ($n = 5$) ranging in age from 14 to 19 gw was obtained from Advanced Bioscience Resources (ABR, Alameda, CA, USA) and StemEx (Diamond Springs, CA, USA) with proper parental consent and the approval of the Ethics Committees. Brain tissue was collected in oxygenized Hank's balanced salt solution (HBSS; LifeTechnologies, Grand Island, NY, USA) and transported on ice. Dissociated cell cultures were prepared from dorsal and ventral regions of the telencephalon as described previously (Zecevic et al., 2005). Isolated tissue of interest was mechanically dissociated and enzymatically degraded at 37°C for 30 min with 0.025% trypsin (Gibco). Afterward, DNase (Sigma Aldrich, St. Louis, MO, USA; 2 mg/ml) was added to the cell suspension and cells were washed in HBSS (LifeTechnologies, Grand Island, NY, USA). Cells were resuspended in the proliferation medium consisting of DMEM/F12 (LifeTechnologies) with 10 ng/ml basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ, USA), 10 ng/ml epidermal growth factor (EGF Millipore, Billerica, MA, USA) and supplemented with B27 (LifeTechnologies). Cells were kept in the proliferating medium 7–10 days until 80% confluence was achieved. A surface marker CD15 (Lex) was used for immunomagnetic cell sorting of RGCs using MACS columns (Miltenyi Biotec, Auburn, CA, USA) which resulted in 96% purity of RGCs (Mo et al., 2007; Yu and Zecevic, 2011). For immunocytochemistry approximately 250,000 cells were plated on coverslips coated with poly-D-lysine (Sigma–Aldrich). For total protein and RNA isolation approximately 2 million cells were plated in poly-D-lysine coated wells. In order to confirm the identity of isolated cells, 24 h after isolation, live immunocytochemistry was performed using markers for radial glia, CD15 and brain lipid binding protein (BLBP). After 3 days *in vitro* (DIV), cells were transferred from proliferation to differentiation medium (DM; DMEM/F12/B-27 without bFGF and EGF) and kept for an additional 7 DIV.

TREATMENT OF CELL CULTURES AND IMMUNOCYTOCHEMISTRY

Cells were treated for 7 DIV (every third day) in DM with a combination of recombinant human Shh (C24II), N-terminus (200 ng/ml; R&D systems, Minneapolis, MN, USA) and purmorphamine (PMM; 1 μM; Calbiochem Millipore), an agonist of Smoothened receptor and cyclopamine, an antagonist of Smo receptor (2.5 μM; EnzoLife Sciences, NY, USA). Control cells were kept in DM. Cells were fixed in 4% paraformaldehyde 24 h after isolation of RGCs and 7 DIV after pharmacological treatment. Primary antibodies diluted in blocking solution (1% bovine serum albumin, 5% normal goat serum, and 0.5% Tween-20 in PBS) were applied overnight at +4°C, followed by corresponding secondary Alexa 488- or Alexa 555- conjugated antibodies (Life Technologies) for 2 h at RT. Nuclei were counterstained with the nuclear stain bis-benzimide. Cells were visualized with a Zeiss fluorescence microscope using Axiovision software and photographed with a digital camera. Ten pre-designated adjacent optical fields

of view were examined at magnification 10× (0.5 mm^2 surface area); counts of immunolabeled cells were pooled together, expressed as means ± SEMs (Standard Error of the Means) and analyzed using Student's *t*-test. The criterion for significance was set at 5%.

WESTERN BLOT

Cells were homogenized in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulphonyl fluoride, and protease inhibitor cocktail) on ice for 30 min, centrifuged at 14,000*g* for 15 min at 4°C, and the supernatants were collected as the cell lysates. Equal amounts of protein from each sample were separated by SDS-PAGE on 12% gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Used primary antibodies are listed in **Table 2**. Membranes were incubated with the primary antibodies overnight at 4°C, and then with their corresponding secondary HRP-conjugated antibodies (1:15000, Thermo Fisher Scientific, Temecula, CA, USA). Protein signal was detected using SuperSignal West Pico Chemiluminescent system (Thermo Fisher Scientific, Temecula, CA, USA). Western blots were scanned and densitometric analysis was performed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Statistical analyses were performed using paired *t*-test. The criterion for significance was set at 5%.

REAL-TIME PCR

Real-time PCR (RT-PCR) was used to determine mRNA expression of GAPDH, Gsx2, Ascl1 and Sp8. Total RNA was extracted from cells using TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately 1 μg of RNA was used in the reverse transcription reaction using M-MuLV reverse transcriptase with random hexamers (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. RT-PCR was performed in a Realplex^2 Mastercycler (Eppendorf, Hamburg, Germany) using 96-well reaction plates (Eppendorf, Hamburg, Germany). The reactions were prepared according to the standard protocol for one-step QuantiTect SYBR Green RT-PCR (Applied Biosystems, Cheshire, UK). The sequences of the forward and reverse primers are presented at **Table 3**. The thermal cycle conditions were 95°C for 2 min followed by 40 cycles of 15 s at 95°C, 15 s at 55°C, and 20 s at 68°C. All assays were performed in triplicates. Averaged cycle of threshold (Ct) values of GAPDH triplicates were subtracted from Ct values of target genes to obtain ΔCt, and then relative gene expression was determined as $2^{-\Delta Ct}$. The results were presented relative to the control value, which was arbitrarily set to 1.

RESULTS

Gsx2 IS EXPRESSED IN THE HUMAN DEVELOPING CEREBRAL CORTEX

In order to establish if Gsx2 is present in the cortex of human developing telencephalon at midgestation, we dissected fresh dorsal (cortical; 16–19 gw) and ventral (GE) telencephalon (16–18 gw) and determined Gsx2 mRNA expression levels (**Figure 1A**). RT-PCR demonstrated that Gsx2 mRNA levels in the GE at 16 and 18 gw were 345 folds and 104 folds higher respectively compared to the cortex. Notably mRNA Gsx2 was present in the cortical tissue in all investigated samples (**Figure 1A**). An increase of the cortical Gsx2 mRNA levels was observed during development, changing three to nine folds from 16 to 18 and 19 gw, respectively (**Figure 1A**). Protein levels for Gsx2 in fetal cerebral cortex at 17–19 gw followed the trend seen with mRNA (**Figure 1B**). Thus, in addition to regional differences, Gsx2 expression in the human fetal brain seems to vary with gestational age, although this point needs to be confirmed on more cases.

Next, we sought to determine the expression pattern of Gsx2 in human fetal brain and compare it with the mouse using ISH. At E12.5 mouse brain characteristic Gsx2 expression was detected in the medial (MGE), lateral (LGE), and CGE but not in the pallium, in agreement to previous reports (Hsieh-Li et al., 1995; **Figures 1C,D**). However, in the human fetal cerebral cortex Gsx2 transcript was observed in the cortical ventricular/subventricular zone (VZ/SVZ), intermediate zone (IZ), subplate (SP), and cortical plate (CP) regions in all studied ages, from 18 to 24 gw ($n = 10$, **Figures 1E,F**). At this developmental stage, the highest expression was observed in the SP (**Figure 1F**). Occasionally $Gsx2^+$ cells were also observed on the ventricular surface which suggested their proliferation (**Figure 1E**). To explore this possibility, we performed fluorescent *in situ* hybridization (FISH) for Gsx2 combined with immunoreaction to anti-Ki67 antibody, which labels cycling cells. We estimated that 20% of all $Gsx2^+$ cells in the VZ/SVZ were proliferating as they were co-labeled with Ki67. Hence, not only that $Gsx2^+$ cells were present in the human developing cortex at midgestation, but a fraction of these cells were proliferating locally. Our findings differ from previously published reports where $Gsx2^+$ cells have been exclusively described in the subpallium in both rodents (Corbin et al., 2003; Xu et al., 2010) and humans (Ma et al., 2013). The observed difference in these studies could be due to the different methodological approaches followed. Here, in addition to immunolabeling with commercially available antibodies, we performed ISH, a high stringency conditioned method, to detect a specific signal for Gsx2 mRNA in the cortex.

After demonstrating the expression of Gsx2 in the human developing cortex, we stained cryosections with anti-Gsx2 and

Table 3 | List of primer sequences used in the study.

Gene	Forward	Reverse
Ascl1(Mash1)	TCTCATCCTACTCGTCGGACGA	CTGCTTCCAAAGTCCATTCGCAC
GAPDH	ACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA
Gsx2	GGAGATTCCACTGCCTCACCAT	CGGAGTCGAGACAGGTACATGT
Sp8	GAGGCTACAACTCGGATTACTCG	GTAGCACTGGCTTGAAGCCGTC

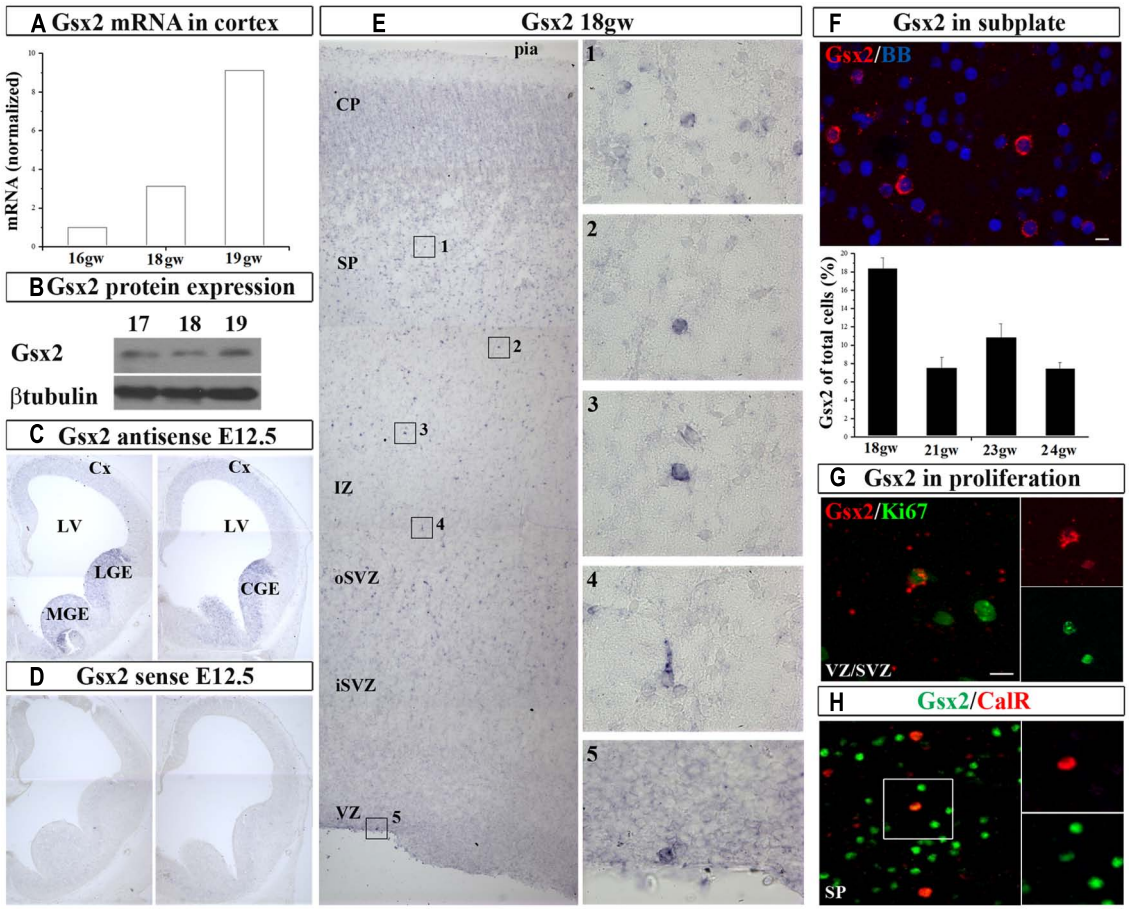

FIGURE 1 | Gsx2 in the human and mouse developing brain at mid-gestation. Expression of Gsx2 mRNA **(A)** and protein **(B)** in the human fetal cortex. *In situ* hybridization in E12.5 mouse with Gsx2 anti-sense **(C)** and sense **(D)** probe. **(E)** ISH in human fetal cerebral cortex (18 gw); insets- high magnification of Gsx2$^+$ cells; **(F)** Representative Gsx2$^+$ cells in subplate (FISH) for quantification (23 gw); percentage of Gsx2$^+$ cells from total cells is shown in the histogram in the different stages of development (18–24 gw). **(G)** *In situ* signal for Gsx2$^+$ cells (red) co-labeled with anti-Ki67 antibody (green) in cortical VZ (optical sectioning); **(H)** CalR$^+$ cells (red) in subplate colabeled with Gsx2 (green; 18 gw). VZ, ventricular zone; iSVZ, inner subventricular zone; oSVZ, outer subventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; Cx, cortex; LV, lateral ventricle; CGE, caudal ganglionic eminence; MGE, medial ganglionic eminence. Scale bars 10 μm.

anti-CalR antibodies and quantified the number of co-labeled cells. Immunolabeling of Gsx2$^+$ cells confirmed ISH results, with the highest number of cells observed in the SP and CP, accounting for about 10% of all cells in those regions at 17–19 gw ($n = 8$). Importantly, approximately one-third of all CalR$^+$ cells during midgestation were co-labeled with Gsx2 (30% at 15–16 gw, 29% at 17–19 gw; **Figure 1H**), suggesting that Gsx2 expression is important for the CalR lineage during this time of development and in this cortical region.

DISTRIBUTION OF Ascl1 AND Sp8 IN THE HUMAN DEVELOPING FOREBRAIN

We next studied the expression of TF Ascl1 in the human cortex, a reported downstream target of Gsx2 in rodents. We detected Ascl1 mRNA in fetal cortical VZ/SVZ (**Figure 2A**), which confirmed previous results obtained by immunolabeling in fetal human (Letinic et al., 2002; Jakovcevski et al., 2011) and non-human primate brains (Petanjek et al., 2009a). Expression of Ascl1 was higher in the GE compared to the cortex: 22 and 5-folds at 16 and 18 gw, respectively. Similar to our findings on Gsx2, the expression levels of Ascl1 mRNA increased in the course of development, from 16 to 18 gw (**Figure 2A**). Ascl1 expression was also demonstrated at the protein level (**Figure 2B**). To compare our results to mouse, we immunolabeled Ascl1 in mouse developing brain (E12.5) and observed the presence of Ascl1$^+$ cells mainly in the subpallium (**Figure 2C**). We next performed a similar experiment in the human forebrain at 24 gw, and showed that, besides the strong immunolabeling of the GE previously shown (Jakovcevski et al., 2011; Hansen et al., 2013), considerable immunoreactivity was present in the cortical regions (**Figure 2D**). The density of Ascl1$^+$ cells in the human cortex at midgestation was the highest in the VZ/SVZ where it reached almost 20% of all nuclei, followed by around 14% in the CP and 10% in the IZ and SP (**Figure 2E**). Notably, in the CP Ascl1$^+$ cells were preferentially distributed in the upper cortical layers (II/III; **Figure 2D**). We previously co-labeled cells with either CalR or GABA and Ascl1 antibodies

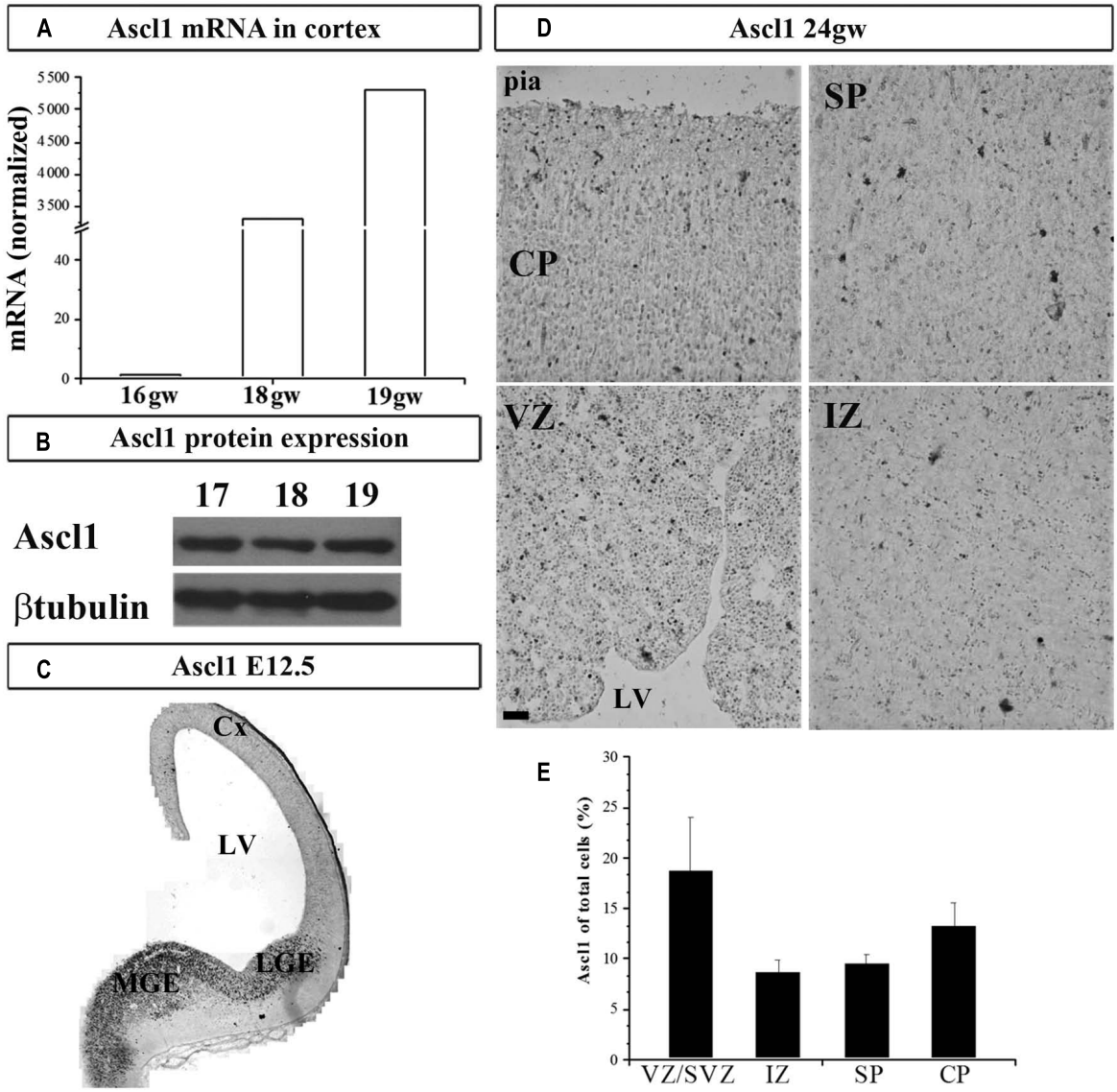

FIGURE 2 | Ascl1 in the human and mouse developing brain at mid-gestation. Expression of Ascl1 mRNA **(A)** and protein **(B)** in the human fetal cortex. Immunohistochemistry in E12.5 mouse with anti-Ascl1 antibody **(C)**. **(D)** Representative immunolabeled Ascl1$^+$ in human fetal cerebral cortex (24 gw). **(E)** Percentage of Ascl1$^+$ cells in all nuclei (labeled with bis-benzimide) in the human fetal neocortex at midgestation (21–24 gw). Data are presented as mean values + standard error of the mean (minimum three sections from two brains were studied). VZ/SVZ, ventricular/subventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; Cx, cortex; LV, lateral ventricle; CGE, caudal ganglionic eminence; MGE, medial ganglionic eminence. Scale bar 50 μm.

and found that in the CP up to 50% of Ascl1$^+$ cells could be co-labeled with GABA (Jakovcevski et al., 2011).

Next, we studied the expression of the zinc finger TF Sp8 required for the normal development of CalR interneurons in the olfactory bulb (Waclaw et al., 2006). In the human fetal cortex immunoreactivity for Sp8 has been observed in the dorsal LGE (dLGE) and dCGE, and a weak expression in the cortical VZ/SVZ (Ma et al., 2013). In addition, the same group reported that some migrating neurons in the CP were co-labeled with Sp8, GABA and chicken ovalbumin upstream promoter TF II (CoupT-FII) antibodies, indicating their CGE origin (Ma et al., 2013). In the current study we extended these results by demonstrating not only Sp8 protein, but also the Sp8 mRNA expression in human fetal cortical tissue (**Figures 3A,B**). In the two cases studied (16 and 18 gw) Sp8 mRNA was higher in the GE than in cortex six and twofolds, respectively, and as previously observed with Gsx2 and Ascl1, cortical levels increased approximately two times from 16 to 18 and 19 gw (**Figure 3A**). In comparison to the E12.5 mouse brain where Sp8$^+$ cells were revealed in the MGE, LGE and cortex as reported previously (Sahara et al., 2007; Borello et al., 2013; **Figure 3C**), in human, Sp8$^+$ cells were also packed in the cortical SVZ/VZ and scattered in SP and CP

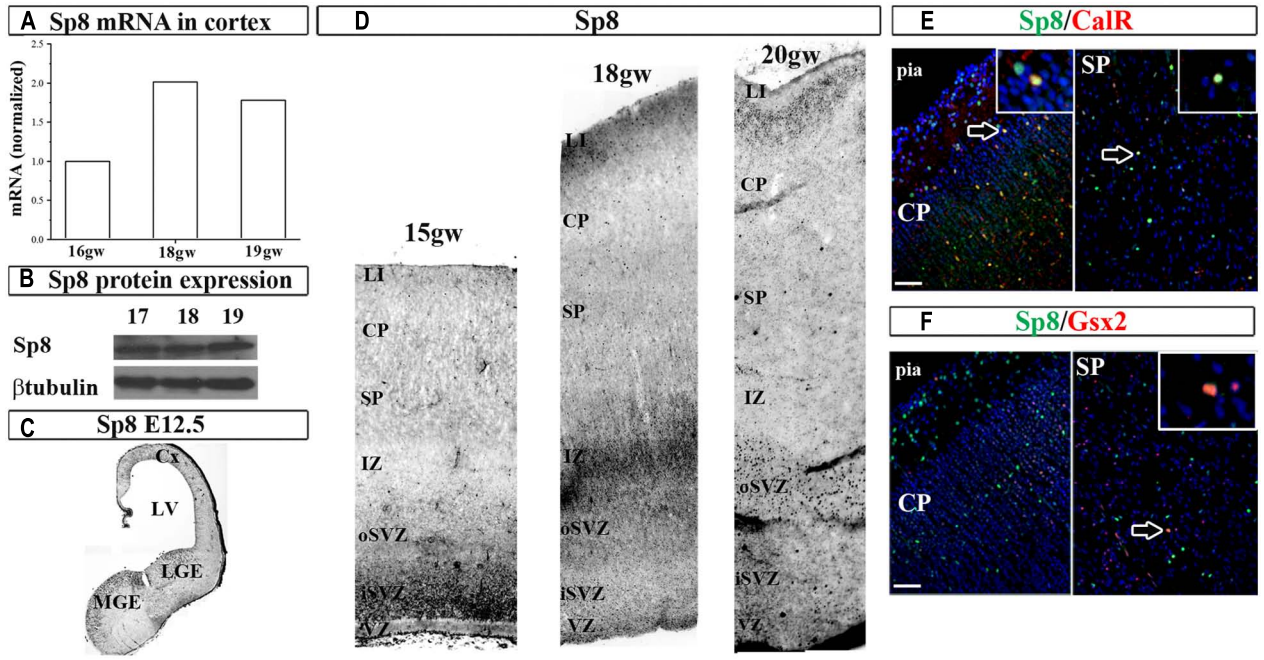

FIGURE 3 | Sp8 in the human and mouse developing brain at mid-gestation. Expression of Sp8 mRNA **(A)** and protein **(B)** in the human fetal cortex. Immunohistochemistry in E12.5 mouse with anti-Sp8 antibody **(C)**. **(D)** Representative immunolabeled Sp8$^+$ cells in the human fetal cerebral cortex (15–20 gw). Sp8$^+$ cells (green) co-labeled with **(E)** CalR (red) and **(F)** Gsx2 (red) in cortical plate and subplate. VZ, ventricular zone; iSVZ, inner subventricular zone; oSVZ, outer subventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; Cx, cortex; LV, lateral ventricle; CGE, caudal ganglionic eminence; MGE, medial ganglionic eminence. Scale bar, 50 μm.

(**Figure 3D**). Double-labeling experiments have shown that subpopulations of cortical Sp8$^+$ cells co-expresses CalR or Gsx2, suggesting that a subpopulation of them are cortical interneurons (**Figures 3E,F**). However, further studies are needed to establish a direct lineage relationship of Gsx2 to CalR$^+$ cells in the human cortex.

CORTICAL AND GE HUMAN RGCs HAVE DIFFERENT POTENTIAL TO GENERATE CalR$^+$ CELLS

Previously, we have shown that cortical RGCs *in vitro* have the potential to generate CalR$^+$/GABA$^+$ interneurons and Nkx2.1$^+$ progenitors (Yu and Zecevic, 2011; Radonjic et al., in press). Here, we explore if cortical and GE RGCs retain their expression of regional characteristic markers *in vitro* and compare their capacity to produce GABA and CalR$^+$ cortical interneurons.

To this end, we dissected cortical VZ/SVZ and ventral telencephalon (GE) and established dissociated cell cultures. To isolate RGCs, we immuno-sorted CD15$^+$ cells, a specific surface marker of RGCs (Mo et al., 2007; Yu and Zecevic, 2011; Ortega et al., 2013). Cortical and GE RGC cultures were differentiated for 7 DIV and the expression of characteristic markers for RGCs (Sox2), projection neurons (Tbr1 and Tbr2) and interneuronal progenitors (Gsx2, Sp8, Ascl1, and Nkx2.1) were analyzed. Expression of typical markers of glutamatergic neurons Tbr1 and Tbr2 was higher in cortical RGC cultures than in GE RGC cultures (**Figure 4A**). In contrast, the expression of Sp8, Ascl1, and Nkx2.1 was higher in GE RGC cultures (**Figure 4A**), but no difference in Gsx2 expression was observed in these two regions. After 7 DIV both cortical and GE RGCs generated CalR$^+$ and GABA$^+$ cells, but with a different capacity. Namely, more CalR$^+$ (22 vs.12%) and GABA$^+$ (22 vs.16%) cells were generated in the GE RGC cultures than in dorsal cultures (**Figures 4B,C**). These results are in agreement with our previous reports (Mo et al., 2007; Mo and Zecevic, 2008). Hence, we concluded that cortical and GE RGC cultures retain *in vitro* regional identities as well as the potential to generate GABAergic interneurons.

EXOGENOUS Shh AFFECTS THE EXPRESSION OF TRANSCRIPTION FACTORS UPSTREAM OF CalR

The morphogen Shh has numerous functions in the developing central nervous system (Dahmane and Ruiz-i-Altaba, 1999). One of function is the induction of the ventral TFs Nkx2.1 (thyroid transcription factor-1, TTF1), Dlx1/2 and Ascl1 and specification of cortical interneurons in the ventral telencephalon of rodents (Anderson et al., 1997; Kohtz et al., 1998; Sussel et al., 1999; Xu et al., 2004, 2005; Butt et al., 2005). In the human fetal cerebral cortex, we previously observed Shh expression in the Map2$^+$ neurons in the fetal CP/SP and Sox2$^+$ RGCs in the VZ (Radonjic et al., in press). Treatment of cortical RGC cultures with exogenous Shh suppressed the generation of CalR$^+$ interneurons in favor of Nkx2.1$^+$ progenitors, suggesting that in humans Shh differentially affects subgroups of cortical progenitors (Radonjic et al., in press).

Here, using *in vitro* enriched RGCs we examined the effect of Shh on TFs upstream of CalR$^+$ cells and demonstrated that

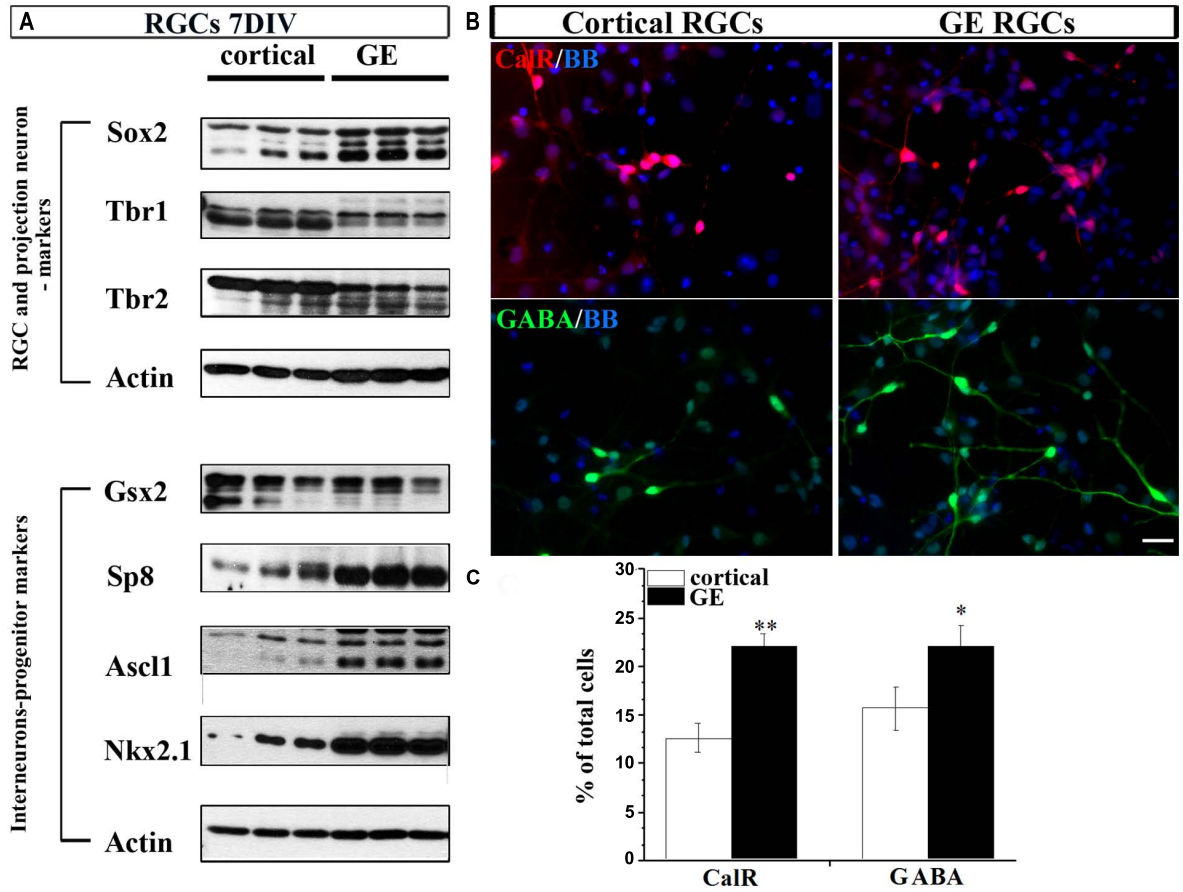

FIGURE 4 | Differentiation of RGCs enriched from cortical or GE regions. (A) Markers of RGCs, projection neurons and interneurons expressed in cortical and GE enriched RGCs after 7 DIV. **(B)** Representative staining of CalR$^+$ (red) and GABA$^+$ (green) cells after 7 DIV in cortical and GE RGCs. **(C)** Histogram shows the percentage of CalR$^+$ and GABA$^+$ cells from the total cells differentiated after 7 DIV in cortical and GE RGC cultures. $*p < 0.05$, $**p < 0.01$; *t*-test. Scale bar, 20 μm.

treatment of cortical RGCs with PMM/Shh reduced protein levels of Ascl1 and Sp8, but not Gsx2 levels (**Figure 5**). However, treatment with cyclopamine, an inhibitor of Shh signaling, did not affect expression levels of these TFs (**Figures 5A,B**). The lack of effect of cyclopamine suggests that additional Shh independent pathways might control cortical interneurongenesis, as it is a case with oligodendrocyte progenitors (Nery et al., 2001; Ortega et al., 2013) and Nkx2.1$^+$ progenitors (Radonjic et al., in press). The mRNAs levels of Gsx2, Ascl1 and Sp8 obtained by PCR analysis showed similar trend, but the differences did not reach significance (**Figure 5C**). Although additional experiments are needed to formulate final conclusions, these results suggest that in human cortical development Shh could differentially modulate distinct TFs implicated in the generation of CalR$^+$ interneurons.

DISCUSSION

The potential origin and progenitors of CalR$^+$ interneurons in the human cerebral cortex are still under debate. Here, we demonstrated the expression pattern of three TFs, Gsx2 and its downstream effectors Ascl1 and Sp8, both in the GE and in the cortex of the fetal human forebrain at midgestation. A fraction of cells expressing these TFs were co-labeled with CalR, suggesting their lineage relationship. Notably, we identified a potentially important species difference in the expression of Gsx2. While in the mouse cortex, Gsx2 mRNA was not observed, human fetal cortical cells express this transcript and importantly a subset of these cells was proliferating in the cortical VZ/SVZ. Thus, our results support the view that both cortical and GE regions of the human fetal telencephalon have the capacity to give rise to cortical CalR$^+$ cells. Finally, we show that the mechanism underlying the effects of Shh on the reduction of CalR interneurons involves the down-regulation of Ascl1 and Sp8 expression.

TRANSCRIPTION MECHANISMS INVOLVED IN THE GENERATION OF CalR CELLS

In mice, bipolar CalR$^+$ interneurons originate mainly from Gsx2$^+$ germinal zones of LGE and CGE (Fogarty et al., 2007; Sahara et al., 2007; Miyoshi et al., 2010; Xu et al., 2010). It is estimated that the CGE gives rise to approximately 30–40% of all cortical interneurons (Grateron et al., 2003; Miyoshi et al., 2010). Use of inducible genetic fate mapping techniques demonstrated that 75%

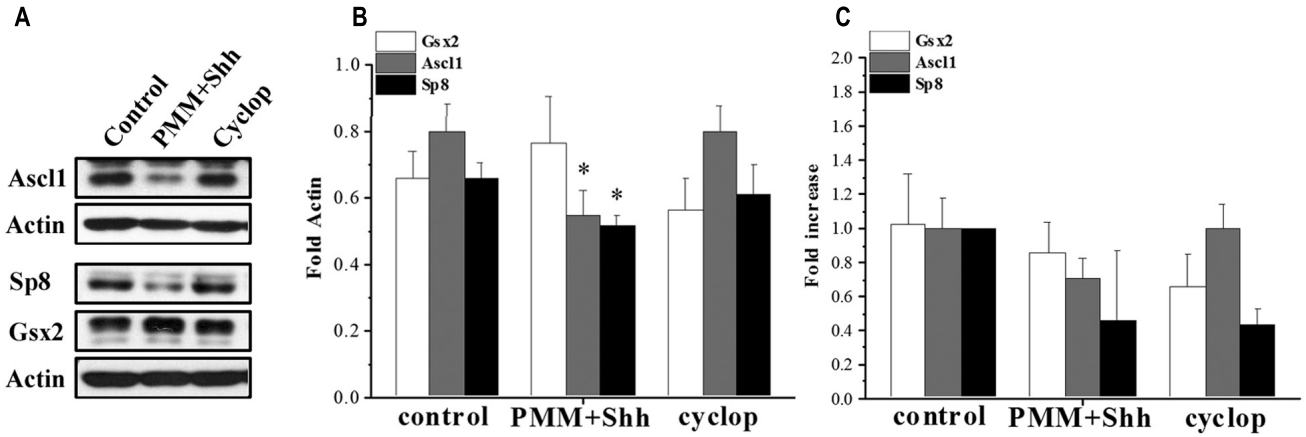

FIGURE 5 | Effect of Shh signaling on Gsx2, Ascl1, and Sp8 in cortical RGC cultures after 7 DIV. (A) Representative immunoblots and **(B)** quantification on the protein expression and **(C)** mRNA levels after treatment with PMM/Shh and cyclopamine. $*p < 0.05$, *t*-test.

of labeled precursors from the CGE contribute to the superficial cortical layers regardless of their birth date (Miyoshi et al., 2010). A required downstream effector of Gsx2 is Ascl1 (Wang et al., 2009). Both Gsx2$^+$ and Ascl1$^+$ neural progenitor cells express CoupTFII (Kanatani et al., 2008), a TF that labels cells in the CGE (Yozu et al., 2005; Kanatani et al., 2008; Miyoshi et al., 2010). In human cortex at mid-gestation 60–75% of CalR$^+$ cells, depending on the cortical layer, co-express CoupTFII (Reinchisi et al., 2012). Other studies in human and monkey fetal brains found that nearly all cortical CalR$^+$ cells co-express either CoupTFII or/and Sp8 and thus originated in the CGE and dLGE (Hansen et al., 2013; Ma et al., 2013). Another downstream effector of Gsx2 is Sp8, a TF that plays a role in cortical patterning, proliferation and differentiation of cortical progenitors (O'Leary and Sahara, 2008; Waclaw et al., 2009; Wang et al., 2009; Cai et al., 2013). In rodents, Sp8 is present in 20% of cortical interneurons that originate from dLGE and CGE and express Reelin, VIP, NPY, and CalR (Ma et al., 2012). Similar to rodents, in the human fetal telencephalon Sp8 immunoreactivity was demonstrated in the dLGE and CGE, and in postmitotic neurons in the cortex (Ma et al., 2013; Hansen et al., 2013). Our results show that at midgestation cortical Sp8$^+$ cells were mainly localized to the SVZ/VZ, but also in upper cortical regions. Notably, a subpopulations of cortical Sp8$^+$ cells co-express CalR or Gsx2, suggesting that they are cortical interneuron progenitors.

Although recent results point to the CGE as the major source of CalR$^+$ cells, a fraction of proliferating CalR$^+$ cells was observed in the human cortical VZ/SVZ at midgestation (Jakovcevski et al., 2011; Zecevic et al., 2011). Further corroboration that a subtype of CalR$^+$ cells has cortical origin came from our result that 20% of Gsx2$^+$ cells, presumably progenitors of CalR$^+$ cells, are dividing in the cortical VZ/SVZ. Additionaly, both *in vivo* and *in vitro* approaches showed the potential of human cortical Pax6$^+$/BLBP$^+$ RGCs to produce CalR$^+$ cells. We previously reported that in human fetal cortical VZ/SVZ, CalR is co-localized in a subpopulation of Pax6$^+$ cells, and CalR$^+$ cells can be produced from genetically labeled BLBP$^+$ cortical RGCs *in vitro* (Mo and Zecevic, 2008; Yu and Zecevic, 2011). Moreover, knocking down Pax6 in cortical RGCs, greatly reduced the generation of CalR$^+$ cells (Mo and Zecevic, 2008). Taken together, these results suggest an important role of Pax6 not only for genesis of glutamatergic neurons, but also CalR$^+$ cells. However, we did not observe GABA in the Pax6$^+$ progenitors, suggesting that GABA is expressed only in postmitotic cells that already down-regulated Pax6.

Thus, our results reinforced the observation that in primates a number of progenitors related to cortical interneurons have a mitotic origin in the cortical VZ/SVZ at midgestation. These include CalR$^+$, Ascl1$^+$, and Nkx2.1$^+$ (Jakovcevski et al., 2011), CoupTFII$^+$ (Reinchisi et al., 2012) and Gsx2$^+$ cortical progenitors (this study). Other groups have also found that cortical cells expressing Ascl1 and co-labeled with interneuron markers proliferate in human and the non-human primate VZ/SVZ (Letinic et al., 2002; Petanjek et al., 2009b).

These findings are consistent with the idea that the cortical VZ/SVZ is an additional source of interneurons in the primate developing cortex. However, this issue is still under debate since other groups reported either no expression of those TFs (Gsx2) in cortical areas or the expression only in postmitotic cells (Sp8, CoupTFII, Nkx2.1; Hansen et al., 2013; Ma et al., 2013). These discrepancies might be methodological, due to the different tissue preparation, *post mortem* time or antibodies used. To minimize the inherent variability in human tissue sampling, we tried to confirm our results at both the mRNA and protein level using a combination of histological techniques (ISH and immunohistochemistry, respectively) as well as Western blot and RT-PCR analysis of the tissue homogenates. Due to scarce availability of human fetal brain tissue, it is not unusual that such discrepancies arise and we hope that further studies with larger samples and better standardized methods would resolve the still open questions about the origin of cortical interneurons in primates. We must emphasize, however, that the finding of interneuron progenitors in cortical regions of the primate brain does not preclude the possibility that some of these cells have originated in the GE, migrated as intermediate progenitors and continue to proliferate in the cortical VZ/SVZ, as already suggested in mice (Wu et al., 2011). Both mechanisms

could be present simultaneously with a goal to increase the number of cortical interneurons in the enlarged primate cerebral cortex.

Notably, the cortical and GE VZ/SVZ are not the only sources of cortical interneurons in later stages of human brain development. Still another probable source is a primate-specific subpial granular layer (SGL), transiently present under the pia in humans (Brun, 1965; Gadisseux et al., 1992; Meyer and Wahle, 1999; Zecevic and Rakic, 2001) and monkey developing brains (Zecevic and Rakic, 2001). Various sources for CalR$^+$ cells are probably necessary to supply increased upper cortical layers with higher numbers of CalR$^+$ interneurons in primates compared to other species. Importantly, different origins of CalR$^+$ cells are likely to contribute to the diversity of this subpopulation of interneurons in primates. The increased complexity of cortical progenitors is considered to be an evolutionary adaptation necessary for the development of higher brain functions in primates, and particularly in humans.

EXTERNAL FACTORS AFFECTING CalR EXPRESSION

Clonal lineage studies of non-pyramidal neurons suggested that the expression of calcium binding proteins is not genetically programmed and is likely to be induced by functional activity (Alcántara et al., 1993; Grateron et al., 2003) and external factors (Mione et al., 1994). For example, bFGF stimulates the generation and differentiation of CalR$^+$ neurons, and its effects are enhanced by retinoic acid (Pappas and Parnavelas, 1998). Another external factor affecting CalR$^+$ cells in mice is Shh (Butt et al., 2005; Xu et al., 2005, 2010). Exogenous Shh treatment of MGE progenitors in mice resulted in a down-regulation of both CalR and Gsx2, whereas down-regulation of Shh in the MGE resulted in conversion of interneuron fate from PV and Sst$^+$ to bipolar CalR$^+$ (Xu et al., 2005, 2010; Carney et al., 2010). Human stem cells are used by many groups as a model for studying the development of human cortical interneurons (Mariani et al., 2012; Maroof et al., 2013). In our study, we used instead RGCs isolated from the human developing cortex to study the molecular mechanisms underlying interneuron generation and specification. We observed that cortical and GE RGCs *in vitro* retain different potential to generate interneurons. This result is in line with previous *in vitro* results demonstrating that enriched RGCs maintain their regional identity (Radonjic et al., in press). Treatment of cortical human RGCs resulted in down-regulation of Ascl1 and Sp8, while there was no effect on Gsx2 expression. We hypothesize that this decrease is either due to re-specification to Nkx2.1$^+$ progenitors or maintenance of Gsx2$^+$ progenitor state. We demonstrated recently that treatment with Shh reduced the number of CalR$^+$ cells generated in RGC cultures (Radonjic et al., in press), thus it is tempting to speculate that Shh arrests further differentiation of Gsx2 progenitors into CalR$^+$ cells.

TRANSIENT CalR EXPRESSION IN CORTICAL PROGENITORS DURING DEVELOPMENT

In this study we describe the expression pattern of CalR$^+$ cells during human brain development in presumably interneuron progenitors. However, the question remains if CalR expression during development is transient or stable. In the primate cortex, CalR$^+$ cells can be detected already at 5 gw with a gradient from subcortical GE to the neocortex (Rakic and Zecevic, 2003; Zecevic et al., 2011). However, the earliest CalR$^+$ cells in the preplate zone cannot be considered interneurons (Meyer et al., 2000; González-Gómez and Meyer, 2014). Later during development, at midgestation, CalR$^+$ cells almost entirely overlap with immunoreactivity to GABA (Rakic and Zecevic, 2003; Zecevic et al., 2005). During development however, the number of CalR$^+$ cells decreases while at the same time levels of other calcium binding proteins expressed in GABAergic neurons, such as calbindin or parvalbumin, increase (Yan et al., 1995, 1996; Schlösser et al., 1999; Brandt et al., 2003). In addition, the percentage of CalR$^+$/GABA$^+$ cells is reduced from 96% at midgestation to 37% in adulthood in primates (Del Río and DeFelipe, 1994; Yan et al., 1995), suggesting that CalR could have an important role in other cortical cell types during development.

CONCLUSION

Although many molecular and cellular mechanisms in brain development are shared between humans and rodents, considerable differences stress the need to expand studies of human cortical development. Evolutionary adaptations resulted in the development of the outer SVZ in primates (Smart et al., 2002; Zecevic et al., 2005; Hansen et al., 2010; Lui et al., 2011), which without doubt has a critical role in the expansion and unique organization of the complex human cerebral cortex. The complexity of the CalR$^+$ progenitors pool shown here can be translated into a higher diversity of cortical CalR$^+$ cells, which might be essential for balanced cortical function. Further studies on the origin and specificity of different interneuron subtypes in the human cerebral cortex are needed to better understand and eventually prevent or treat numerous human-specific psychiatric and neurological disorders.

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Spatio-temporal extension in site of origin for cortical calretinin neurons in primates

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The vast majority of cortical GABAergic neurons can be defined by parvalbumin, somatostatin or calretinin expression. In most mammalians, parvalbumin and somatostatin interneurons have constant proportions, each representing 5–7% of the total neuron number. In contrast, there is a threefold increase in the proportion of calretinin interneurons, which do not exceed 4% in rodents and reach 12% in higher order areas of primate cerebral cortex. In rodents, almost all parvalbumin and somatostatin interneurons originate from the medial part of the subpallial proliferative structure, the ganglionic eminence (GE), while almost all calretinin interneurons originate from its caudal part. The spatial pattern of cortical GABAergic neurons origin from the GE is preserved in the monkey and human brain. However, it could be expected that the evolution is changing developmental rules to enable considerable expansion of calretinin interneuron population. During the early fetal period in primates, cortical GABAergic neurons are almost entirely generated in the subpallium, as in rodents. Already at that time, the primate caudal ganglionic eminence (CGE) shows a relative increase in size and production of calretinin interneurons. During the second trimester of gestation, that is the main neurogenetic stage in primates without clear correlates found in rodents, the pallial production of cortical GABAergic neurons together with the extended persistence of the GE is observed. We propose that the CGE could be the main source of calretinin interneurons for the posterior and lateral cortical regions, but not for the frontal cortex. The associative granular frontal cortex represents around one third of the cortical surface and contains almost half of cortical calretinin interneurons. The majority of calretinin interneurons destined for the frontal cortex could be generated in the pallium, especially in the newly evolved outer subventricular zone that becomes the main pool of cortical progenitors.

Keywords: interneurons, calretinin, ganglionic eminence, ventricular zone, GABA, epilepsy

INTRODUCTION

Neurons of the mammalian neocortical network can be roughly divided in glutamatergic excitatory and GABAergic inhibitory neuron population (Nieuwenhuys, 1994; DeFelipe et al., 2002, 2013). The first class, often referred as pyramidal neurons, is characterized by dendrites covered with spines and by long axon that projects away from pial surface and travels through white matter to target other cortical (corticocortical projections) or subcortical regions (corticofugal projections). Nearly all pyramidal neurons also send recurrent collateral branches which end in the local cortex (DeFelipe and Farinas, 1992; Spruston, 2008; Thomson, 2010; Zaitsev et al., 2012). During development, some features common to the typical pyramidal neurons are transformed by reduction, but analogy with the dendritic morphology of typical pyramidal cell is obvious. These neurons can be defined as modified pyramidal neurons (Braak and Braak, 1985). The spiny stellate cells in layer IV of the primary sensory areas are glutamatergic neurons that retract their output axon branch during development but retain only spiny dendrites and an intracortical axonal arbor (Vercelli et al., 1992; Nieuwenhuys, 1994; Markram et al., 2004). Glutamatergic cortical neurons are usually defined as principal, while in mammals they are (depending on region and species) 3–5 times more numerous than those synthesizing GABA. Still less abundant, cortical GABAergic neurons form extremely heterogeneous population with regard to their connectivity, molecular features, and physiological properties (DeFelipe et al., 2013). The vast majority of GABAergic neurons has axon branching only inside the cortex and are therefore defined as local circuit neurons (interneurons). A small amount of cortical GABAergic neurons has axons turning into the white matter (Tomioka and Rockland, 2007; Melzer et al., 2012). They are remnants of the earliest functional fetal neocortical network (Kostovic and Rakic, 1990; Super and Uylings, 2001; Judas et al., 2013).

Cortical GABAergic neurons are internal modulators of cortical output that is achieved through direct and indirect control of principal neuron compartments (Jones, 1993). Some of the cortical GABAergic neurons subtypes are targeting the axon initial segment of principal neurons, some are making synapses mainly on cell bodies and proximal dendrites, whereas others are targeting more distal parts of principal neurons dendritic tree (DeFelipe et al., 2013). Interestingly, some cortical GABAergic

neurons preferentially target other cortical GABAergic neurons (Caputi et al., 2009).

Each of the main cortical GABAergic neurons group defined on the basis of efferent connectivity is characterized by remarkable physiological diversity and could be divided into distinct subtypes (Markram et al., 2004; Ascoli et al., 2008; DeFelipe et al., 2013). Additional heterogenity is showed by expression of molecular markers. Nevertheless, molecular classification is suitable to study regional and species differences in laminar distribution and site of origin for different cortical GABAergic neurons subtypes. High expression of three calcium-binding proteins, parvalbumin, calbindin and calretinin is present only in cortical GABAergic neurons, and not in neurons expressing glutamate. More than 80% of all cortical GABAergic neurons express one of these three calcium-binding proteins without significant overlap, making these molecules a good marker for identification of different cortical GABAergic neuron subpopulations (Van Brederode et al., 1990; Kubota et al., 1994; Yan et al., 1995; Gonchar and Burkhalter, 1997; Tamamaki et al., 2003; Gonchar et al., 2007; Burkhalter, 2008; Uematsu et al., 2008; Xu et al., 2010). It was found that calbindin-positive cells also express somatostatin (Rogers, 1992; Kubota et al., 1994; Gonchar and Burkhalter, 1997; Kawaguchi and Kubota, 1997). However, around one third of somatostatin-positive cells do not express detectable level of calbindin. They represent a larger proportion of cortical GABAergic neurons and also include neuronal nitric oxide synthase (nNOS) expressing cells, as well as neuropeptide Y positive but calbindin-negative neurons. Therefore, the neuropeptide somatostatin was shown to be a more eligible marker than calbindin, enclosing a larger subset of cortical GABAergic neurons without overlap to other two major subpopulations. Neurochemical features of interneurons can be coupled with their synaptic targets. The parvalbumin-expressing cells are mainly targeting soma and perisomatic region, including axon initial segment of principal cells. The calretinin-expressing cells are targeting proximal parts of dendritic tree, whereas somatostatin-expressing are targeting the distal dendritic regions of pyramidal neurons.

Recent data suggest that the most numerous cortical GABAergic neurons subpopulation in rodent is parvalbumin subpopulation. The parvalbumin-expressing neurons account for about 40% of total cortical GABAergic neurons population in rodents, around 30% of cortical GABAergic neurons are somatostatin-expressing and 25% are calretinin-expressing neurons (Gonchar et al., 2007; Uematsu et al., 2008; Xu et al., 2010). Part of the calretinin neurons co-express vasoactive intestinal peptide or reelin. However, some cells express vasoactive intestinal peptide or reelin only and represent around 5% of the total cortical GABAergic neurons (Burkhalter, 2008; Ma et al., 2013; **Figure 1**). In addition, recent data suggest that population of calretinin, vasoactive intestinal peptide and reelin-expressing cells in rodents can be identified by expression of the serotonin receptor 5HT3aR. Heterogeneous 5HT3aR-expressing population comprise approximately 30% of all interneurons, and together with parvalbumin- and somatostatin-expressing population account for nearly 100% of all mouse cortical GABAergic neurons (Rudy et al., 2011). As recently discovered, 5HT3aR population has to be further characterized.

PRONOUNCED INCREASE IN PROPORTION OF CALRETININ NEURONS DISTINGUISH GABAergic CORTICAL NETWORK OF PRIMATES

Numerous studies analyzing laminar distribution and density of cortical GABAergic neurons subtypes were performed in various species (Hendry et al., 1987; Van Brederode et al., 1990; Rogers, 1992; Beaulieu, 1993; Kubota et al., 1994; Micheva and Beaulieu, 1995; Yan et al., 1995; Del Rio and DeFelipe, 1996; Gabbott and Bacon, 1996a,b; Del Rio and DeFelipe, 1997; Gabbott et al., 1997a,b; Gonchar and Burkhalter, 1997; Tamamaki et al., 2003; Zaitsev et al., 2005; Gonchar et al., 2007; Sherwood et al., 2007; Tomioka and Rockland, 2007; Uematsu et al., 2008; Xu et al., 2010; Barinka et al., 2012). Due to different methodological approaches, it is very difficult to perform consistent comparative analysis of regional, laminar and species differences that surely exist. Intention to make collective conclusion about proportion of cortical GABAergic neurons and their subtypes in animals of particular order is therefore oversimplified (Burkhalter, 2008). Nevertheless, reports analyzing multiple regions suggest considerable consistency in the proportion and laminar distribution between various cortical regions inside the species of the same order (Tamamaki et al., 2003; Xu et al., 2010). It seems that even the main pattern of laminar and regional distribution of different cortical GABAergic neurons subtypes is highly conserved through mammalian evolution. However, there is no proportional increase in number of all cortical GABAergic neurons subtypes.

The vast majority of studies performed in rodents demonstrated that GABAergic neurons represent less than 20% of cortical neurons (Beaulieu, 1993; Micheva and Beaulieu, 1995; Gabbott et al., 1997a; Tamamaki et al., 2003). Parvalbumin is the most dominant population, whereas the calretinin is the less numerous (**Figure 1A**). Most of the studies suggest that in the human, where higher order associative areas comprise at least 50% of the neocortical surface, calretinin becomes the dominant population representing almost 50% of cortical GABAergic neurons (Conde et al., 1994; Gabbott and Bacon, 1996b; Zaitsev et al., 2005; Barinka and Druga, 2010). The number of GABAergic neurons increases more than is the case for principal neurons during mammalian evolution, so they exceed 20% of all cortical neurons in the primate neocortex (Hendry et al., 1987; Del Rio and DeFelipe, 1996; Gabbott and Bacon, 1996a,b; Jones, 2009). The proportion of cortical GABAergic neurons seems to increase for about 50% when primates (where cortical GABAergic neurons represent around 24% of total neuron number) are compared to rodents (where cortical GABAergic neurons represent around 16% of total neuron number). In parallel with increase of total neuron number, the number of parvalbumin- and somatostatin-expressing neurons increases linearly. Each of these subtypes represents between 5 and 7% of neurons in both, rodent and primate species. This would imply that increase in proportion of cortical GABAergic neurons is principally related to calretinin subtype which number increases exponentially, representing about 4% of total number of neurons in rodents and about 12% in primates (**Figure 1B**).

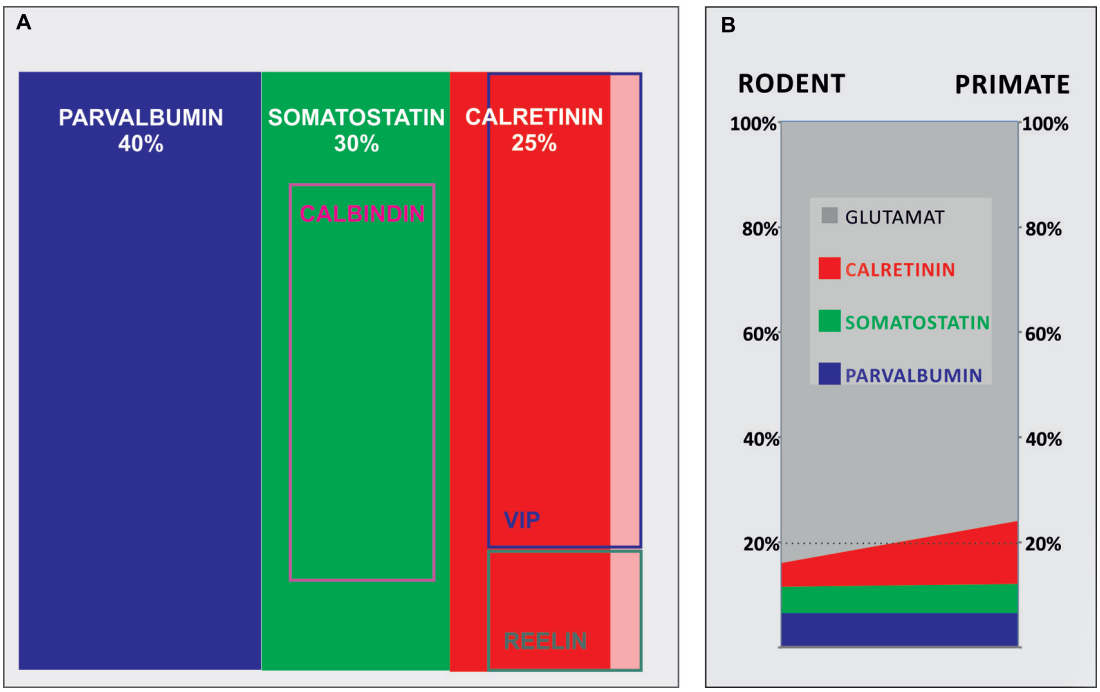

FIGURE 1 | A contribution of main cortical GABAergic neuron subpopulations to total number of GABAergic neurons in rodents and their contribution to total number of neurons in rodents and primates. **(A)** Demonstrates contributions of different subpopulations of GABAergic neurons in rodents, showing that parvalbumin, somatostatin, and calretinin comprise more than 90% of all GABAergic neurons. **(B)** Demonstrates 50% of increase of the contribution of cortical GABAergic neurons from rodents (≈16%) to primates (≈24%), which can be attributed to threefold increase in number of calretinin neurons (shown in red; from ≈4% in rodents to ≈12% of the total neuron number in primates).

We expect that such a disproportional increase in number of one GABAergic neurons subtype will provoke significant changes in neuronal network organization and substantially different modes of signal processing (Burkhalter, 2008). The connection between exponentially increased number of calretinin neurons through human associative areas and tremendous increase in cognitive capability is speculative (Forbes and Grafman, 2010), but apparent (**Figure 2**). The majority of neurological and psychiatric disorders that involve cortical pathology are suggested to have some level of disorganization in GABAergic network (Friocourt and Parnavelas, 2011; Marin, 2012). Most of these disorganizations have developmental origin. While some disorders can be found only in humans, such as schizophrenia and autism, other disorders (e.g., epilepsy) have in humans more complex and specific symptomatology. Therefore, it is important to understand human-specific features in organization and development of cortical GABAergic network, especially for those showing main differences, as is calretinin neuron population. We suggest that evolution is partially changing rules about origin of this neuron subtypes.

GE IS A MAIN SOURCE OF RODENT CORTICAL GABAergic NEURONS AND EARLY FETAL PRIMATE CORTICAL GABAergic NEURONS

During corticogenesis in mouse, from embryonic day (E) 11 to E19 vast majority, if not all cortical GABAergic neurons originate from moleculary and morphologically distinct regions of ventral telencephalon (Flames et al., 2007; Gelman and Marin, 2010) and migrate tangentially into the cerebral cortex (Van Eden et al., 1989; Parnavelas et al., 2002; Faux et al., 2010; Hernandez-Miranda et al., 2010; Antypa et al., 2011; **Figure 3**). A primary source is the medial ganglionic eminence (MGE), producing approximately 50–60% of the cortical GABAergic neurons with a peak of proliferation around E12–E13 (Wichterle et al., 2001; Wonders and Anderson, 2006; Butt et al., 2007; Gelman and Marin, 2010). The MGE is defined by the expression of homeobox transcription factor Nkx2.1 (Sussel et al., 1999; Butt et al., 2008). The parvalbumin-expressing subpopulation originate from Nkx2.1 progenitors in the ventral part of MGE, while the Nkx6.2 co-expressing progenitors from the dorsal part of MGE produce somatostatin subpopulation of cortical GABAergic neurons (Flames et al., 2007; Fogarty et al., 2007; Wonders et al., 2008). After leaving the MGE, migratory cortical GABAergic neurons downregulate Nkx2.1 but maintain Sox6 expression necessary for their normal positioning and maturation (Batista-Brito et al., 2009).

The second greatest source of GABAergic neurons progenitors is the caudal ganglionic eminence (CGE), producing in mouse about 30% of all cortical GABAergic neurons with a later peak of neurogenesis, around E16.5 (Miyoshi et al., 2010). Progenitors from the CGE express orphan nuclear factors COUP-TF I/II (Kanatani et al., 2008) and generate diverse subtypes of cortical GABAergic neurons that preferentially occupy superficial cortical layers (Miyoshi et al., 2010). The CGE-derived interneurons include two large groups: bipolar interneurons that express

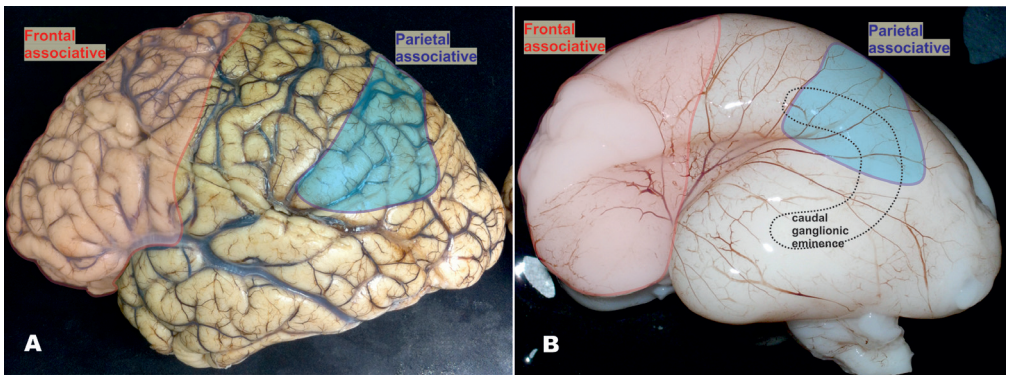

FIGURE 2 | Mature and fetal human brain around mid-gestation, showing that higher order multimodal areas enclose the frontal granular cortex and majority of parietal lobe (excluding postcentral gyrus). (A) In the human brain, frontal granular cortex occupies 80% of the frontal lobe without clear correlate in mouse. In the humans, this cortex represents almost one third (red) of total cortical surface, as well as 80% of multimodal associative areas including associative areas of the parietal cortex (blue). In the higher order associative areas located in the frontal (red) and parietal lobe (blue), calretinin-expressing neurons represent almost 50% of GABAergic neuron population and more than 10% of total neuron population. Due to presumably smaller proportion of calretinin neurons in temporal and occipital cortices (Ma et al., 2013) and taking into the consideration the size of frontal granular cortex, we assumed that almost half of cortical calretinin neurons are located in the frontal lobe. **(B)** The proportions in the size of higher order associative cortices are already established around mid-gestation. In the recent study, Ma et al. (2013) concluded that vast majority of calretinin neurons in human originate from the caudal ganglionic eminence (CGE, dashed line showed a contour of its position) and that there is no abundant production of GABAergic neurons in the pallial proliferative zones as it was suggested before (Letinic et al., 2002). The anatomical relations between anteriorly located prefrontal cortex (that comprises almost half of cortical calretinin neurons) and postero-laterally located CGE (as a main source of calretinin neurons) do not concur with this presumption (see **Figure 5B**). We suggest that presumption of Ma et al. (2013) can be correct in humans for all cortical regions only during early fetal stage, and later on for the all lobes excluding frontal cortex (Al-Jaberi et al., 2013).

calretinin and/or vasoactive intestinal peptide (Xu et al., 2004; Butt et al., 2005; Wonders and Anderson, 2006), as well as neurogliaform reelin-positive cells (Miyoshi et al., 2010).

The third source giving nearly 10% of GABAergic neurons in murine cerebral cortex is the preoptic area. Different lineages within the preoptic area, such as Nkx5.1 and Dbx1 lineage, generate a great diversity of cortical GABAergic neurons (Gelman et al., 2011).

The remaining major part, the lateral ganglionic eminence (LGE), is a source of striatal GABA projection neurons, olfactory bulb and amygdala interneurons (Corbin et al., 2008). The LGE serves as a migratory route for interneurons derived from adjacent MGE (Wichterle et al., 2001) and in smaller extent from the CGE (Miyoshi et al., 2010). In contrast to the largest part of the LGE, the most dorsal part (dLGE) that is adjacent to proliferative zone of the dorsal telencephalon is characterized by strong expression of the zinc finger transcription factor Sp8 and produces calretinin neurons for olfactory bulb and amygdala. In the mouse, only a small fraction of calretinin-expressing cortical GABAergic neurons is found to be derived from progenitors within the dLGE. In the humans, it is suggested that Sp8 progenitors from the dLGE give more important contribution to calretinin-expressing cortical GABAergic neurons due to smaller needs for olfactory bulb neurons (Ma et al., 2013).

Recent studies showed conserved pattern underlying the expression of transcription factors in the subpallium of monkey and human fetuses (Hansen et al., 2010; Ma et al., 2013; Pauly et al., 2013). In addition, pattern of origin for main subpopulations of cortical GABAergic neurons derived in the subpallium is also conserved. Sox6-expressing progenitors within cells leaving the MGE give somatostatin and parvalbumin subpopulations whereas COUP-TFII and/or Sp8-expressing cells leaving the CGE and dLGE contribute to cortical calretinin subpopulation (Ma et al., 2013). Although, a considerable increase in proportion and number of calretinin neurons in human cortex requires expanded pool of progenitors. Several studies showed that a novel pool of GABAergic neuron progenitors appears in the pallial (cortical) proliferative zones (Jakovcevski et al., 2011; Al-Jaberi et al., 2013) and becomes one of the main sources for cortical GABAergic neurons after the early fetal period (Letinic et al., 2002; Rakic and Zecevic, 2003; Petanjek et al., 2009a,b). Even though evidence for early fetal production of cortical GABAergic neurons is strong (Petanjek et al., 2009a; Jakovcevski et al., 2011; Al-Jaberi et al., 2013), most of available data suggest that during the early fetal period in primates [E47–55 in monkey and from 8 to 13 postconceptional weeks (pcw) in human] the majority of cortical GABAergic neurons is generated in the GE (**Figure 3**). Laminar and cellular organization during the early fetal stage in primates (Ip et al., 2010) corresponds to whole neurogenetic stage of rodents (Rakic, 2009). Later on during development, primate proliferative compartments reveal novel features of organization and do not have clear similarities with rodents (see the next chapter).

However, already during the early fetal period a largely increased pool of GE progenitors was observed in both monkey and human (Hansen et al., 2013). Previously unknown type of non-epithelial neural stem cell lacking radial fibers populated massively expanded subventricular zone (SVZ) of the GE. The MGE exhibited unique patterns of progenitor cell organization and clustering. The important production of cortical interneurons in the dLGE was observed and CGE generated a higher proportion of cortical GABAergic neurons than in rodent. The

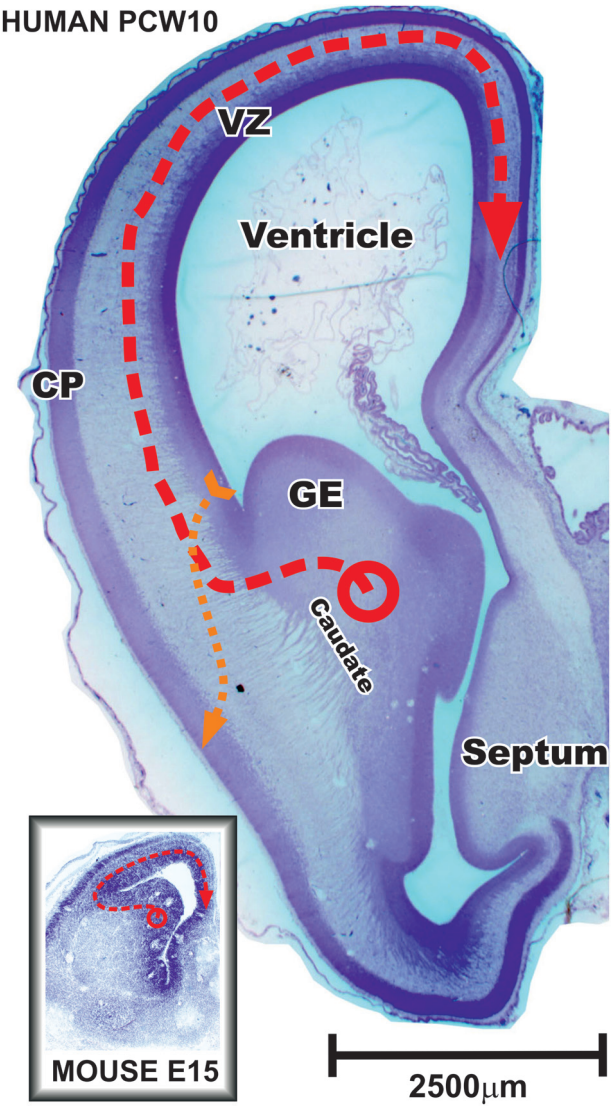

FIGURE 3 | Frontal section through the anterior part of the fetal human and mouse brain during early stages of cortical neurogenesis. In primates during the early fetal period and in rodents during whole neurogenesis vast majority of cortical plate (CP) GABAergic neurons are produced in the ganglionic eminence (GE) and migrate tangentially into the cerebral cortex (red dashed line). Note that neurons tangentially migrating to the most distant positions in the mouse brain might pass the same distance as certain radially migrating glutamatergic principal cells in human brain (yellow dashed line) that originate in the pallial ventricular and subventricular zone (VZ). During middle trimester of gestation, when majority of cortical neurons are born, discrepancy in scaling between human and mouse is much more pronounced. Modified from Petanjek et al. (2009b).

increased early production of cortical GABAergic neurons within this expanded compartment undoubtedly contributes to considerable higher proportion of calretinin neurons. But, considerably more cortical neurons are generated after the early fetal period when the peak of neurogenesis occurs (Rakic, 2002). Even if increased pool in the CGE continues with proportionally higher production of calretinin neurons later on during second trimester of gestation, we do not believe that it could account for increased need of calretinin neurons in all cortical regions of the human brain.

PALLIAL PRODUCTION OF CORTICAL GABAergic NEURONS IN PRIMATES DURING SECOND TRIMESTER OF GESTATION

Several reports suggested that during the second trimester of gestation in primates (E64–E75 in monkey and 15–24 pcw in humans) neocortical proliferative zones produce a substantial percentage of cortical GABAergic neurons (Letinic et al., 2002; Rakic and Zecevic, 2003; Petanjek et al., 2009b; Yu and Zecevic, 2011; Zecevic et al., 2011). Reports from rodents showed that the dorsal proliferative zones account for only a very small fraction (if any) of cortical GABAergic neurons present at maturity (Xu et al., 2004; Molnar et al., 2006; Wonders and Anderson, 2006). From present studies performed in non-primate species, the notable fraction of pallialy derived cortical GABAergic neurons was observed in ferret, an animal that has more convoluted cortex with enlarged proportion of cortical projection neurons in layers II and III. In ferret up to 5% of layer II and III cortical GABAergic neurons originate from the pallium (Anderson et al., 2002).

The proportion of dorsally derived cortical GABAergic neurons dramatically increases in a larger human brain (**Figure 4**) in parallel with the demand of upper cortical layers (Rakic, 2009). Using retroviral labeling in organotypic slice cultures of the human fetal forebrain, Letinic et al. (2002) demonstrated the existence of two distinct lineages of cortical GABAergic neurons. The Dlx1/2-expressing lineage originates from the GE and accounts for 35% of cortical GABAergic neurons. The second lineage that expresses Dlx1/2 and Mash1 transcription factors is produced in the neocortical ventricular/subventricular zones (VZ/SVZ) at later stages (15–22 pcw) and represents around 65% of cortical GABAergic neurons. It was suggested that massive dorsal production of GABAergic neurons in larger brains might be an answer to facilitate migratory mechanisms and simplify migratory routes through exponentially expanding neocortex (Tan, 2002; Molnar et al., 2006). In those brains, migration from the GE at later developmental stage will become very complex and vulnerable.

Most likely, a main source of pallialy produced cortical GABAergic neurons is evolutionary new, outer proliferative compartment within the subventricular zone (oSVZ) that is not found in rodents (**Figure 5A**). The oSVZ becomes the main site of neuron production in primates, allowing huge expansion of upper cortical layers (Dehay and Kennedy, 2007; Bayatti et al., 2008; Clowry et al., 2010; Molnar and Clowry, 2012; Petanjek and Kostović, 2012). The oSVZ appears at the end of early fetal period and contains the vast majority of pallial progenitors during main stage of neurogenesis, the second trimester of gestation (E60–E90 in monkey, 15–24 pcw in humans). In holoprosencephaly, syndrome with severe striatal hypoplasia and atrophy of the GE, calretinin neurons are preserved, while there is a high decrease in number of other cortical GABAergic neurons subclasses (Fertuzinhos et al., 2009). This suggests that pallial GABAergic neuron progenitors mainly produce calretinin subpopulation. In favor of this conclusion, it has been showed that many of late-born dorsally derived interneurons are calretinin cells that also include double-bouquet

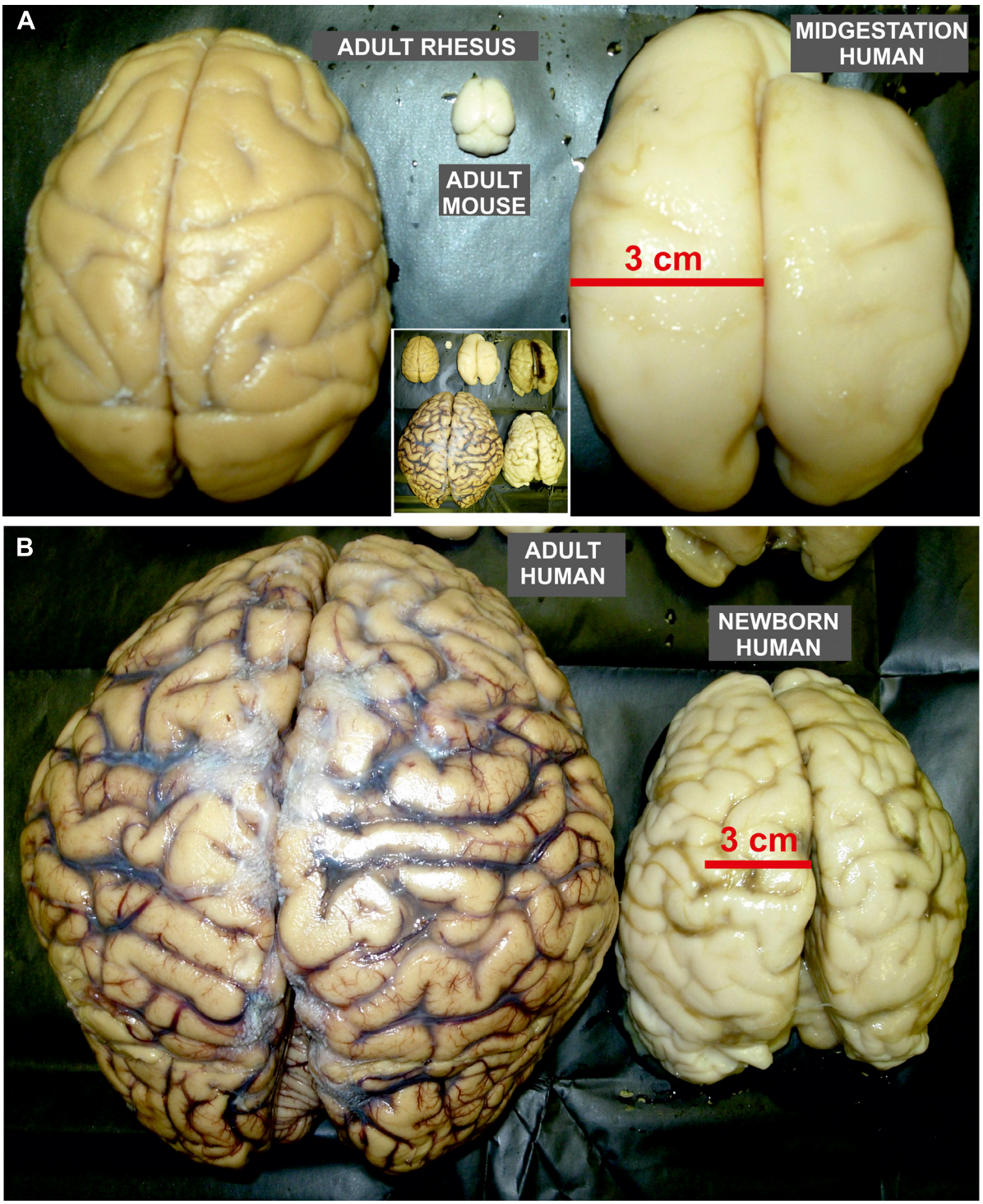

FIGURE 4 | Size proportion of mature rodent and non-human primate brain as well as developing and mature human brains. Dorsal view of adult mouse **(A)**, rhesus monkey **(A)**, and human brain **(B)**, as well as human fetal brain around mid-gestation **(A)** and at term **(B)**. **(A)** The size of human fetal brain already at mid-gestation has reached the size of the adult rhesus monkey brain. Nevertheless, adult rhesus monkey brain is almost 100 times larger than brain of adult mouse. **(B)** The adult human brain is around 3–4 times larger than newborn brain which size reaches the size of adult chimpanzee brain. Note that the pattern of gyrification in human newborn brain is close to the one observed in adult. The inlet in the middle of the figure is the integrative photo of all brains shown in **A** and **B** that demonstrate their actual proportions (the right brain in the upper row of insertion is from fetus at the beginning of third trimester of gestation).

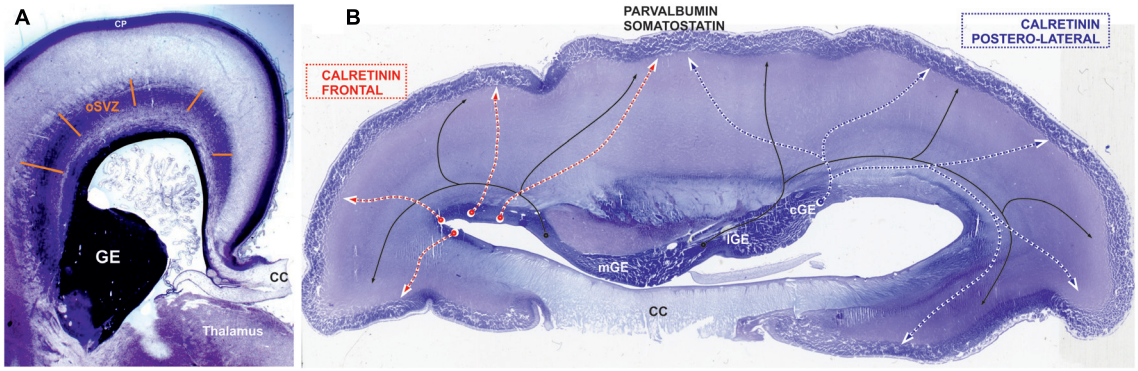

FIGURE 5 | Places of origin for different populations of GABAergic neurons. Nissl-stained sections: **(A)** frontal section at the level of thalamus and **(B)** horizontal section through the body of corpus callosum (CC) at the end of middle trimester of gestation. **(A)** The section shows main proliferative compartment of the human pallium, the outer subventricular zone (oSVZ), that is the most plausible candidate for a major source of additional pool of progenitors of GABAergic neurons (Petanjek and Kostović, 2012). In favour of this view, already during the early fetal period human cortical progenitor cells in frontal lobe have capacity to generate inhibitory interneurons (Al-Jaberi et al., 2013). **(B)** In the mouse vast majority of parvalbumin and somatostatin (black line)-expressing GABAergic cortical plate (CP) neurons originate from the medial ganglionic eminence (mGE), while calretinin expressing originate from the caudal ganglionic eminence (CGE). The progenitors from lateral ganglionic eminence (lGE) give GABAergic neurons mainly for the subcortical structures. This spatial pattern is preserved during early trimester of gestation in primates, but with exponential increase in the pool of cGE progenitors (Hansen et al., 2013). We propose that during middle trimester of gestation, when majority of cortical neurons are produced, cGE remains the main source of cortical calretinin neurons (blue dashed line) for parietal, occipital, and temporal lobes (postero-lateral telencephalon), whereas the frontal lobe (red dashed line) is supplied with calretinin neurons from the oSVZ (see **Figure 2B**). Not only the anatomical relations (rather distant postion of frontal lobe from cGE) that do not concur with exclusive production of calretinin neurons from cGE (Ma et al., 2013), but also a threefold increase in proportion of calretinin neurons in the frontal cortex cannot be achieved through increase in pool and protracted production of progenitors in cGE.

cells abundant only in the upper cortical layers of the human neocortex (DeFelipe, 2011; Jakovcevski et al., 2011). In addition, recent *in vitro* study suggests that human, but not mouse Nkx2.1 expressing dorsal radial glial cells have the potential to generate cells of interneuronal lineage labeled with calretinin (Yu and Zecevic, 2011). Taking into consideration those findings, we would like to point that calculation of Letinić (suggesting 2/3 of cortical GABAergic neurons derived in the pallium) might be inaccurate due the fact that the dLGE and CGE are also important source for calretinin neurons. Therefore, if pallial proliferative zones produce mainly calretinin neurons, the amount of the dorsally derived cortical GABAergic neurons proposed by Letinic is overestimated and it would hardly exceed half of proposed amount (1/3 of cortical GABAergic neurons).

By labeling newborn neurons in slice culture and mapping of proliferating interneuron progenitors, as well as studying expression patterns of several key transcription factors in the developing human and monkey telencephalon, Hansen et al. (2013) and Ma et al. (2013) were not able to demonstrate extensive pallial production. They proposed that the majority of primate cortical GABAergic neurons originate from the GE. Protracted presence of proliferation in the primate GE during the second trimester of gestation and increased pool of calretinin progenitors in the CGE might explain increased proportion of calretinin neurons. However, it is difficult to imagine that this source can be sufficient to supply calretinin neurons of the human frontal cortex (**Figures 2B** and **4**). The granular frontal cortex, that includes mainly higher order associative areas and occupies one third of human cortical surface (**Figure 2A**), is showing the most prominent evolutionary expansion without a correlation in rodents (Uylings and van Eden, 1990; Groenewegen and Uylings, 2000; Uylings et al., 2003; Rakic, 2009; Petanjek et al., 2011; Teffer and Semendeferi, 2012). In the regions abundant with cortico-cortical neurons, as are the higher associative region of the frontal and parietal cortex (Ma et al., 2013), calretinin neurons represent almost half of cortical GABAergic neuron population (Gabbott et al., 1997b). Therefore, we found unlikely that the CGE, even with expanded pool of progenitors and highly protracted neurogenesis, could be able to provide calretinin neurons if there is threefold increase in their proportion. In addition, newly evolved higher order associative areas are not in close range of the CGE, what logically assumes that production should occur more closely (**Figure 2B**). The primate dLGE, as a rostral extension of the CGE becomes in primate more cortical than subcortical proliferative pool (as is the case in rodent). While it is closer to the frontal lobe, it could primarily produce calretinin neurons for the frontal cortex. However, taking into account amount of progenitors in the dLGE and needs for calretinin neurons in the frontal cortex, it is hard to imagine that the dLGE could be their main source.

We propose that contradictory results observed in recent studies about cortical GABAergic neurons origin in primates, those suggesting abundant pallial production of cortical GABAergic neurons (Letinic et al., 2002; Rakic and Zecevic, 2003; Zecevic et al., 2005, 2011; Mo et al., 2007; Fertuzinhos et al., 2009; Petanjek et al., 2009a; Al-Jaberi et al., 2013) and those suggesting only minor dorsal production of cortical GABAergic neurons (Hansen et al., 2013; Ma et al., 2013), might be explained by regional variation in the site of calretinin neuron origin. Al-Jaberi et al. (2013) strongly suggest that human cortical progenitor cells in the frontal lobe have capacity to generate inhibitory interneurons. Therefore, in the primates, the CGE might be

the main source for posterior and lateral regions of the cerebral cortex, whereas frontal lobe is supplied mainly by dorsally derived calretinin neurons (**Figure 5B**). While regional variations in cortical GABAergic neurons production were not analyzed in the studies performed until now, these hypotheses need further confirmation.

PROTRACTED PRODUCTION OF CORTICAL GABAergic NEURONS FROM THE GE DURING THE LAST TRIMESTER OF GESTATION

If massive dorsal production of cortical GABAergic neurons in human is evolutionary answer to keep migratory routes shorter and simpler, one could expect that migration from the GE at later developmental stage will be exhausted. Comparing E55/E64 with E75 in monkey fetuses we could not qualitatively observe a decrease in amount of tangentially migrating GAD65 positive, prospective cortical GABAergic neurons arising from the GE (Petanjek et al., 2009a). There was also a preserved pool of GAD positive progenitors in the GE. In human fetuses at 24–26 pcw, extended stream of densely populated Golgi impregnated migratory like cells leaving GE was observed. Qualitative impression of amount in cells leaving GE together with its size suggests that peak of neurogenesis in the human GE is close to the end of second trimester of gestation (Petanjek et al., 2008). These data suggest that pallial production is not an answer to keep migratory routes shorter and simpler. Pallial production is most likely novel pool of progenitors that produces specific subtypes of cortical GABAergic neurons, such as the calretinin neurons.

BrDU studies in primates showed that the vast majority of cortical neurons are already born by the beginning of the second half of gestation (Rakic, 2002). Therefore, it is interesting that proliferation and migration of prospective GABAergic neurons from the GE are not declining at this period. In the recent study, Ma et al. (2013) observed numerous migratory like cells in the SVZ of the human neonatal brain what suggests that the GE as a proliferative compartment in human fetuses is preserved up to last trimester of gestation (Letinic and Kostovic, 1997). By evaluating human material of Zagreb neuroembryological collection (Judaš et al., 2011), we observed during the late fetal period many MAP-2 and calretinin-positive, non-radially oriented migratory like cells in the dorsal telencephalon, as in the stream leaving the GE (Hladnik et al., 2011, 2014). These preliminary observations are consistent with the hypothesis of protracted production of cortical GABAergic neurons. However, some reports suggest that cell proliferation is almost exhausted in VZ/SVZ of the GE by the last trimester of gestation (Zecevic et al., 2011). But if even neurogenetic potential in the GE decreases sharply during second half of gestation, additional 10–12 weeks of subtle neuron generation might still significantly contribute to single population of cortical GABAergic neurons, especially if this production is directed to specific cortical regions.

Interestingly, some experimental support for this hypothesis is coming from mouse. Riccio et al. (2012) showed the accumulation of proliferating GABAergic progenitors close to the anterior cingulate region in the postnatal mouse. These cells produce calretinin neurons destined for lower cortical layers of anterior cingulate cortex. The proliferation is present until P21 and is notably abundant during first postnatal week. Fate mapping analyses suggest that these neuronal precursors originate from the CGE and LGE.

The first postnatal weeks in rodents are characterized by the completion of migration and onset of intensive differentiation, as well as ingrowth and outgrowth of fibers. These developmental events also characterize last trimester and early postnatal development in primates (Uylings, 2000; Kostovic et al., 2014). If production of calretinin neurons from the GE continues for additional 10–12 weeks after peak of neurogenesis in humans, it might significantly contribute to its proportion. Protracted production of important fraction of cortical GABAergic neurons subpopulation could have large importance in prematurely born infants, where neuron production is altered (Malik et al., 2013). This might lead to neurological and psychiatric disturbances, or alter psychomotor capability. Regarding incidence of premature deliveries in developing countries, such an event could not be only medical problem but could also have significant social impacts (Petanjek and Kostović, 2012; Raznahan et al., 2012).

CONCLUDING REMARKS

Calretinin neurons become in primates the most prominent population of cortical GABAergic neurons. Pronounced increase in the number of calretinin neurons is supplied by changes in the developmental rules regarding their origin. Already during the early fetal period, an increased pool of calretinin neuron progenitors is present in the CGE, as well as clear signs of onset of cortical GABAergic neurogenesis. During the middle trimester of gestation, calretinin neurons destined for the frontal cortical regions originate mainly from the frontal cortical proliferative zones and at lower extent from the dLGE. This period is also characterized by the peak of neurogenesis in the GE, where calretinin neurons destined for posterior and lateral cortical regions originate mainly from the CGE. There seems to be ongoing production of calretinin neurons into the last trimester of gestation.

Increased pool of progenitors in the CGE during the early and middle fetal period, abundant cortical production during middle fetal period together with protracted production from the GE during the last trimester of gestation, might explain such an increase in the proportion of calretinin neurons in primates.

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Neocortical calretinin neurons in primates: increase in proportion and microcircuitry structure

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In this article we first point at the expansion of associative cortical areas in primates, as well as at the intrinsic changes in the structure of the cortical column. There is a huge increase in proportion of glutamatergic cortical projecting neurons located in the upper cortical layers (II/III). Inside this group, a novel class of associative neurons becomes recognized for its growing necessity in both inter-areal and intra-areal columnar integration. Equally important to the changes in glutamatergic population, we found that literature data suggest a 50% increase in the proportion of neocortical GABAergic neurons between primates and rodents. This seems to be a result of increase in proportion of calretinin interneurons in layers II/III, population which in associative areas represents 15% of all neurons forming those layers. Evaluating data about functional properties of their connectivity we hypothesize that such an increase in proportion of calretinin interneurons might lead to supra-linear growth in memory capacity of the associative neocortical network. An open question is whether there are some new calretinin interneuron subtypes, which might substantially change micro-circuitry structure of the primate cerebral cortex.

Keywords: GABA, calretinin, neocortex, pyramidal neurons, species differences

INTRODUCTION

The main biological substrate for mammalian mental abilities is the neuronal circuitry of the cerebral cortex. Tremendous evolutionary increase in the neuron number and cortical connections (DeFelipe, 2011; Charvet and Finlay, 2012) allowed humans to adopt language and mathematical skills, to make affective modulation of emotional cues, possess self-conceptualization, mentalization, as well as to have high capacity of cognitive flexibility and working memory (Rakic, 2009). Such complex functioning is strongly related to distinct expansion of multimodal – high order associative areas, particularly the granular areas of the frontal lobe (i.e., associative prefrontal cortex; Teffer and Semendeferi, 2012). These areas have no clear correlate in mice and rats (Uylings and van Eden, 1990). In addition to expansion in size, there are significant changes in intrinsic organization of cortical circuitries (**Figure 1**). There are novel neuronal elements that appear in the human cerebral cortex making organization of microcircuitry (and consequently functional properties) substantially different when compared to non-primate mammals (Clowry et al., 2010).

In this article we first give a short overview of evolutionary changes in the connectivity of a specific class of principal (glutamatergic) cortico-cortical projecting neurons, as well as a possible functional significance of those changes regarding increase in cognitive capabilities. We also found that present comparative anatomical data suggest a distinct role in reorganization of cortical microcircuitry for one of the GABAergic local circuit neuron classes, the calretinin expressing neurons that in primates have much higher proportion. We propose a possible mechanism how calretinin neurons might contribute to reorganization of microcircuitry in the human associative cortex and how this might be related to an increase of cognitive capabilities.

MICROCIRCUITRY CHANGES IN THE PRIMATE PREFRONTAL CORTEX AND INCREASE IN PROPORTION OF CALRETININ NEURONS

It is well recognized that upper layer pyramids (DeFelipe, 2011; Shepherd, 2011; Teffer and Semendeferi, 2012) are cortico-cortical projecting neurons (Elston et al., 2011; DeFelipe et al., 2012). It is less recognized that in primates, large deep layer III pyramids are long distance cortico-cortical neurons which establish in parallel connections with several cortical areas (Barbas et al., 2005; Yeterian et al., 2012). Experimental studies in rhesus monkey show that they are key elements in the circuitry involved in working memory and other prefrontal cortex-dependent associative cognitive functions (Wang et al., 2006; Verduzco-Flores et al., 2009). Data from various psychiatric disorders showed that selective alteration of large layer III pyramidal cells correlates with a decline in higher cognitive functions (Morrison and Hof, 2002; Selemon et al., 2003; Dean, 2009; Dorph-Petersen et al., 2009; Courchesne et al., 2011; Jacot-Descombes et al., 2012; Teffer and Semendeferi, 2012; Selemon et al., 2013) and developmental studies found that prominence in size of neurons in the upper cortical layers and peak in synaptic number appear by the end of infancy, stage when human specific mental capacities appear (Petanjek et al., 2008, 2011). Altogether, it can be concluded that large layer III pyramidal neurons in the high order associative areas are the main integrative

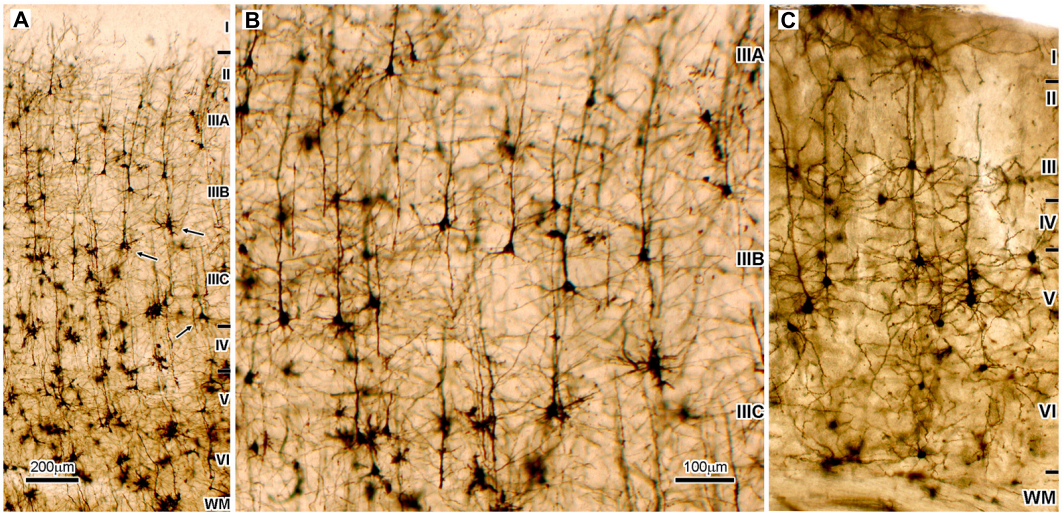

FIGURE 1 | Microphotography of the Golgi Cox impregnated sections of the associative areas in the human (A,B) and mice neocortex (C). (A) Dorso-lateral part of the frontal granular cortex (area 9) shows that supragranular cortical layers (II/III), which contain cortical projecting neurons, are two times thicker than infragranular layers (V/VI), which contain subcortical projecting neurons. In addition, pyramidal neurons (arrows) located deep inside layer III (sublayer IIIC) have largest cell body as well as most complex and extended dendritic arborization. Therefore they are the most prominent neurons found on Golgi staining, and on the Nissl staining they produce distinct cytoarchitectonic feature found only in high order associative areas of the cerebral cortex of human and apes, the magnocellularity (magnopyramidality) of the layer III (Petrides et al., 2012). Those neurons are on Nissl stained sections not only prominent by cell body size, they also have most intense cytoplasmatic staining showing high metabolic activity (Rajkowska and Goldman-Rakic, 1995a). They also have the most intense SMI32 staining, that indicates a very long and ramified axon tree (Morrison and Hof, 2002). The mentioned morphological features are a mark of associative cortico-cortical neurons. **(B)** Enlarged part of panel **(A)** is shown to be of the same magnification as panel **(C)**. **(C)** The highest order associative areas in the mice neocortex are located in the parieto-occipital region. When compared to highest order associative areas of the human neocortex **(A,B)** proportion of cortico-cortical projecting neurons is smaller than cortico-subcortical projecting neurons, and the largest neurons are layer V pyramids. Therefore, thickness of layers II/III in mice is less than half of the thickness of layers V/VI, which is opposite to human. In the parasensory associative areas (that do not have developed sublayer IIIC) of the human temporal cortex, layers II/III contain 44% of total number of neurons and have 30% more neurons than are located in layers V/VI (DeFelipe, 2011). It can be assumed that in areas with developed layer IIIC upper cortical layers contain more than 50% of neurons. In mice, upper cortical layers contain only 22% of neurons that is less than half of amount located in layers V/VI.

elements ("associative" neurons) between different cortical areas (Goldman-Rakic, 1999; see discussion from Petanjek et al., 2008).

Pyramidal neurons located in upper layers of the primate prefrontal cortex also provide rich intracortical projections. From large layer III pyramids 80% of synaptic output belongs to local connections coming from axonal side branches (Melchitzky and Lewis, 2003). They extend several millimeters around, with dense columnar termination through layers II and III.

Human brain evolution is characterized by an increase in the number and width of minicolumns, but also in the increase of space available for interconnectivity between neurons, especially in the human prefrontal cortex where associative layer III pyramidal neurons are particularly abundant (DeFelipe et al., 2012; Spocter et al., 2012). Except to primary regions, prefrontal cortex established connections with all other cortical areas (Groenewegen and Uylings, 2000). That way efficacy of inter- and intra-areal integration within prefrontal cortex correlates with overall level of information processing, influencing consequently level of individual cognitive capability (Petanjek and Kostovic, 2012). Inter-individual differences in internal structure of upper cortical layers of human prefrontal cortex (Rajkowska and Goldman-Rakic, 1995a,b) make additional support that associative layer III neuron class has the major role in increasing the efficiency of cortico-cortical network (Buckner and Krienen, 2013; Hofman, 2014).

In parallel with evolutionary changes in connectivity of cortico-cortical network, significant changes appear in the organization of GABAergic network. This network acts as intrinsic modulator of cortical output since it is composed of local circuit neurons (interneurons; DeFelipe et al., 2013). Numerous studies analyzing laminar distribution and density of cortical GABAergic neuron subpopulations were performed in various species (**Table 1A**). It is curious that only rarely the same group performed a systematic analysis of several species, using the same methodology, making it difficult to conclude about interspecies differences. Most of the studies performed in rat and mouse found that cortical GABAergic neurons represent around 15% (14–16%) of the overall population. In monkey and human their proportion mostly exceeds 20% (20–29%) suggesting an increase in proportion for about 30–50%.

This large increase in proportion of GABAergic neurons seems to be principally caused by increase in number of neurons containing calretinin. Another two main classes, those containing parvalbumin and somatostatin, do not show such a robust increase in proportion (Hladnik et al., 2014). In rodents the proportion of calretinin neurons among the total population of GABAergic

Table 1 | Overview of publications quantifying proportion of GABAergic and calretinin neurons in the neocortex of rodents and primates. **(A)** Proportion of GABAergic cells in population of all neurons, and **(B)** proportion of calretinin neurons inside the GABAergic population.

(A) Percentage of GABAergic cells in the total neuron population		
Anatomical area	**Rat and mouse – GABA in total**	**Monkey and human – GABA in total**
Primary visual area (V1)	15% (Beaulieu et al., 1994) – Rat	20.5% (Beaulieu et al., 1992) – Monkey
	14.5% (Meinecke and Peters, 1987) – Rat	15% (Fitzpatrick et al., 1987) – Monkey
	15% (Lin et al., 1986) – Rat	20% (Hendry et al., 1987) – Monkey
Primary somatic sensory	14% (Micheva and Beaulieu, 1995) – Rat	20–29% (Jones et al., 1994) – S1 and primary motor area-Monkey
area (S1)	25% (Ren et al., 1992) – Rat	
Frontal lobe	22% (Santana et al., 2004) – Rat	24.9% (Gabbott and Bacon (1996) – Monkey
	16% (Gabbott et al., 1997) – Rat	21.2% (Hornung and De Tribolet, 1994) – Human
Temporal lobe		37.7% (del Rio and DeFelipe, 1996) – only layers II and III-Human
Multiple lobe analysis	19.5% (Tamamaki et al., 2003) – Mouse	25% (Hendry et al., 1987)
	15% (Beaulieu, 1993) – Rat	
(B) Percentage of calretinin expressing neurons within GABAergic population		
Anatomical area	**Rat and mouse – calretinin in GABA**	**Monkey and human – calretinin in GABA**
Primary visual area (V1)	17% (Gonchar and Burkhalter, 1997) – Rat	20% (Yan et al., 1995) – Monkey
	24% (Gonchar et al., 2007) – Mouse	
Frontal lobe	16.1% (Uematsu et al., 2008) – Rat	28.6% (Zaitsev et al., 2005) – Monkey
	24.7%* (Gabbott et al., 1997) – Rat	28.8%* – Human
		34.2%* – Monkey (Sherwood et al., 2004)
	18% (Kubota et al., 1994) – Rat	33.2–44.8%* (Gabbott and Bacon, 1996; Meskenaite, 1997;
		Melchitzky et al., 2005)* – Monkey
Temporal lobe		46.2% (del Rio and DeFelipe, 1996) – only layers II and III-Human
Multiple lobe analysis	18% (Xu et al., 2010) – Mouse	
	13.9% (Tamamaki et al., 2003) – Mouse	

Values reported with an asterisk have been calculated from values presented in the original papers.

neurons is between 16–18%, whereas in primate the proportion of calretinin reaches in some areas 35–40% (**Table 1B**). del Rio and DeFelipe (1996) have estimated that, within layer II and III of associative temporal cortex in the human, GABAergic neurons represent around 1/3 of the total number of neurons, and almost half of GABAergic neurons express calretinin. In addition, a recent study (Ma et al., 2013) suggests that, in the human and monkey, calretinin neurons are two times more numerous in the frontal and parietal cortical areas. Collectively all these data indicate that the evolution lead to an increase in calretinin proportion in the upper cortical layers of high order associative regions. Our preliminary observations, comparing orbital frontal cortex in the rat and complementary area 14 in the rhesus monkey, showed a four- to fivefold increase in the proportion of calretinin for the upper cortical layers, where calretinin neurons cover almost 15% of the total number of neurons (Džaja et al., 2014).

FUNCTIONAL PROPERTIES OF CALRETININ NEURONS

For efficient functioning of the human cerebral cortex with its complex areal subdivision and increased number of cortical columns, there is a need for enhanced inter-areal and intra-areal integration (Sherwood et al., 2005; Hofman, 2014). Appearance of most likely, evolutionary new associative neurons (DeFelipe and Fariñas, 1992; Nieuwenhuys, 1994; Spruston, 2008) makes substantial changes in the organization of microcircuitry and allows higher level of network integration (Buckner and Krienen, 2013). Inside the primate cortico-cortical network there is also a fivefold increase in proportion of calretinin neurons and it is reasonable to ask how this changes the microcircuitry structure.

Based on electrophysiological properties, two main types of calretinin interneurons can be distinguished in rodents: accommodating and non-adapting non-fast spiking cells (Markram et al., 2004; Butt et al., 2005; Caputi et al., 2009). These electrophysiological features are correlated with expression of a group of membrane voltage gated proteins (Markram et al., 2004), the calretinin cluster (Toledo-Rodriguez et al., 2004; Schwaller, 2014).

Different types of calretinin neurons can be identified based on their morphological features, particularly on the postsynaptic domain targeted by their axon. Double bouquet cells have vertically oriented axons which project mainly to basal dendrites of pyramidal cells (del Rio and DeFelipe, 1995; Yanez et al., 2005), while bipolar (Peters and Kimerer, 1981) and bitufted

cells (Jiang et al., 2013) project to the proximal and middle region of pyramidal cell's apical dendrite. Therefore, calretinin neurons provide direct inhibition, although with a low connectivity rate of ∼10% (Caputi et al., 2009), on mid-proximal dendritic domain of pyramidal cell (i.e., proximal parts of apical and basal dendrites). In addition to this sparse connectivity with nearby pyramids, calretinin neurons provide strong innervation onto somatostatin neurons (Pfeffer et al., 2013) and other calretinin cells (Caputi et al., 2009). These somatostatin neurons are known for providing direct inhibition of pyramidal cell's apical and basal dendrites (Wang et al., 2004; Jiang et al., 2013), as well as for providing an inhibitory influence on parvalbumin neurons (Pfeffer et al., 2013).

Parvalbumin neurons are mostly basket cells, which exert strong inhibitory control over pyramid's soma (Markram et al., 2004; Pfeffer et al., 2013). Optogenetic activation of fast spiking parvalbumin cells induces gamma oscillations in nearby pyramids (Cardin et al., 2009) and, without parvalbumin activity, pyramids would continue to fire but without synchrony (Gulyas et al., 2010). In other words, parvalbumin basket cells phase-lock their target pyramids through hyperpolarization, after which pyramids undergo rebound, but short lived depolarization and fire in synchrony (Cobb et al., 1995). Elimination of inhibitory influences on parvalbumin basket cells prolongs their influence on pyramids (Pouille and Scanziani, 2004), and this is where the potential role of calretinin neurons could reside. By inhibiting somatostatin neurons (Pfeffer et al., 2013; Cauli et al., 2014) they could create a disinhibitory window for parvalbumin baskets. We hypothesize that this, by calretinin neurons provided disinhibition, might prolong the effect of parvalbumin cells on pyramids, allowing longer periods of synchronized gamma oscillations.

This group of cells, including calretinin neurons, their somatostatin targets, parvalbumin neurons, and their pyramidal targets, can be collectively called a neuronal assembly (Borgers et al., 2012; Somogyi et al., 2014). The significance of an assembly is that it can activate its efferent targets with high probability, through the synchronous activity of its pyramids (Harris et al., 2003; Buzsaki and Wang, 2012). Interneurons are needed to segregate, maintain and also establish a temporal sequence of activation between particular assemblies (Somogyi et al., 2014). We hypothesize that the role of increased proportion of calretinin neurons would depend on the criteria of their territorial exclusivity, i.e., their developmental positioning to efferent targets compared to other calretinin interneurons. If newly added calretinin cells show no territorial exclusivity, i.e., their connections significantly overlap with those of pre-existent calretinin neurons, they would be incorporated into already established assemblies, connecting to somatostatin neurons to which some calretinin neurons have previously connected. Hence, there would be an increased number of calretinin cells per assembly, while the number of pyramids would not change, allowing for a more potent overall effect of calretinin neurons. However, in case of the other extreme, newly added calretinin cells will mostly connect to their own group of pyramids and parvalbumin and somatostatin neurons, creating smaller assemblies and allowing for more parallel processing. More parallel units might lead to supra-linear growth in memory capacity of the neocortical network, which is of particular importance in regions involved in planning and executive functions.

Present evidence suggests that the evolutionary path of the primate cortico-cortical network seems to have been an expansion in two aspects. First, there is an increase in proportion of principal neurons located in layers II and III, which would be a way to create the basic excitatory architecture for inter-areal processing. Second, there is an increase in proportion of calretinin expressing GABAergic interneurons, which would be a way to create a gain in synchrony and parallel processing between disparate cortical areas. An open question is whether this jump in proportion of calretinin neurons is based on a simple expansion of already preexistent subtypes of these cells found in the rodents or do we have some new cellular subtypes. If so, this might produce a more profound changes then simple supra-linear increase in their number, similar to changes occurring with appearance of associative principal neurons. These two might have been converging processes, making structure of microcircuitry in the primate neocortex substantially different when compared to other non-primate mammals.

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Lack of functional specialization of neurons in the mouse primary visual cortex that have expressed calretinin

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Calretinin is a calcium-binding protein often used as a marker for a subset of inhibitory interneurons in the mammalian neocortex. We studied the labeled cells in offspring from a cross of a Cre-dependent reporter line with the CR-ires-Cre mice, which express Cre-recombinase in the same pattern as calretinin. We found that in the mature visual cortex, only a minority of the cells that have expressed calretinin and Cre-recombinase during their lifetime is GABAergic and only about 20% are immunoreactive for calretinin. The reason behind this is that calretinin is transiently expressed in many cortical pyramidal neurons during development. To determine whether neurons that express or have expressed calretinin share any distinct functional characteristics, we recorded their visual response properties using GCaMP6s calcium imaging. The average orientation selectivity, size tuning, and temporal and spatial frequency tuning of this group of cells, however, match the response profile of the general neuronal population, revealing the lack of functional specialization for the features studied.

Keywords: calretinin, visual cortex, orientation tuning, spatial frequency, interneurons

INTRODUCTION

Calretinin (gene symbol *Calb2*) is a calcium-binding protein that took its name from its structural similarity to calbindin and to the location where it was first discovered, the retina. In the mammalian neocortex, calretinin is one of the traditionally used markers to categorize interneurons, along with other proteins such as parvalbumin and somatostatin (Gonchar and Burkhalter, 1997). Indeed, in adult rodents, most or perhaps all calretinin-immunoreactive (CR-IR) neurons in the cerebral cortex stain positive for the inhibitory neurotransmitter GABA (100%, Kubota et al., 1994; >93%, Gonchar and Burkhalter, 1997; 100%, Gonchar et al., 2008). In layer II/III of mouse primary visual cortex, CR-IR neurons are even the most abundant class of GABAergic neurons (41%, Gonchar et al., 2008). The exclusively GABAergic nature of cortical CR-IR neurons is not conserved across mammals, though, as in monkey prefrontal and human temporal neocortex about a quarter of the CR-IR neurons are not positive for GABA (del Río and DeFelipe, 1996; Melchitzky et al., 2005). Still, in both monkey and rodent, the class of CR-IR neurons contains morphologically similar groups, in particular the double-bouquet neurons of layer II/III and a subpopulation of Cajal-Retzius neurons in layer I (Condé et al., 1994; see also Barinka and Druga, 2010 for a review).

Despite their abundance, surprisingly little is known about the function of CR-IR cells. The only indirect functional information comes from two anatomical findings. First, calretinin interneurons mainly target the dendrites of other GABAergic neurons in the visual cortex in both rat (Gonchar and Burkhalter, 1999) and monkey (Meskenaite, 1997). They can thus exert a disinhibitory effect on pyramidal neurons. For this reason, CR-IR interneurons are hypothesized to be gating cells (Callaway, 2004) and necessary for persistent activity (Wang et al., 2004). Second, layer I calretinin interneurons are targeted by feedback connections from higher order visual areas (Gonchar and Burkhalter, 2003). They are thus well-positioned to convey feedback information like attentional signals and scene interpretation (Lamme and Roelfsema, 2000). Other than that in rodent cortex CR-IR cells are not fast-spiking (Kawaguchi and Kubota, 1997; Porter et al., 1998; Kawaguchi and Kondo, 2002), we have no physiological information about their function and response properties.

For this reason, our original aim was to study the receptive field properties of CR-IR neurons in the mouse primary visual cortex using calcium imaging. To achieve this, we crossed the CR-ires-Cre mouse line, which expresses the enzyme Cre-recombinase in a fashion similar to endogenous CR-expression (Taniguchi et al., 2011), to a Cre-dependent reporter mouse line expressing the red fluorescent protein tdTomato (Madisen et al., 2010). Rather than marking the current situation of CR expression, the tdTom label will not only be present in all cells that express the *Calb2* gene, but also in all cells that have expressed *Calb2* in their past. Although, in many other interneuron Cre-mouse lines these two populations are not too distinct (Taniguchi et al., 2011), they are not necessarily the same. Using immunohistochemistry and the Allen Institute's *in situ* hybridization data (Lein et al., 2007; Oh et al., 2014), we investigated to which extent these classes overlap, and found that there is extensive transient expression of CR in excitatory neurons during development. The group of tdTomato-positive (tdTom+) cells is thus far larger than the CR-IR interneurons that we originally set out to study. Calcium imaging in these animals, with the genetically encoded calcium indicator GCaMP6s (Chen

et al., 2013) under the neuronal synapsin promoter, showed the tdTom+ neurons to be a large heterogeneous group with response properties similar to the general, tdTomato-negative (tdTom−) population.

MATERIALS AND METHODS

ANIMALS AND ANESTHESIA

We used male and female, 2–4 months old mice from a cross of homozygous B6; 129S6-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J mice (Madisen et al., 2010; Jackson Laboratories), in which a Cre-dependent transgene encoding the tdTomato fluorescent protein is inserted in the ROSA26 locus, with homozygous B6(Cg)-Calb2$^{tm1(cre)Zjh}$/J (Taniguchi et al., 2011; Jackson Laboratories) mice expressing Cre-recombinase following the pattern of *Calb2* expression. For the surgeries (viral injection and window implantation), we anesthetized the mice with isoflurane (1.5–2.5% vol/vol) and administered three subcutaneous injections: dexamethasone (4 mg/kg), metacam (1 mg/kg), amoxicillin (100 mg/kg). We assessed the depth of anesthesia with the pedal reflex. To protect the mice' eyes, we used cavasan ointment. During two-photon calcium imaging, the animals were anesthetized with 0.5–1.5% vol/vol isoflurane. We adjusted the flow rate depending on the response level of the animal in order to have the lowest percentage to keep the animal in an anesthetized state. The temperature of the mouse was maintained at 37°C with heating pad and rectal probe during both surgeries and recordings. All animals were kept in a 12 h day/night cycle with access to food and water ad libitum. Experiments were carried out during the day cycle. All experiments were approved by the institutional animal care and use committee of the Royal Netherlands Academy of Arts and Sciences.

SURGICAL PROCEDURES

For the detection of the calcium changes, we used the genetically encoded calcium indicator GCaMP6s (Chen et al., 2013). Mice were injected in the right V1 (stereotactic coordinates: 2.9 mm lateral, 0.4 mm anterior to lambda), at a depth of 400 μm, with 80 nl of a solution containing the virus AAV1.Syn.GCaMP6s.WPRE.SV40 (virus titer 3.04×10^{13} gc/ml, University of Pennsylvania Vector Core) using a Nanoject volume injection pump (Drummond Scientific Company). Two weeks after the viral injection, the mice were anesthetized and surgically implanted with a glass window over a V1 craniotomy (Van Versendaal et al., 2012). At the start of the surgery, the scalp was anesthetized with Xylocaine and removed. Then a coated iron ring was attached with Loctite 454 over V1 to the bone parallel to the plane of the skull and sealed with black dental cement to reduce the amount of light from the monitor entering the microscope. A craniotomy was drilled with a 2 mm diameter and after opening the brain was kept moist with artificial cerebro-spinal fluid (ACSF), consisting of a solution of 125 NaCl, 10 Hepes, 5 KCl, 2 $MgSO_4$, 2 $CaCl_2$, and 10 Glucose, in mM. The space between the dura and the 5 mm glass window was filled with silicon oil (∼10 mPa · s viscosity, DC 200, Fluka Analytical, UK) and sealed with a type 1 glass coverslip 100 μm thickness fixed with dental cement. With dental cement also a well was created to contain the water for the immersion objective of the microscope. We started the imaging sessions 10 days after the surgery. We generally did not experience any tissue growth under the glass window. The window was cleaned before the imaging session with 70% ethanol.

TWO-PHOTON CALCIUM IMAGING

For imaging we used a converted Olympus BX61WI confocal microscope equipped with a Ti-sapphire laser (Mai-Tai, Spectra-physics, CA, USA), with two non-descanned PMTs with filters optimized for GFP and RFP (Semrock BrightLine FF01-520/70, FF01-625/90, and FF555-Di03 dichroic). The mice were head-fixed under the objective using a magnetic holder connected to the metal ring previously implanted over the skull of the animal (see surgical procedures). The magnet[1] had the following specifications: 21 mm outer diameter, 15 mm inner diameter at top, 9 mm inner diameter bottom, 2 mm thick[2]. A black cloth was used to cover the objective in order to prevent the light coming from the monitor to reach the PMTs. Two-photon laser scanning microscopy was performed using a wavelength of 910 nm and neurons were imaged using a 20× water-immersion objective (Olympus, 0.95 NA). We scanned at seven frames per second. Time series recordings of these neurons were performed while showing visual stimuli.

VISUAL STIMULATION

Stimuli were presented on a gamma-corrected Dell UltraSharp U2312HM 23″ full HD LCD monitor, placed 15 cm in front of the mouse and oriented toward the contralateral eye. Stimuli were made with custom-made Matlab scripts, available at https://github.com/heimel/InVivoTools, a fork from code written by Steve Van Hooser, and employed the PsychoPhysics Toolbox 3 (Kleiner et al., 2007). We first measured orientation tuning, using full screen square-wave drifting gratings with different directions going in steps of 30°. Unless otherwise mentioned, the stimulus duration was 2 s, the interstimulus was an isoluminant gray screen of 3 s, contrast was 90%, temporal frequency was 2 Hz and spatial frequency 0.05 cpd. For each test, stimuli were repeated until 5 min of imaging was reached, i.e., 4–5 repetitions for each stimulus. Circular ROIs were drawn around the cells, and one responsive tdTom+ cell was chosen for which subsequent stimuli were optimized. The analysis was constrained to neurons with similar stimulus preferences. Usually, there were a number of such cells per field of view. The center of the receptive field of a neuron was assessed by presenting a drifting grating of the preferred direction in one of 6 × 3 grid locations on the monitor. Next, a size tuning stimulus was shown centered at the center-of-mass of the responses of the chosen neuron to all patches, at its optimal direction, 20–40–60–80–100° of visual angle, 2 Hz and 0.05 cpd. Spatial frequency tuning curve was assessed using a full screen sinusoidal stimulus of 0.01, 0.021, 0.044, 0.092,

[1] http://www.supermagnetman.net

[2] https://sites.google.com/site/alexanderheimel/protocols/magnetic-head-holder for more details.

0.191, 0.4 cpd at the optimal orientation for the chosen neuron. Determining temporal frequency tuning was done with a full screen sinusoidal grating drifting at 1, 3, 8, 12, 16, 20 Hz.

ANALYSIS OF CALCIUM SIGNALS

Circular ROIs were drawn centered in all cells expressing GCaMP6s using custom Matlab scripts. The changes in fluorescence were divided by the average fluorescence just before stimulus onset to obtain $\Delta F/F$. Response was defined as the average $\Delta F/F$ from 0.5 s after stimulus onset to stimulus offset. Cells were said to be responsive if a one-sided *t*-test of responses versus baseline fluorescence was significant at the 0.1 level. Only cells that were responsive and had a maximum response of at least 5% were included in the analysis. Orientation selectivity index was defined as $\mathrm{OSI} = \sqrt{\{[\Sigma R(\varphi)\sin(2\varphi)]^2 + [\Sigma R(\varphi)\cos(2\varphi)]^2\}}/\Sigma R(\varphi)$, where φ is the angle of the stimulus and $R(\varphi)$ the neuron's response to it. This is equal to 1 – circular variance. Direction selectivity index (DSI) was defined by $\mathrm{DSI} = \sqrt{\{[\Sigma R(\varphi)\sin(\varphi)]^2 + [\Sigma R(\varphi)\cos(\varphi)]^2\}}/\Sigma R(\varphi)$. The suppression index was defined as the (Rp-Rl)/Rp where Rp is the response to the smallest stimulus that reached 95% of the maximum response, and Rl the response to the largest stimulus (Ayaz et al., 2013). Using the red channel, cells expressing tdTomato were identified. One tdTom+ cell was chosen to optimize the stimuli, but for calculating population responses for the subsequent stimuli, we selected only cells whose preferred orientation differed 30° or less from the presented orientation, and had a receptive field center within 100 pixels of the center of the size stimulus. For the spatial frequency tuning we used slightly different criteria in that we selected cells whose preferred orientation differed 60° or less from the presented orientation.

IMMUNOHISTOCHEMISTRY AND *IN SITU* HYBRIDIZATION

After an overdose of pentobarbital (100 mg/kg i.p.), we transcardially perfused the mice with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), and post-fixated the brains for 2 h in PFA at 4°C. After changing the brain to a PBS solution, we cut the brains in sagittal or coronal slices of 50 μm thickness. We incubated the slices for 2 h in 500 μl blocking solution (0.1% Triton X-100, 5% NGS in PBS) on a rotary shaker at room temperature. We then incubated the slices in 250 μl of primary antibody per well and left it overnight at 4°. The next day we discarded the primary antibody solution and proceeded with three washes of 10 min at room temperature on the rotary shaker with 500 μl of washing solution (0.1% Tween in PBS). We added 250 μl per well of the secondary antibody solution and incubated for 1 h at room temperature on the rotary shaker. We washed the slices in washing solution three times for 10 min at room temperature on the rotary shaker.

We used the following primary antibodies: (1) mouse anti-calretinin, Millipore (1:700 in blocking solution); (2) rabbit anti-parvalbumin, Swant (1:1000 in blocking solution); (3) rat anti-somatostatin, Millipore (1:200 in blocking solution); (4) mouse anti-SatB2, Santa Cruz (1:1000 in blocking solution). As secondary antibodies we used: (1) Goat anti-mouse Alexa 647, Invitrogen (1:700); (2) Goat anti-rabbit Alexa 488, Life technologies (1:700); (3) Goat anti-rat Alexa 647, Invitrogen (1:700); (4) goat anti-mouse Alexa 488, Invitrogen (1:700). Stained sections were mounted on glass slides with mowiol.

For the imaging of the immunostained sections we used a Leica TCS SP5 Confocal microscope and we mainly imaged the superficial layers of primary visual cortex. *In situ* hybridization image for *Calb2* expression was retrieved from the Allen Mouse Brain Atlas, available from[3] (Lein et al., 2007). The *in situ* hybridization images for the CR-ires-Cre and Ai14 cross are collected from the Allen Mouse Brain Connectivity Atlas, available from[4] (Oh et al., 2014).

STATISTICS

Values in the text are expressed as the mean ± SEM. For the population statistics of comparing the response properties of tdTom+ and tdTom− cells, the distribution were first tested for normality with the Shapiro–Wilk test. In all cases, at least one of the distribution failed and we performed the non-parametric Mann–Whitney *U* test (Kruskal–Wallis).

RESULTS

OVERLAP OF tdTOMATO AND CALRETININ EXPRESSION

To study the function in visual processing of neurons that express calretinin, we used offspring from a cross of two mouse lines: one expressing the tdTomato red fluorescent protein inside the *Rosa26* locus preceded by a floxed stop cassette; and the CR-ires-Cre line in which the *Cre-recombinase* coding sequence follows the *Calb2* promoter and an IRES-element. This resulted in the expression of the tdTom protein in all the cells that sufficiently expressed *Calb2* and thus Cre during their existence.

First, we investigated to which extent the cells expressing tdTomato (tdTom+ cells) overlap with the Calretinin immunoreactive cells (CR-IR cells). In **Figure 1A**, tdTom+ cells are shown in red and the CR-IR cells in green in a slice of adult V1. The levels of CR immunoreactivity varied among the positive cells, but showed a bimodal distribution separated at about 8% of the most intense levels (**Figure 1B**). Although it was possible that even for the very low levels of labeling, there was some calretinin expression, it did suggest a natural classification of CR-IR positive and negative cells. The distribution of tdTomato expression levels was even more clearly bimodal and made a clear distinction between tdTom+ and tdTom− negative cells possible (**Figure 1B**). Using these classifications, we estimated that only 20% of tdTom+ cells were positive for calretinin (**Figure 1C**). By contrast, 96% of the CR-IR cells expressed tdTomato. To understand whether the protein expression differences between CR and tdTomato came from a difference of translation of the proteins, we checked the Allen Brain Atlas, where *in situ* hybridization images are available for the CR-ires-Cre mice (Oh et al., 2014). A co-hybridization for tdTomato mRNA and CR mRNA showed a pattern similar to the protein immunohistochemistry images (**Figure 1D**). This suggested that the differences were not due to translational

[3] http://mouse.brain-map.org

[4] http://connectivity.brain-map.org

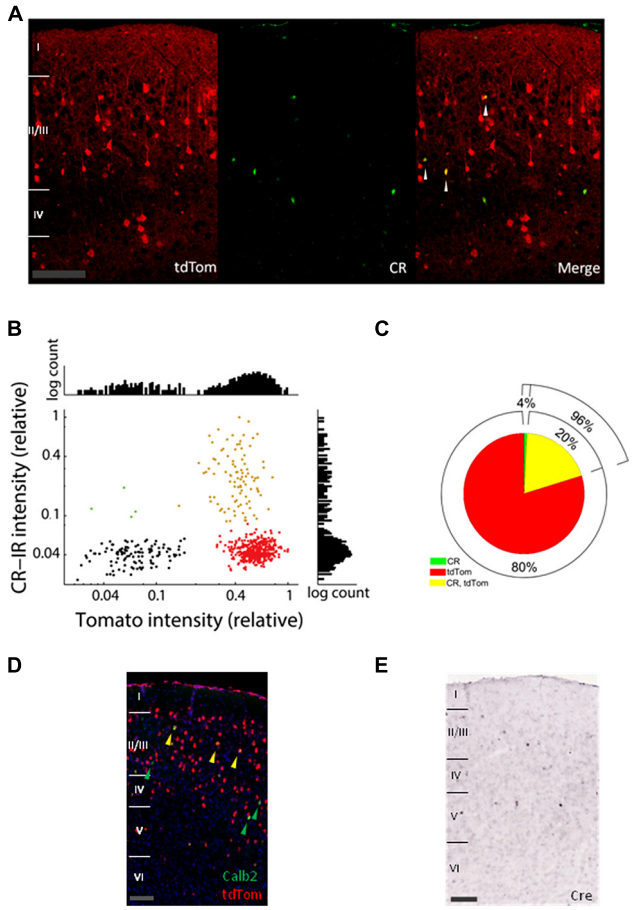

FIGURE 1 | Expression of tdTomato does not match mRNA and protein expression of calretinin. (A) Confocal images of coronal slices of V1 from a tdTomato× CR-ires-Cre mouse immunostained with Millipore mouse Calretinin (CR) antibody (green). White arrows show colocalization of CR and tdTom. Scale bar is 100 μm. **(B)** Average label intensities for CR-IR and tdTom+ expressing cells. Red points are from cells classified as tdTom+, green points belong to CR-IR cells, and yellow belong to cells where both proteins colocalize. Black shows a collection of background expression levels. Intensity is normalized per slice to the maximum average intensity found for any cell in each slice. Graphs at right and top show histograms of the intensities for the *y*- and *x*-axis, respectively. **(C)** Pie graph showing the colabeling of CR immunoreactive and tdTomato protein. **(D)** *In situ* hybridization images of V1 from a P56 mouse from the same cross (Allen Mouse Brain Connectivity Atlas, experiment 267223174, image 31). In red tdTomato mRNA expressing cells, in green cells expressing the *Calb2* mRNA (green arrows) and in yellow colocalization of the two mRNAs (yellow arrows). Scale bar is 100 μm. **(E)** *In situ* hybridization of Cre-recombinase mRNA in P56 visual cortex is consistent with CR expression (image from Allen Mouse Brain Atlas, experiment 100146208, image 31). Scale bar is 100 μm.

regulation of CR or tdTomato. Indeed, *in situ* hybridization of Cre-recombinase mRNA showed a pattern remarkably similar to the expression pattern of *Calb2* mRNA and CR protein (**Figure 1E**). Cre-recombinase is thus not expressed at P56 in most of the tdTom+ cells. It must have been expressed earlier in the history of these cells, when it irreversibly activated the *tdTomato* gene. Together with the reported high specificity of the CR-ires-Cre line when checked as adults with a Cre-dependent marker virus (Taniguchi et al., 2011), this shows that the tdTom+ cells that are not CR-immunoreactive in the adult mouse have transiently expressed CR in their past.

tdTOMATO COLOCALIZES WITH DIFFERENT INTERNEURONAL AND PYRAMIDAL MARKERS

We next wanted to understand the nature of the tdTomato-positive cells, which clearly contained more cells than just the CR-IR interneurons. The *in situ* hybridization data of the Allen Institute for this mouse cross showed a low colocalization between the tdTomato and the GABA synthesizing enzyme *Gad67* mRNAs (**Figure 2A**, *Gad67* shown in green). We wondered if the overlap of tdTomato with *Gad67* was completely due to CR expressing interneurons or if parvalbumin-positive or somatostatin-positive interneuron classes had a transient expression of CR during their development or migration. Our immunostainings showed no colocalization with the parvalbumin protein and a low colocalization with somatostatin (**Figure 2B**). This showed that parvalbumin interneurons do not transiently express CR. The fraction of somatostatin neurons that expressed tdTomato was consistent with previous reports of SST interneurons with CR immunoreactivity (Xu et al., 2006; Gonchar et al., 2008). Given the relatively low overlap with *Gad67*, we wondered whether the tdTomato positive cells were pyramidal neurons and stained for SatB2 which labels a large group of pyramidal cells (Britanova et al., 2008). We found that indeed 60% of tdTom+ cells are SatB2 positive and 5% of SatB2-IR cells are positive for tdTomato (**Figure 2C**, quantification not shown). Thus, we conclude that most of the tdTom cells are pyramidal neurons, and indeed many tdTom+ cells have pyramidal morphology (for example, **Figure 2D**).

VISUAL RESPONSE PROPERTIES OF tdTOM+ NEURONS MATCH THOSE OF tdTOM− NEURONS

Although the tdTom+ cells formed a larger group than the CR interneurons, the cells all expressed calretinin in their past, and this may be an indication of not only a common history but also of a common functional role. To explore whether they showed any specific response to visual stimulation, we imaged the visual response properties of tdTom+ cells in primary visual cortex using two-photon imaging of the genetically encoded calcium indicator GCaMP6s (Chen et al., 2013), virally expressed behind the neuronal *Synapsin* promoter.

We started by measuring orientation tuning. The tdTom+ group contained a variety of orientation or direction selective and unselective cells (some examples shown in **Figure 3A**). The tuning of tdTom+ group did not stand out of the population as a whole. The tdTom+ and the tdTom− neurons were equally orientation tuned, as assessed by the orientation selectivity index, which was equal to 1-circular variance, (mean OSI = 0.37 ± 0.03, $N = 118$ and 0.41 ± 0.02, $N = 396$, respectively; $p = 0.27$, Kruskal–Wallis test, $K[1] = 1.2$; six mice, **Figures 3B,C**). The distribution of direction selectivity of the two groups were also very similar (mean DSI was 0.23 ± 0.02, $N = 118$ and 0.27 ± 0.01, $N = 396$ respectively; $p = 0.31$, Kruskal–Wallis test, $K[1] = 1.0$; **Figures 3D,E**). After showing all orientations, we picked one and varied the temporal frequency. Next, we selected the responses of all cells that had

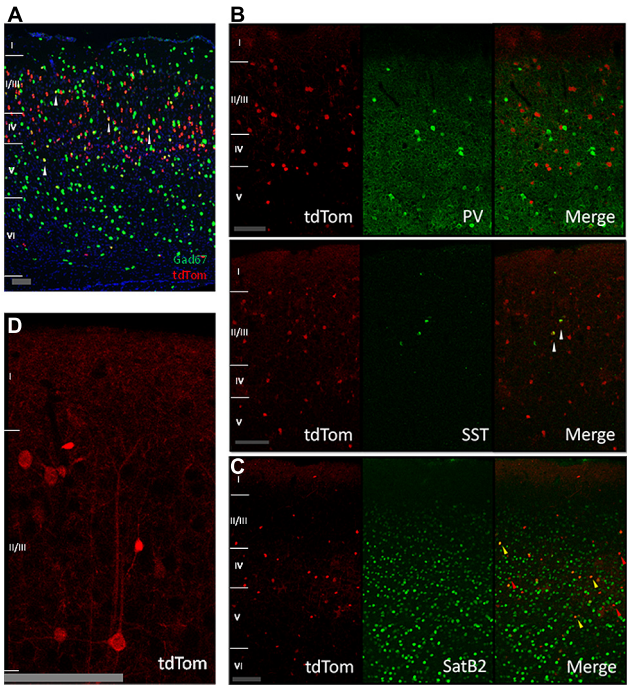

FIGURE 2 | Cre-recombinase in inhibitory and pyramidal neurons. **(A)** *In situ* hybridization image showing tdTomato expressing neurons in red, *Gad67* positive neurons in green, and in yellow neurons expressing both mRNAs (highlighted by white arrows; image from Allen Mouse Brain Connectivity Atlas, experiment 180304494, image 33). Scale bar is 100 μm. **(B)** Expression of tdTomato in other inhibitory neurons. First row: immunostained sagittal sections from V1 showing no colocalization between tdTom and parvalbumin (PV). Second row: immunostained sagittal sections from V1 showing low colocalization of tdTom and somatostatin (SST) protein (white arrow). Scale bar is 100 μm. **(C)** Expression of tdTomato in pyramidal neurons. Immunostained coronal slices from V1 for the pyramidal marker SatB2. Red arrows show cells that are positive only for tdTomato, yellow arrows show cells that express both proteins. Scale bar is 100 μm. **(D)** Close up of a tdTomato neuron with a pyramidal shape. Scale bar is 100 μm.

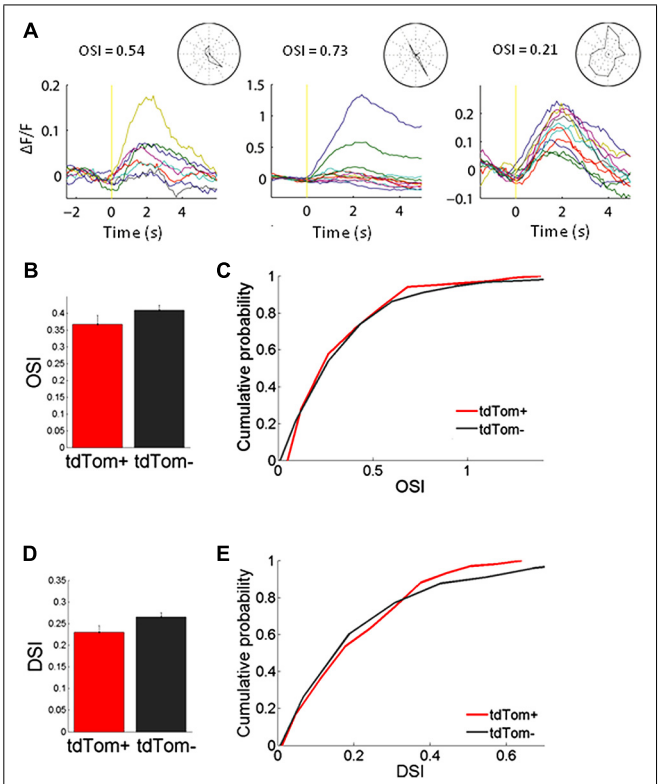

FIGURE 3 | Orientation tuning of tdTom+ neurons is like the general population. **(A)** Example peristimulus time histograms for tdTom+ neurons during visual stimulation with drifting gratings. Yellow vertical line represents the start of the visual stimulation. Normalized fluorescence changes to different grating orientations are shown by different colors. Stimulus duration is 2 s. **(B)** No difference in mean orientation selectivity index (OSI) for the tdTom+ and tdTom− neurons. Error bars show SEM. **(C)** Distributions of OSI are equal for tdTom+ and tdTom− neurons. **(D)** No difference in mean direction selectivity index (DSI). Error bars show SEM. **(E)** Direction selectivity index distributions of tdTom+ and tdTom− population are not different.

an orientation preference within 30° of the presented orientation. We found both lowpass and bandpass cells, for both the tdTom+ and tdTom− population. We found no differences in temporal frequency tuning. Both groups responded on average up to about 15 Hz (**Figure 4A**). The optimal temporal frequency was equal for the two groups (tdTom+, 6.4 ± 1.2 Hz, $N = 16$, tdTom−, 6.4 ± 0.9 Hz, $N = 36$; $p = 0.95$, Kruskal–Wallis test, $K[1] = 0.003$; four mice).

In the same way, we measured the response to sinusoidal gratings of different spatial frequencies. Also in this respect did the two populations not differ from each other. The average spatial frequency tuning curve overlapped and the mean optimal spatial frequencies were equal for tdTom+ and tdTom− (0.034 ± 0.01 cpd, $N = 6$ and 0.044 ± 0.01 cpd, $N = 20$, respectively; $p = 0.9$, Kruskal–Wallis test, $K[1] = 0.03$; in four mice, **Figure 4B**). The curves for both temporal and spatial frequency reached values slightly lower than zero at high frequencies. This was due to the slow kinetics of the GCaMP6s indicator. The 3 s of interstimulus interval following the 2 s of visual stimulation were not enough for the fluorescence to completely return to the baseline level, meaning that when the subsequent stimulus evokes no or very little response the response may appear slightly negative (see also **Figure 3A**). Because of the feedback connections from higher areas onto CR interneurons (Gonchar and Burkhalter, 2003) and the hypothesized role of these feedback connections in surround suppression (Bair et al., 2003), we were interested in the size tuning properties of the tdTom+ cells. One might expect perhaps that neurons receiving feedback from cells with larger receptive fields, or interneurons that are involved in surround suppression, show larger responses with increasing stimulus size (Adesnik et al., 2012). In the tdTom+ group, we found cells showing a strong suppression and cells that lacked suppression (**Figure 5A**). The average size tuning curve and the distribution of suppression indices did not show a difference between the tdTom+ and tdTom− neurons (mean SI 0.38 ± 0.05, $N = 21$ and 0.43 ± 0.07, $N = 38$, respectively; $p = 0.4$, t-test, $t[33] = -0.90$; four mice, **Figures 5B,C**). We conclude that in none of the studied visual response properties the tdTom+ population is significantly different from the tdTom− population.

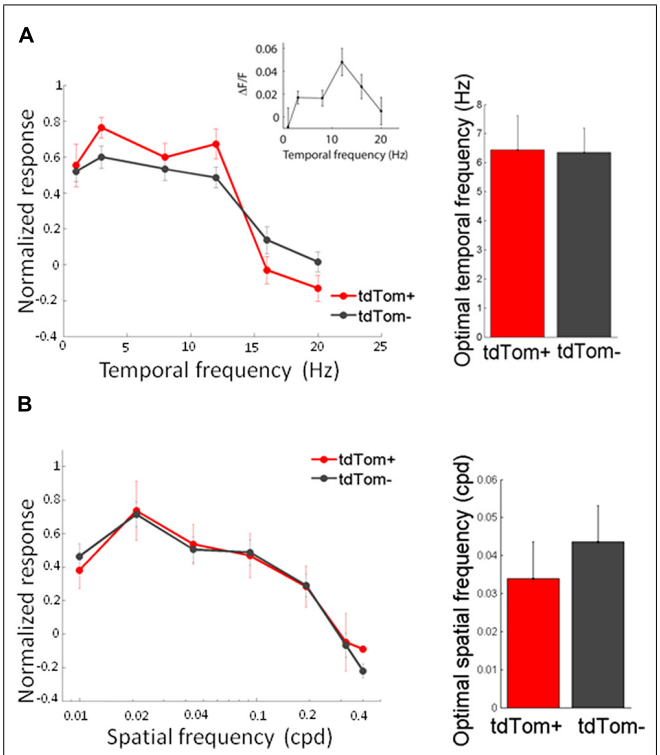

FIGURE 4 | Temporal and spatial frequency tuning of tdTom+ neurons is like the general population. (A) Left, population average tuning to temporal frequency are not different for tdTom+ and tdTom− groups. Inset, example tuning of a tdTom+ neuron. Right, mean optimal temporal frequencies are identical. Error bars show SEM. **(B)** Left, population response tuning to spatial frequency is the same for tdTom+ and tdTom− groups. Right, mean optimal spatial frequencies are not different. Error bars show SEM.

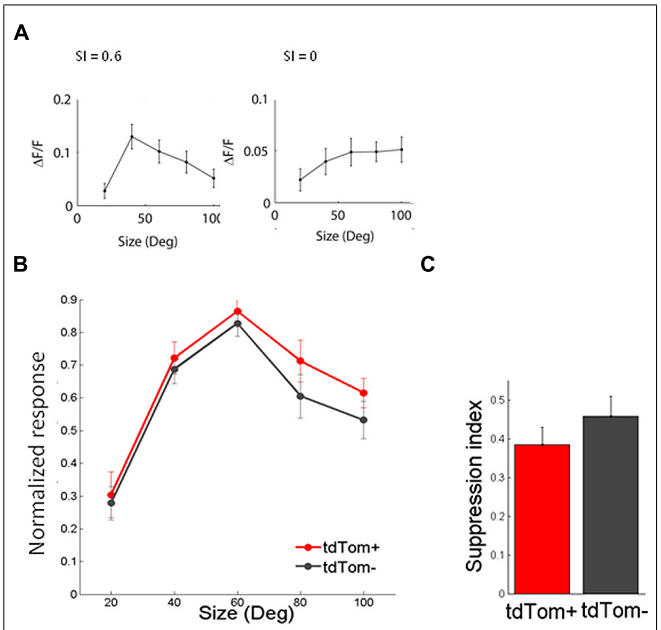

FIGURE 5 | Size tuning of tdTom+ is like the general population. (A) Example tuning of two tdTom+ neurons to visual stimulation of a circular patch of different sizes, and their corresponding Suppression Index values. **(B)** Population average of normalized response to differently sized gratings are not different for tdTom+ and tdTom− groups. Error bars are SEM. **(C)** Mean suppression index is not different for the two groups. Error bars are SEM.

DISCUSSION

We studied the visual response properties of a class of labeled neurons in the primary visual cortex that resulted from a cross of a Cre-dependent tdTomato reporter line and a line in which Cre-recombinase was expressed like calretinin. These neurons that we called tdTom+ do not seem to have different response properties compared to the general population (tdTom−).

Originally, the aim of the cross was to study the function of CR-immunoreactive interneurons. However, in visual cortex, only a fifth of the tdTom+ cells showed CR-immunoreactivity, and mRNA levels showed a similarly low overlap between tdTomato and *Calb2*. In contrast, when cells were labeled in the mature CR-ires-Cre animal with a virus with a Cre-dependent fluorescent reporter vector, the specificity was as high as 91% (Taniguchi et al., 2011). *In situ* hybridization also showed that the pattern of expression of Cre-recombinase is very similar to the pattern of CR expression in these animals. We must conclude that the high number of tdTom+ cells that do not express CR is due to transient expression of CR. The tdTom+ cells have expressed CR and thus cre-recombinase in their past. This has activated the *tdTomato* transgene and the cells have expressed tdTomato ever since, even when they no longer expressed CR. There is the possibility that some or all tdTom+ cells that we judged negative for CR-IR, in fact do express calretinin at a very low level, but this is not detectable above the background level in our staining.

We investigated whether the tdTom+ cells perhaps included another set of interneurons besides the CR-interneurons. We found, however, no colocalization with parvalbumin. We did find some colocalization with somatostatin (SST), which was consistent with the reported overlap of SST and CR expressing interneuron populations (Xu et al., 2006; Gonchar et al., 2008). Available *in situ* hybridization data showed that many tdTom+ cells did not express the GABA-synthesizing enzyme *Gad67* and would thus not be interneurons. Indeed, 60% of tdTom+ neurons were positive for the pyramidal marker SatB2 and pyramidal shaped tdTom+ neurons were recognizable in confocal images.

Our findings are consistent with the report that CR immunoreactivity starts to be widely present in the cortical anlage from embryonic day 14 in rats, and continues in the first two postnatal weeks. Many of the CR-IR neurons at these early stages of development show undifferentiated non-pyramidal shapes, but there is also transient expression in some pyramidal-like neurons in layers V, VI and layer II/III (Fonseca et al., 1995). We showed that the extent of this transient expression is very high. Transient expression of CR protein in pyramidal-like neurons has also been reported in rat hippocampus during development (Jiang and Swann, 1997) and adult neurogenesis (Brandt et al., 2003).

There is a considerable sequence homology between the promoters of the mouse *Calb2* and the human *CALB2* genes (Strauss

et al., 1997), and thus the regulation of CR expression in primate and mouse may be quite similar. It is thus not inconceivable that the CR-IR pyramidal neurons that have been reported in monkeys and human (del Río and DeFelipe, 1996; Melchitzky et al., 2005) are homologous to cells that are contained in our tdTom+ group.

The investigation of the visual response properties of the heterogeneous group of neurons with a shared CR-IR history revealed the absence of specificity in their response to the studied features. Unlike parvalbumin neurons, which show a reduced orientation selectivity (Kerlin et al., 2010; Hofer et al., 2011), tdTom+ cells showed an orientation selectivity identical to the general population. The mean OSI was slightly lower than the mean OSI found for excitatory neurons alone in another study. Although measurements of OSI are dependent on recording conditions, this would suggest that the group of interneurons included in our tdTom+ sample have a lower orientation selectivity than the pyramidal cell population. The tdTom+ cells also showed no more or less direction selectivity than the general population. We cannot rule out that we would have missed a small difference, but our study had about an 80% probability to detect a difference in orientation or direction selectivity of 20% with 95% confidence. A halving of the mean OSI for the tdTom+ group compared to that of the tdTom− group, like the difference that was reported for all GABAergic inhibitory neurons compared to excitatory neurons (Kerlin et al., 2010), we would have been able to detect with 100% certainty.

Previously, it was found that the spatial frequency tuning can vary with cortical cell type (Kerlin et al., 2010) and in particular putative fast-spiking interneurons show a preferred frequency only about half of that of excitatory neurons (Niell and Stryker, 2008). The tdTom+ and tdTom− groups, however, did not differ significantly in spatial frequency. We had, however, less statistical power for the spatial frequency tuning than for orientation and direction selectivity, and had only 30% chance to pick up a similar difference for the preferred spatial frequency of the two groups at the 95% confidence level. We also did not find any difference in temporal frequency tuning. We know no reference study where a difference in temporal frequency between neuron types within one area was reported, but we had a power of 80% to find a 65% difference in temporal frequency. The size tuning of tdTom+ was also not different from the other cells, unlike somatostatin neurons which show considerably less surround suppression (Adesnik et al., 2012). Our study would have had enough power to detect if there was a similarly large difference between the tdTom+ and tdTom− population, and even a 91% certainty of detecting only a halving of the suppression index as was seen for PV interneurons (Adesnik et al., 2012).

We could thus not discern any particular feature of this group of cells, other than their common CR history. The underlying reason for the common CR expression during their development remains unclear. For investigating the visual response properties of CR-IR interneurons, viral transfection with a vector for cre-dependent expression of GCaMP6s or another fluorescent protein after the second postnatal week will be needed.

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Calretinin immunoreactivity in the claustrum of the rat

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The claustrum is a telencephalic structure which consists of dorsal segment adjoining the insular cortex and a ventral segment termed also endopiriform nucleus (END). The dorsal segment (claustrum) is divided into a dorsal and ventral zone, while the END is parcellated into dorsal, ventral and intermediate END. The claustrum and the END consist of glutamatergic projection neurons and GABAergic local interneurons coexpressing calcium binding proteins. Among neurons expressing calcium binding proteins the calretinin (CR)-immunoreactive interneurons exert specific functions in neuronal circuits, including disinhibition of excitatory neurons. Previous anatomical data indicate extensive and reciprocally organized claustral projections with cerebral cortex. We asked if the distribution of cells immunoreactive for CR delineates anatomical or functional subdivisions in the claustrum and in the END. Both segments of the claustrum and all subdivisions of the END contained CR immunoreactive neurons with varying distribution. The ventral zone of the claustrum exhibited weak labeling with isolated cell bodies and thin fibers and is devoid of immunoreactive puncta. Within the medial margin of the intermediate END we noted a group of strongly positive neurons. Cells immunoreactive for CR in all subdivisions of the claustrum and END were bipolar, multipolar and oval with smooth, beaded aspiny dendrites. Small number of CR-immunoreactive neurons displayed thin dendrites which enter to adjoining structures. Penetration of dendrites was reciprocal. These results show an inhomogenity over the claustrum and the END in distribution and types of CR immunoreactive neurons. The distribution of the CR-immunoreactive neurons respects the anatomical but not functional zones of the claustral complex.

Keywords: calcium-binding proteins, calretinin, claustrum, endopiriform nucleus, rat

INTRODUCTION

The claustrum is a pallial/subcortical structure which, in the rat, consists of a dorsal segment (CLD), termed also insular claustrum adjoining the insular cortex, and a ventral segment also termed endopiriform nucleus (END) situated deep to piriform cortex. The CLD is further parcellated into a dorsal part of claustrum (DCl) and a ventral part of claustrum (VCl; Paxinos and Watson, 2007). Paxinos and Watson (1997) divided the END into two parts, the dorsal endopiriform nucleus (DEn) and the ventral endopiriform nucleus (VEn). In the most recent edition of their stereotaxic atlas (Paxinos and Watson, 2007) END is divided into three parts: the DEn, the VEn and the intermediate endopiriform nucleus (IEn). This recent subparcellation of END was used also in our present study. The claustrum is reciprocally and topographically connected with most if not all neocortical and allocortical areas (Edelstein and Denaro, 2004; Druga, 2014). Studies on the expression of developmental regulatory genes specific to the lateral and ventral pallial histogenetic divisions of mammalian brain indicate that CLD and END, together with the pallial part of the amygdala, may be regarded as a single entity, named the "claustroamygdaloid complex" (Medina et al., 2004).

The CLD and END consist of glutamatergic projection neurons and GABAergic local interneurons coexpressing calcium-binding proteins (CBPs) and neuropeptides in various combinations (Kowiański et al., 2009). Calcium-binding proteins are classified as "buffer proteins," which may act as modulators of cytosolic calcium levels. The expression of CBPs is often used for revealing functionally distinct subdivisions of the CNS and for the detection of various classes of inhibitory interneurons. Calretinin (CR) is one calcium-binding protein with complex Ca2+ -binding kinetics (Schwaller, 2014). In addition to its buffer function it might also serve as a Ca2+ sensor. Further, CR is involved in the regulation of neuronal excitability and synaptic plasticity (Camp and Wijesinghe, 2009). CR-immunoreactive (CR-ir) interneurons have been shown to influence the activity of other interneurons (Gulyás et al., 1996), in some cortical areas a gating function of CR-ir interneurons has been presumed (Callaway, 2004; Barinka et al., 2012). There are some data indicating resistence of CR-ir

neurons in several neurological and psychiatric disorders (Camp and Wijesinghe, 2009; Barinka and Druga, 2010; Schwaller, 2014). While the various functions of CR interneurons have been partly elucidated, its expression in tumor cells (e.g., mesothelioma, soft tissue tumors) has proven to be enigmatic (Barak et al., 2012).

Although Paxinos et al. (1999) reported that the rat claustrum is defined by the absence of CR staining, subsequent studies in the mouse have reported CR-immunoreactivity in both subdivisions of the claustrum (Real et al., 2003, 2006). In addition, Wójcik et al. (2004) reported CR-ir neurons in the dorsal claustrum of the rabbit, Reynhout and Baizer (1999) in the monkey claustrum and Rahman and Baizer (2007) in the cat claustrum. Recently, Cozzi et al. (2014) described CR-ir neurons in the claustrum of the dolphin. The objective of the present study is to define the distribution of CR-ir neurons in all subdivisions of the claustrum of the rat, while searching for patterns related to connectivity, functional characteristics and histogenetic parcellation. Specifically, we sought to analyze the distribution of dendritic arborizations of CR-ir neurons and their extensions to adjacent cortical and subcortical structures. Our results complement the mouse and guinea pig data of Real et al. (2003) and Edelstein et al. (2010).

MATERIALS AND METHODS

Experiments were carried out in six adult male Wistar rats (350–400 g). All animals were cared for in accordance with the regulations and laws of the European Union (86/609/EEC) as well as NIH guidelines (Assurance No. A5820-1). Experiments were approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic. Animals were housed under standard conditions (12 h light/12 h dark cycle, 22 ± 1°C, humidity 50–60%, free access to food and water).

Rats were irreversibly anesthetized (urethan 2 g/kg i.p.) and transcardially perfused with 0.01 M phosphate-buffered saline (PBS; pH 7.4), immediately followed by 4% paraformaldehyde in 0.1 PBS. The brains were removed from the skull, postfixed in buffered 4% paraformaldehyde for 3 h and then cryoprotected via a standard sucrose gradient in PBS at 4°C. The brains were sectioned in the coronal plane at 50 μm with a cryocut Leica CM 1900. Four sets of sections through anterior—posterior extent of the claustrum were processed; three sets were used for immunocytochemistry to CBP (Parvalbumin, Calbindin, Calretinin), and a fourth was stained with cresyl violet. Intervals between sections immunostained for CR was 200 μm. Free-floating sections were incubated in 0.15% hydrogen peroxide in PBS for 10 min, rinsed with PBS five times, permeabilised with 0.3% Triton-X100 for 5 min and then incubated with a blocking solution containing 2% horse serum (Vector Laboratories, Burlinghame, CA, USA) for 1 h at room temperature. The sections were then incubated with the primary antibody (mouse anti-CR monoclonal, Millipore, dilution 1:8000, catalogue No. MAB1568) in PBS containing 1.5% normal horse serum and 0.1% Triton-X100 for 48 h at 4°C and then rinsed five times in PBS. Thereafter sections were then incubated for 1 h at room temperature with secondary antibody (biotinylated anti-mouse made in horse, Vector, dilution 1 : 50) in PBS containing 1.5% normal horse serum. Lastly, a standard ABC kit was used (Sigma-Aldrich). The peroxidase reaction was carried-out by using as the chromogen DAB (Sigma-Aldrich).

No immunostaining was seen in the control sections where incubation with primary antibody was omitted.

In each animal 25–28 sections immunostained for CR were analyzed.

Measurements of neuronal diameter and cross-sectional area were performed under a 40 × objective by means of the imaging acquisition application cell^{F} (Olympus). The average values of these parameters along with standard error of mean (S.E.M.) are provided. In each animal (6 animals), 20 neurons in each subdivision were evaluated. Hence, together 120 neurons in each subdivision were analyzed (alltogether, 600 neurons were analyzed). Selected CR-ir neurons with extensive dendritic trees were drawn using a camera lucida attachment. Light-microscopic images were photographed with an Olympus Bx51 microscope equipped with an Olympus digital camera DP 72. Digital images were loaded into Adobe Photoshop.

DENSITOMETRIC ANALYSIS

Densitometric analysis of CR immunopositivity in areas DCl, VCl, DEn, VEn and IEn was performed in seven coronal sections (2.1 mm, 1.2 mm, 0.2 mm, −0.8 mm, −1.8 mm, −2.8 mm and −3.8 mm AP relative to bregma (Paxinos and Watson, 2007); sections A, B, C, D, E, F and G in Tab. 2) evenly spaced along the rostrocaudal axis. The pictures were captured using an image analyzing software (QuickPHOTOMICRO 2.3, Promicra) and digital kamera (OlympusDP 72) attached to the microscope Olympus BX51. 4 × objective was used. To avoid differences in light intensity of the captured images, all images were captured at the same light intensity in the microscope (6 on the microscope scale). The images were converted to grayscale. Densitometric analysis was performed using software Densita, MBF Bioscience (MicroBrightField, Inc.), an integrated system for quantitative analysis of optical density in biological tissue. To get true scaling of the acquired images we calibrated grid using scale bar image acquired under the same conditions as the immunohistology images. Then we load the calibration strip image with solid blocks of defined grayscale values to capture calibration luminance values and to calculate related relative optical density (ROD) values. The ROD was calculated using formula:

$$ROD_{AVG} = \frac{1}{N}\sum_{i=0}^{n} \log_{10}\left(\frac{F}{P_i}\right),$$

where F is the background gray level (F = 255), Pi is the pixel gray level and N is the number of the pixels. A lower ROD value means that more light is coming through the tissue and a higher value means more light is blocked in the tissue and therefore not detected. The linear (first order) regression line was fitted to approximate the data. Using the regression line we calculated ROD for each studied area and section. To eliminate the possible biasing influence of slightly different intensity of immunopositivity between different sections and animals, the ROD were corrected dividing the values measured in individual areas under study by the values from the corpus

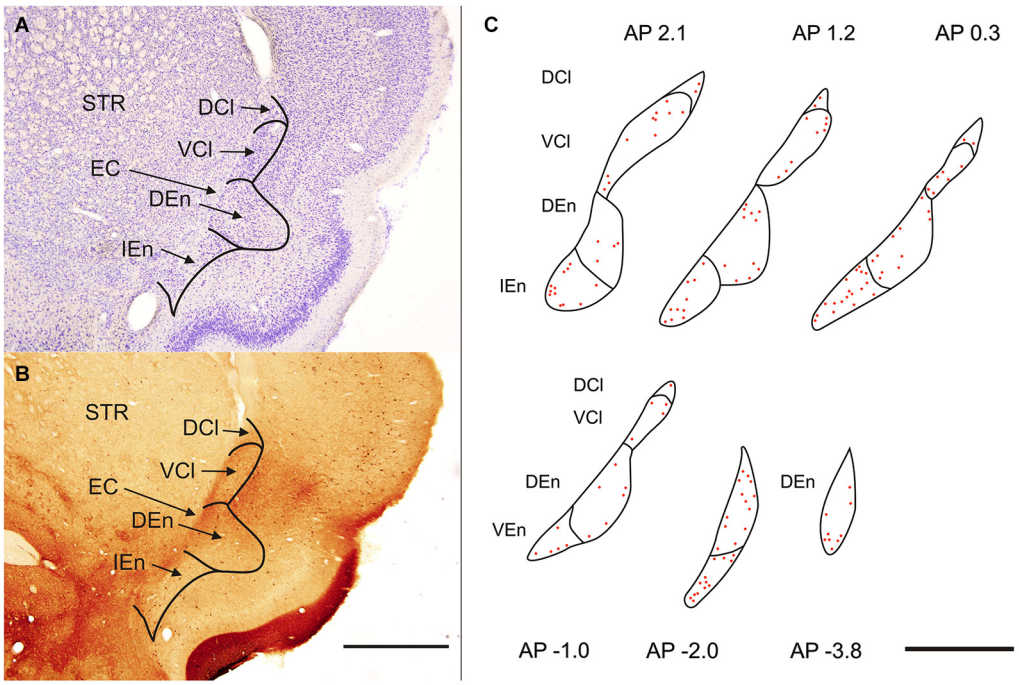

FIGURE 1 | (A) Transverse section of rat hemisphere showing claustrum and the endopiriform nucleus (END) stained with cresyl violet. **(B)** Transverse section of rat hemisphere showing the calretinin (CR) immunoreactivity in the dorsal claustrum and in the END. DCl—dorsal part of the claustrum. An oval central region with a weakly stained neuropil and small number of CR-ir neurons corresponds with the ventral part of the claustrum (VCl). DEn—dorsal endopiriform nucleus, IEn—intermediate endopiriform nucleus. EC—external capsule, STR—striatum. Scale 1000 μm. **(C)** Distribution of CR-ir neurons is shown on camera lucida drawings. Abbreviations as in **(A)** and **(B)**. Scale 1000 μm.

callosum on the corresponding sections. This corrected staining index (corrected relative optical density, cROD) allowed comparison among different sections and animals. The corpus callosum was selected as the reference structure because it is weakly immunolabeled by antibodies to CR and has well-defined borders.

STATISTICAL ANALYSIS

Analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test was used for the comparison of corrected relative optical densities as well as for the differences in morphometric parameters of neuronal somata between subdivisions of claustral complex. Probability (p) values < 0.05 were considered significant.

RESULTS

NISSL STAINING

In the rat the claustrum (CLD) is located underneath the insular cortex and shows elliptic or drop-like shape. Its major axis is oriented from dorsolateral to ventromedial. Anteroposteriorly claustrum extends from rostral pole of the striatum to posterior part of thalamus (from AP 2.5 to AP −1.9). The END exhibits elliptic configuration its major axis is vertically oriented and paralel with external capsule Anteroposterior extent of the DEn was from AP 2.5 to AP −4.3 while the anteroposterior extent of the IEn and VEn was shorter 3.1 mm and 2.2 mm.

Polymorph, small and medium sized round, eliptic and multipolar cells can be seen in the rat's claustrum and in the END. The cells are tightly grouped in the VCl, while they are less densely packed in the DCl (**Figure 1A**). In the END its dorsal part (DEn) exhibits lower density of neurons and difference in density enables to demarcate it against the VCl. For the IEn is characterisic lower density of cells and presence of larger and more intensely stained perikarya. In comparison with the DEn the cells within the VEn are less densely packed and less intensely stained.

CALRETININ IMMUNOREACTIVITY

Claustrum

Sections processed for CR-ir exhibited densely stained cells against a backround of stained fibers and puncta. CR-ir neurons were present in all subdivisions of the claustrum as well as in END (**Figures 1B,C**).

In the CLD of the rat, two zones are evident which differ relative to the CR-immunoreactivity of their neuropil (**Figure 1B**). One zone, which corresponds to the central part of the VCl, exhibited weak CR-immunoreactivity. This "pale nucleus" contains isolated thin fibers oriented in various directions and is practically devoid of CR-ir puncta (**Figure 2**). It was also seen to contain isolated CR-ir cell bodies primarily in its

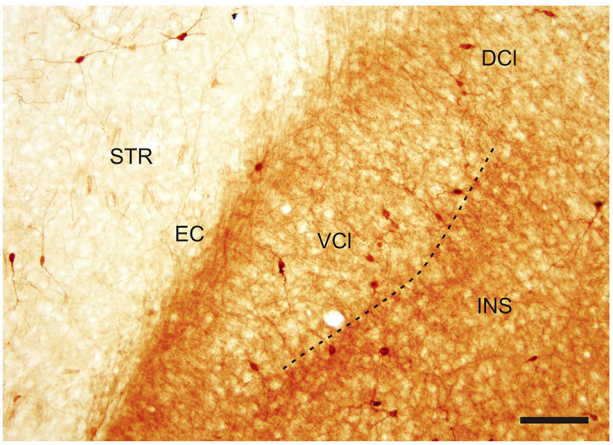

FIGURE 2 | Photomicrograph demonstrating CR-ir in the DCl and in the VCl. Dashed line indicates boundary between insular cortex and VCl. DCl—dorsal part of the claustrum, EC—external capsule, INS—insuluar cortex, STR—striatum, VCl—ventral part of the claustrum. Scale 100 μm.

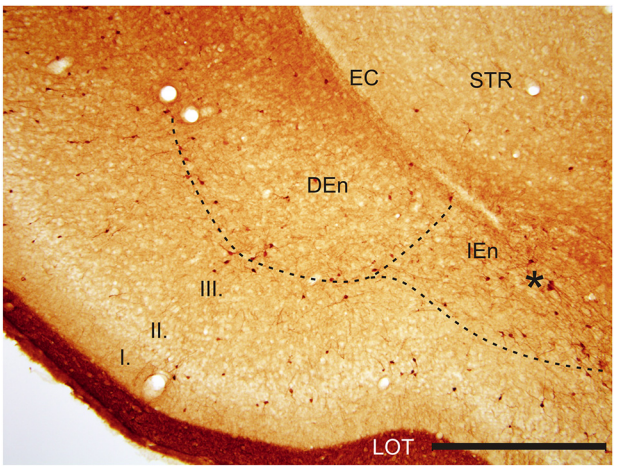

FIGURE 3 | Photomicrograph showing CR-ir in the DEn and in the IEn. Dashed line indicates boundary between piriform cortex and DEn and IEn. I–III—layers of the piriform cortex, EC—external capsule, LOT—lateral olfactory tract, STR—striatum. *—cluster of strongly CR-ir neurons within the medial margin of the IEn. Scale 500 μm.

periphery (7–14 per section). A second zone, which corresponds partly to the DCl of Paxinos and Watson (2007), surrounds the VCl in its dorsal, medial and basal aspects with a rim of moderately CR-ir neuropil and fibers and contained small number of CR-ir neurons (2–6 per section). A neighboring zone which medially delineates the VCl and DCl from the margins of the external capsule was seen to have numerous moderately stained CR-ir puncta, perikarya and thin fibers. Lateral to the VCl and DCl, a band of moderate-to-strong CR-immunoreactivity was seen corresponding to the infragranular layers of the insular cortex. (**Figures 1B**, **2**). Although CR-ir neurons were distributed in both zones of the CLD, they were most prevalent at VCl and in deep layers of the insular field (GI, DI, AID, AIV; see Paxinos and Watson (2007).

The endopiriform nucleus

With regard to CR-immunoreactivity in the DEn and IEn, both displayed weak-to-moderate labeling of neuropil containing a relatively small number of thin positive fibers and puncta. In the DEn, few scattered strongly positive neurons were noted (4–9 per section) (**Figure 3**). The medial border of DEn and IEn was indentifed by a thin rim of strongly positive neuropil which was seen to be continuous with the moderate-to-strongly positive medial border of the CLD. Ventral to the DEn and caudal to stereotaxic coordinate AP 0.5 within the medial margin of the IEn, we noted a cluster of strongly CR-ir neurons embedded within weakly or moderately stained neuropil, discernible throughout the caudal extent of the nucleus (**Figure 3**). Majority of neurons within this cluster exhibited medium sized or large multipolar morphology (up to 30 μm) and emerging dendrites were thicker than in other types of CR-ir neurons (**Figure 4**). The IEn contained 8–15 CR-ir neurons per section. As seen with the DEn and IEn, the VEn displayed moderately immunoreactive neuropil with a relatively small number of positive neurons.

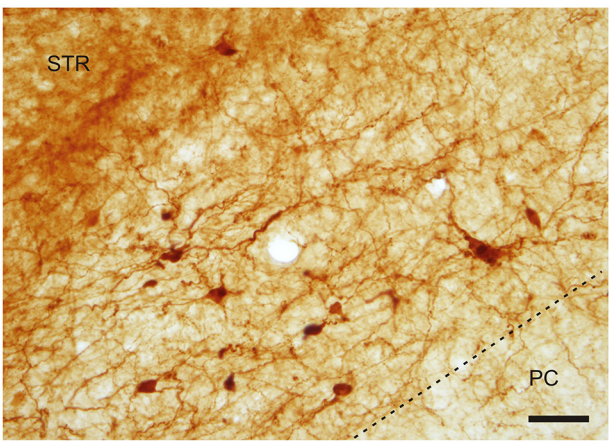

FIGURE 4 | A cluster of CR-ir neurons in the IEn. Dashed line indicates the boundary between the IEn and the third layer of the piriform cortex (PC). Scale 50 μm.

Morphology of CR-ir neurons

With regard to generally observed somatodendritic patterns in the rat claustrum, CR-ir multipolar, oval and fusiform densely stained neurons were evident in all subdivisions. The multipolar neurons displayed smooth and occasionally beaded radially-oriented dendrites, while oval and fusiform cells typically exhibited smooth bipolar dendrites. Dendrites of all of the examined CR-ir neurons were seen to be of the aspiny type. Proximal segments of some dendrites exhibited undulated appearance. The larger diameter of positive DCl and VCl neurons averaged 13.5 ± 0.3 μm (±S.E.M.) and 14.0 ± 0.2 μm respectively, with a cross-sectional area of 73.7 ± 2.0 μm^2 and 80.7 ± 2.2 μm^2 respectively. The average diameter of CR-ir DEn neurons was 14.5 ± 0.2 μm, with a cross-sectional area of 85.1 ± 1.9 μm^2.

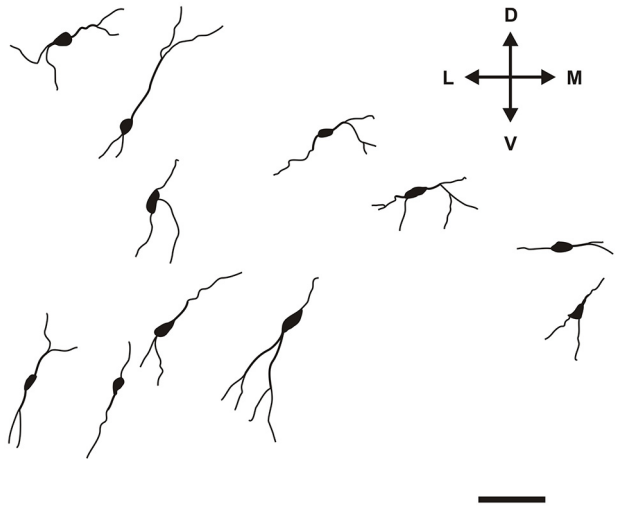

FIGURE 5 | Camera lucida drawings of various types of CR-ir neurons in the DCl. Scale 50 μm. D, L, V, M—dorsal, lateral, ventral and medial direction.

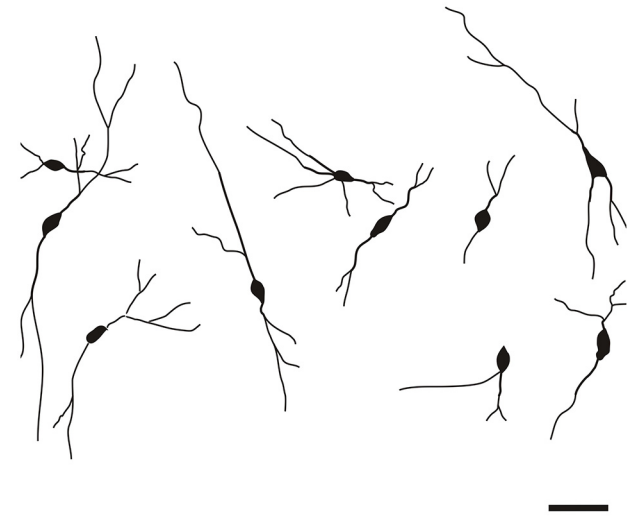

FIGURE 6 | Camera lucida drawings of CR-ir neurons in the VCl. Scale 50 μm.

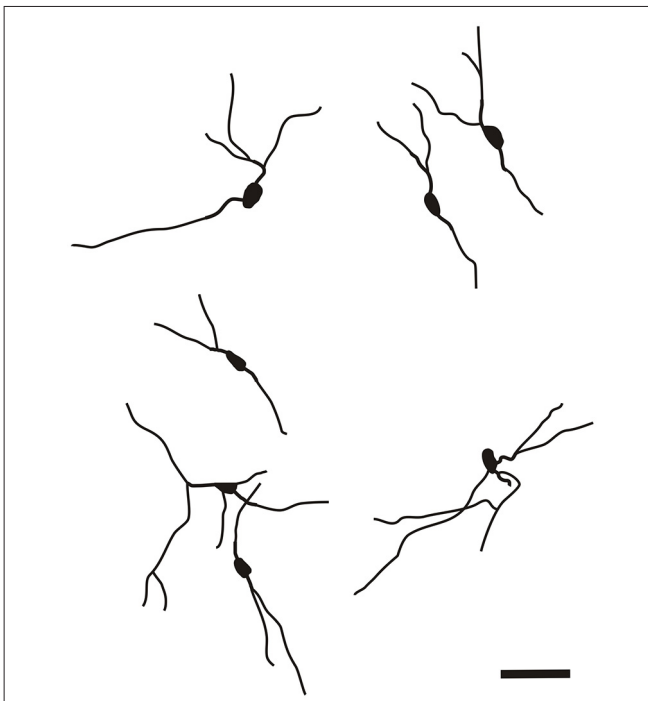

FIGURE 7 | Camera lucida drawings of CR-ir neurons in the DEn. Scale 50 μm.

The average diameter of CR-ir neurons within the IEn was 15.6 ± 0.3 μm, with cross sectional area of 104.2 ± 2.9 μm^2. Neurons in the VEn were bigger with average larger diameter 16.9 ± 0.4 μm amd cross-sectional area 112.3 ± 3.8 μm^2. In the case of the average diameter, the differences DCl-IEn, DCl-Ven, VCl-IEn, VCl-VEn, DEn-IEn as well as DEn-Ven reached the statistical significance ($p < 0.05$). With exception of IEn-VEn, DCl-VCl and VCl-DEn, all interareal comparisons of cross-sectional area of neuronal perikaryon showed statistically significant differences between studied claustral subdivisions ($p < 0.05$).

Specific to the morphology of the DCl, oval and fusiform cell bodies were most prevalent, with vertically-oriented bipolar dendrites (with the occasional horizontally-oriented type). Sparsely-branched dendrites were seen to extend 100–150 μm from the cell body (**Figure 5**). As for the VCl, oval and multipolar cell bodies were most commonly seen, fusiform cell bodies were observed less frequently (**Figure 6**). Bipolar cells were noted in close proximity to its border with the external capsule.

The CR-ir neurons in the DEn exhibited oval, fusiform (spindle) or multipolar perikarya with slight prevalence of oval perikarya (**Figure 7**).

The CR-ir neurons within the IEn were comprised of a combination of multipolar, triangular and oval perikarya with thicker primary dendrites (**Figure 4**).

In the VEn oval and spindle CR-ir neurons prevailed.

Very small number of CR-ir spindle neurons with bipolar morphology and long dendrites were located within the external capsule. Terminal segments of their dendrites leave the external capsule and enter the dorsal claustrum or marginal part of the striatum. In addition to vertically oriented CR-ir fibers, the external capsule contained small number of obliquelly or horizontally passing thin fibers.

It is of interest to note that a very small number of CR-ir striatal neurons displayed thin dendrites which traversed the external capsule and terminated in the medial marginal zone of the CLD (**Figure 8**). Further, in the CLD were seen dendrites branching from scattered CR-ir neurons within the medial and lateral marginal zones of the CLD, and then entering the adjoining marginal zone of the striatum (**Figures 9**, **11**) as well as layer VI of agranular insular cortex (**Figure 12**). In addition small number

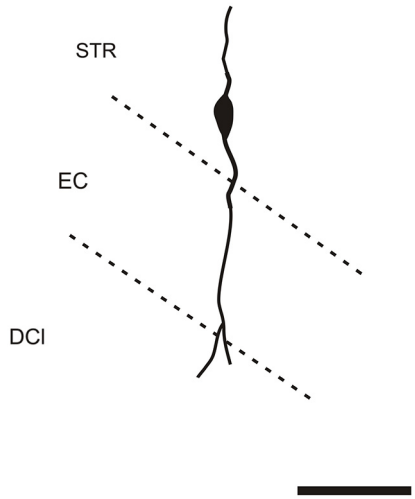

FIGURE 8 | Camera lucida drawing of striatal CR-ir neuron sending dendrite to the margin of the DCl. EC—external capsule, DCl—dorsal part of the claustrum, STR—striatum. Scale 50 μm.

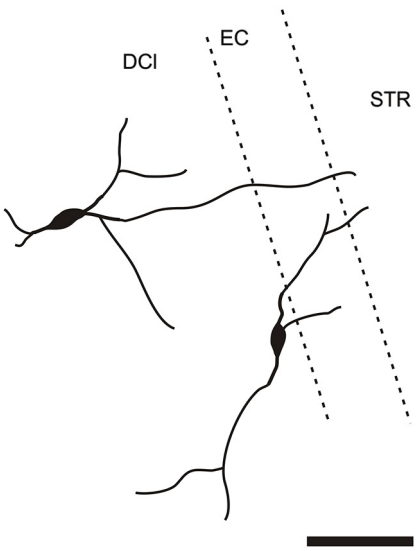

FIGURE 9 | Camera lucida drawing of two claustral CR-ir neurons sending dendrites to the margin of the striatum. DCl—dorsal part of the claustrum, EC—external capsule, STR—striatum.

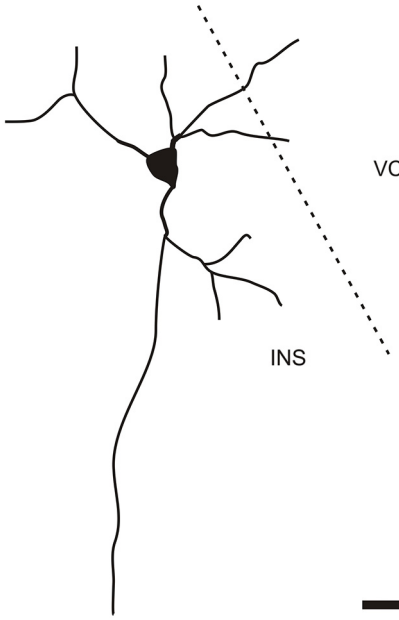

FIGURE 10 | Camera lucida drawing of CR-ir neuron located in the 6th layer of the insular cortex sending dendrites to the ventral part of the claustrum. INS—insular cortex, VCl—ventral part of the claustrum. Scale 50 μm.

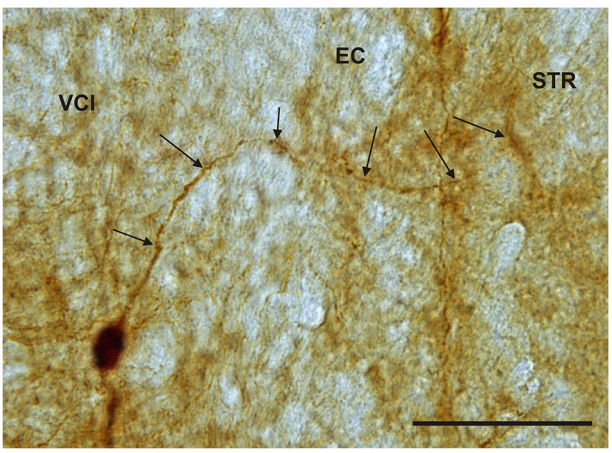

FIGURE 11 | Photomicrograph showing CR-ir neuron within the medial marginal part of the VCl sending dendrite (arrows) through external capsule (EC) to the marginal lateral part of the striatum (STR). Scale 50 μm.

of CR-ir neurons in layer VI of the insular cortex send processes (possibly dendrites) into the lateral marginal zone of the CLD (**Figure 10**).

Densitometry

A densitometric analysis was performed to quantify the observed differences in the CR-immunopositivity of neuropil between DCl, VCl, DEn, VEn and IEn. In all five subdivisions under study, the cROD was measured. The cROD values acquired in the DCl, VCl, DEn, VEn and IEn were then compared among each other. The results are summarized in **Table 1**. The highest cROD was observed in VEn (7.6 ± 1.0; dimensionless number ± standard error of mean, S.E.M.). The lowest density was measured in DCl (5.3 ± 0.4); however the difference between VEn and DCl did not reach statistical significance. To examine possible differences in the immunopositivity for CR along the rostrocaudal axis, we further compared the cROD between seven different rostrocaudal levels in examined areas. The results are summarized in **Table 2**. No significant differences in the cROD were found.

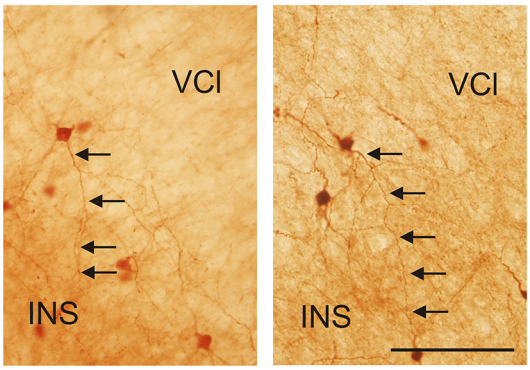

FIGURE 12 | Photomicrographs demonstrating CR-ir neurons located in the lateral marginal part of the VCl sending dendrites into 6th layer of the insular cortex (INS). Scale 100 μm.

Table 1 | Corrected optical density ± S.E.M.

DCl	VCl	DEn	IEn	VEn
5.3 ± 0.4	7.2 ± 0.5	5.4 ± 0.4	5.7 ± 1.0	7.6 ± 1.0

Table 1—demonstrates corrected optical density in the subdivisions of the claustrum (DCl, VCl) and in the subdivisions of the endopiriform nucleus (DEn, IEn and VEn).

DISCUSSION

This is the first study with detailed description of the distribution of CR-ir neurons in all subdivisions of the claustral complex in the rat.

To our knowledge, our findings are the first to demonstrate the existence of: (1) neurons in layer VI of the insular cortex sending processes (possibly dendrites) into the lateral marginal zone of the CLD, (2) CR-ir neurons in the CLD sending dendrites to the striatum; and (3) CR-ir neurons in the marginal area of the striatum sendings dendrites to the CLD.

Real et al. (2003) described moderate-to-dense immunoreactive neuropil throughout the DEn and VEn in the mouse, with a paucity of CR-ir neurons. The rat END consists of three subdivisions: the DEn, VEn and IEn. While the DEn and VEn shared pattern of labeling similar to the situation described in the mouse, the IEn displayed strongly positive medium-sized neurons, typically multipolar in shape.

If we compare CR-immunoreactivity in the claustrum of rodents such as the mouse and rat with other mammals, differences are clearly seen. There are fewer CR-ir neurons in the dorsal and ventral claustrum of the rabbit compared to the mouse (Real et al., 2003) and to the rat in the present study.

In the dorsal claustrum of the rabbit, CR-ir neurons were predominant in the medial half of the nucleus along with a moderate number of of thin positive fibers and puncta. Also, densely-stained fibers were noted along its medial border. The obvious subdivision of the dorsal claustrum into CR-positive and-negative sectors we report here in the rat is not described in the rabbit. It can also be said that CR-ir neurons in the ventral claustrum of the rabbit are extremely rare. CR-immunoreactivity in the claustrum of the cat (Rahman and Baizer, 2007) and monkey (Reynhout and Baizer, 1999) is comparable, with positive neurons homogenously distributed throughout the nucleus. While in monkey prevailed CR-ir neurons with bipolar morphology in the cat claustrum, in addition to elongated bipolar neurons also CR-positive neurons with large irregularly shaped somas were seen. Interestingly, the diffuse distribution of CR-positive neurons and fibers was also reported in the dorsal claustrum of the dolphin (Cozzi et al., 2014); the ventral claustrum was not described.

Structurally speaking, CR-ir neurons in the rabbit, cat and monkey claustrum are similar, with a preponderance of oval-shaped bipolar cells. In general, studies on rabbit and cat claustrum commonly report an abundance of multipolar neurons (Reynhout and Baizer, 1999; Wójcik et al., 2004; Rahman and Baizer, 2007). By way of comparison, the dolphin claustrum was reported to have small monopolar and bipolar neurons with round or fusiform cell bodies (Cozzi et al., 2014). With regard to functional considerations, the parcellation of the claustrum into several zones on the basis of reciprocal connectivity with sensory cortices suggests that it is a complex of modality-dependent units with upstream impact. This view is supported by the fact that majority of claustral cells are projection neurons, with but 7–12% comprised of GABAergic interneurons (Spahn and Braak, 1985). Based on corticoclaustral and claustrocortical projection patterns, studies indicate that CR-immunoreactivity in the claustrum of the rat and rabbit is not reflective of its functional organization (Sloniewski et al., 1986; Sadowski et al., 1997; Kowiański et al., 1998).

Immunohistochemical localization of the vesicular glutamate transporter (VGLUT2) in the mouse claustrum enables one to differentiate a VGLUT2-negative central core which corresponds

Table 2 | Corrected optical density ± S.E.M. at different rostrocaudal levels.

	A	B	C	D	E	F	G
DCl	4.7 ± 0.7	5.7 ± 0.7	4.8 ± 1.1	5.4 ± 1.1	6.2 ± 1.3		
VCl	6.6 ± 0.6	7.4 ± 1.0	5.8 ± 1.0	6.1 ± 1.1	7.4 ± 1.4		
Den	5.7 ± 0.8	5.9 ± 0.9	4.6 ± 1.1	4.7 ± 0.8	5.7 ± 1.2	5.5 ± 1.8	6.2 ± 1.2
IEn	4.4 ± 0.9	5.7 ± 0.8	6.7 ± 2.0				
VEn	7.0 ± 1.3	8.2 ± 1.8	7.5 ± 2.4				

Table 2—contains corrected optical density in sections at different rostrocaudal levels: section A (AP 2.1 mm), B (AP 1.2 mm), C (AP 0.2 mm), D (AP −0.8 mm), E (AP −1.8 mm), F (AP −2.8 mm) and G (AP −3.8 mm) relative to bregma (Paxinos and Watson, 2007).

to the VCl in the rat, and which is surrounded both dorsally and medially with a VGLUT2-positive plexus of fibers and puncta (Real et al., 2003). This VGLUT2-positive "shell" partly corresponds to the strongly CR-ir dorsal and medial marginal zones seen in the mouse and rat claustrum (Real et al., 2003; present results). Interestingly, the weakly CR-positive central core in the rat claustrum is strongly parvalbumin-positive and seen to contain a high density of parvalbumin-ir neurons, fibers and puncta (Druga et al., 1993). Additionally, projections from the ventroposterolateral parvicellular and ventroposteromedial parvicellular thalamic nuclei (which are considered to be relay nuclei for gustatory and visceral signals) terminate not only in the insular cortex but also in the dorsal and medial claustrum (Allen et al., 1991). This is seen as corresponding to the CR-positive zone in the DCl. Lastly, similar termination fields within the claustrum exhibited projections from the parabrachial nucleus (Allen et al., 1991).

Of particular note, our present data indicate that the dendrites of CR-ir neurons in the medial and lateral margins of the rat claustrum demonstrate the presence of direct reciprocal connectivity with neighboring structures, such as the striatum and insular cortex. This intriguing pattern of interconnectivity is consistent with the concept of a global processing and synchrony detection system, perhaps relative to the competitive actions of often disparate sensory afferents flowing into the claustrum (Smythies et al., 2012, 2014), and is in agreement with the proposal of some authors who suggested the importance of the claustrum for complex functions like perception, consciousness and cognitive processes (Crick and Koch, 2005).

CONCLUSIONS

The calcium-binding protein CR was clearly and abundantly in evidence throughout the claustrum of the rat, comprised of immunopositive neurons, fibers and puncta. Both segments of the claustrum—the dorsal claustrum as well as all subdivisions of the END—contained CR-ir neurons, albeit with varying population densities. The majority of these positive neurons displayed small- or medium-sized round, oval or elongated cell bodies with aspiny dendrites, and were unevenly distributed. The DCl and the marginal parts of the VCl were noted to have the higher density of CR-ir neurons. In contrast, the central core of the VCl contained a paucity of CR-ir neurons with weakly immunoreactive neuropil. With respect to the END and its three subdivisions, CR-ir neurons were most prevalent in the IEn.

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Revisiting enigmatic cortical calretinin-expressing interneurons

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Cortical calretinin (CR)-expressing interneurons represent a heterogeneous subpopulation of about 10–30% of GABAergic interneurons, which altogether total ca. 12–20% of all cortical neurons. In the rodent neocortex, CR cells display different somatodendritic morphologies ranging from bipolar to multipolar but the bipolar cells and their variations dominate. They are also diverse at the molecular level as they were shown to express numerous neuropeptides in different combinations including vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), neurokinin B (NKB) corticotrophin releasing factor (CRF), enkephalin (Enk) but also neuropeptide Y (NPY) and somatostatin (SOM) to a lesser extent. CR-expressing interneurons exhibit different firing behaviors such as adapting, bursting or irregular. They mainly originate from the caudal ganglionic eminence (CGE) but a subpopulation also derives from the dorsal part of the medial ganglionic eminence (MGE). Cortical GABAergic CR-expressing interneurons can be divided in two main populations: VIP-bipolar interneurons deriving from the CGE and SOM-Martinotti-like interneurons originating in the dorsal MGE. Although bipolar cells account for the majority of CR-expressing interneurons, the roles they play in cortical neuronal circuits and in the more general metabolic physiology of the brain remained elusive and enigmatic. The aim of this review is, firstly, to provide a comprehensive view of the morphological, molecular and electrophysiological features defining this cell type. We will, secondly, also summarize what is known about their place in the cortical circuit, their modulation by subcortical afferents and the functional roles they might play in neuronal processing and energy metabolism.

Keywords: neuropeptides, neocortex, neocortical circuits, embryonic and fetal development, neuroenergetics

INTRODUCTION: WHAT ARE THE CORTICAL CALRETININ-EXPRESSING INTERNEURONS?

Cortical calretinin (CR) expressing interneurons represent a heterogeneous subpopulation of about 10–30% of GABAergic interneurons (Kubota et al., 1994; Gonchar and Burkhalter, 1997; Tamamaki et al., 2003). They display different somatodendritic morphologies ranging from bipolar to multipolar (Jacobowitz and Winsky, 1991; Kubota et al., 1994; Gonchar and Burkhalter, 1997; Gonchar et al., 2008; Caputi et al., 2009). They are also diverse at the molecular level as they express numerous neuropeptides in different combinations including vasoactive intestinal polypeptide (VIP) (Kubota et al., 1994; Cauli et al., 1997), cholecystokinin (CCK) (Cauli et al., 1997; Gonchar et al., 2008), neurokinin B (NKB) (Kaneko et al., 1998; Gallopin et al., 2006) corticotrophin releasing factor (CRF) (Gallopin et al., 2006; Kubota et al., 2011), enkephalin (Enk) (Taki et al., 2000; Férézou et al., 2007), but also to a lesser extent and in a species-dependent manner neuropeptide Y (NPY) (Cauli et al., 1997; Wang et al., 2004; Gonchar et al., 2008) and somatostatin (SOM) (Cauli et al., 1997; Wang et al., 2004; Xu et al., 2010b). CR-expressing interneurons also exhibit different firing behaviors such as adapting, bursting or irregular (Kawaguchi and Kubota, 1996; Cauli et al., 1997, 2000; Porter et al., 1998; Wang et al., 2002; Karagiannis et al., 2009). They mainly originate from the caudal ganglionic eminence (CGE) (Xu et al., 2004; Butt et al., 2005) but a subpopulation also derives from the dorsal part of the medial ganglionic eminence (MGE) (Fogarty et al., 2007; Xu et al., 2008). Cortical GABAergic CR+ interneurons can be divided in two main populations: VIP-bipolar interneurons deriving from the CGE and SOM-Martinotti-like interneurons originating in the dorsal MGE. In the rodent neocortex, bipolar cells account for the majority of CR+ interneurons, therefore, we consider them as our main focus of the review. Due to the relatively few and scattered studies of CR+ interneurons, their inputs and outputs, the roles they play in cortical neuronal circuits as well as neuroenergetics of the brain remained elusive and enigmatic until quite recently. The aim of this review is, firstly, to provide a comprehensive view of the morphological, molecular and electrophysiological features defining cortical bipolar CR-expressing interneurons. We will, secondly, also review their places in the

cortical circuit, their modulations by subcortical afferents and the functional roles they might play in neuronal processing and energy metabolism. We are convinced that the synthesis of recent data on bipolar CR-expressing interneurons will show that they are much less enigmatic than they appeared roughly 25 years ago (Peters and Harriman, 1988).

EMBRYONIC ORIGINS

Unlike glutamatergic neurons which are born in the ventricular zone of the telencephalic vesicle and then migrate radially (Molyneaux et al., 2007; Rakic, 2007), most of the GABAergic interneurons derive from one proliferative region called ganglionic eminence (GE) located in the ventral part of the telencephalon (Wonders and Anderson, 2006; Batista-Brito and Fishell, 2009; Bartolini et al., 2013). Once born, interneuron precursors migrate first dorsally, guided by attracting and repulsive molecular cues, then tangentially toward neocortex and hippocampus along two main migratory streams (Chedotal and Rijli, 2009; Marin, 2013). They eventually acquire their final laminar location by penetrating the cortical plate. Anatomically, the GE is subdivided in three main regions, the medial, lateral, and caudal GE (MGE, LGE, and CGE, **Figure 1**). Cortical and hippocampal interneurons derive mainly from the MGE and CGE, while the LGE is the major contributor of GABAergic interneurons of striatum and basal forebrain structures. The preoptic area (**Figure 1**) has recently been described as another source of cortical interneurons (Gelman et al., 2009) but given that very few CR+ interneurons derive from this zone, we will focus on the MGE and CGE.

Lineage analyses using either grafts or genetic tools such as Cre driver lines that label specifically a subfield of the GE have shown that parvalbumin-expressing (PV+) and SOM+ interneurons are generated in the MGE at different time points (between E9 and E15, **Figure 1**; Xu et al., 2004; Butt et al., 2005; Miyoshi et al., 2007; Tricoire et al., 2011). In contrast, similar approaches revealed that a large portion of the remaining interneuron subtypes, including CR+ interneurons, derive from the CGE and are produced at later embryonic stages (between E12.5 and E16.5, **Figure 1**; Lee et al., 2010; Miyoshi et al., 2010; Tricoire et al., 2011). In addition to being generated by distinct progenitors, CR+ interneuron precursors use a different migratory pathway. While SOM+ interneurons take a more rostrolateral route, CGE-derived interneurons take a caudal path for their tangential migration (Kanatani et al., 2008). The late birth of CGE-derived interneurons has the consequence that they are still migrating at birth and reach their final destination later than MGE-derived interneurons (Miyoshi et al., 2010). However, several refinements have to be considered. SOM+ hippocampal neurons exhibit a dual origin with one expressing type 3 serotonin receptor (5-HT3 R) and the other not (Chittajallu et al., 2013). In the neocortex, such a dichotomy has not been reported yet.

Several transcription factors of CR+ (and SOM+) interneurons are necessary for the proper specification and migration from the GE. Mice lacking the Dlx1 gene show reduction of CR+ and SOM+ interneurons without affecting the PV+ population (Cobos et al., 2005). Removal of the transcription factor Nkx2.1 restricted at early embryonic stages to the MGE domain (**Figure 1**) results in a molecular and cellular switch of MGE-derived cortical interneurons (PV+ and SOM+ subpopulations) to CGE-derived neurons (VIP+ and CR+ cells; Butt et al., 2008).

In contrast with SOM+ interneurons, little is known about the regional and cell type specification of CGE in general and of CR+ bipolar interneurons in particular. Gsx2 (also called Gsh2) is enriched in (but not restricted to) the LGE and CGE from early development (**Figure 1**) and has been directly implicated in promoting the CR+ interneuron identity (Xu et al., 2010a). The orphan nuclear receptor COUP-TFII shows restricted expression in the CGE (Kanatani et al., 2008; Willi-Monnerat et al., 2008) and, together with COUP-TFI, is required for the caudal migration of cortical interneurons (Tripodi et al., 2004). Moreover, in Nkx2.1 mutant mice, a higher number of CR+ and VIP+ cortical interneurons are generated and COUP-TFII is ectopically expressed in the MGE (Butt et al., 2008). Conversely, conditional loss-of-function of COUP-TFII in subventricular precursors and postmitotic cells leads to a decrease in VIP+ and CR+ interneurons, compensated by the concurrent increase of MGE-derived PV+ interneurons. Interestingly, COUP-TFI mutants are more resistant to pharmacologically induced seizures (Lodato et al., 2011). In addition to these genetic factors, electrical activity has been also shown to regulate development of cortical neurons. CR+ but not VIP+ interneurons activity is required before postnatal day 3 for correct migration and Elmo1, a target of Dlx1 and expressed in CR+ neurons, is both necessary and sufficient for this activity-dependent interneuron migration (De Marco Garcia et al., 2011).

Over the past decade, many advances have been achieved in the identification of the genetic factors that influence the specification of cortical and hippocampal interneurons especially for MGE-derived interneurons. However, the program specifying the identity of CGE-derived interneurons still needs to be unraveled. For instance, the cues that regulate the final maturation of the morphological, synaptic and electrophysiological properties have to be determined. Indeed, although embryonic origin is a major contributing factor, immature interneurons arriving at their final destination are likely to encounter local factors such as guidance molecules and specific levels of network activity that will instruct them where to grow dendrites and axons.

ANATOMICAL PROPERTIES

LAMINAR, COLUMNAR, AND AREAL DISTRIBUTION PATTERN

The initial descriptions of the brain-wide distribution of CR+ neurons did not report obvious differences in the number or type of neurons across the different cortical areas (Jacobowitz and Winsky, 1991; Resibois and Rogers, 1992; Gabbott et al., 1997). They observed that within a certain cortical area always supragranular layers II/III show the highest density of CR+ neurons (**Figure 2**). A more fine-grained analysis, however, suggested that there is a subtle gradient from rostral to caudal, with a higher density of CR+ neurons in the visual cortex (Xu et al., 2010b), a finding similar to that of VIP+ neurons, one of the major subtypes of CR+ neurons (Morrison et al., 1984; Rogers, 1992; Gonchar et al., 2008). Interestingly, in the mouse barrel cortex where columnar modules are easily visualized, both, CR+ and VIP+ interneurons showed a preference for septal compartments

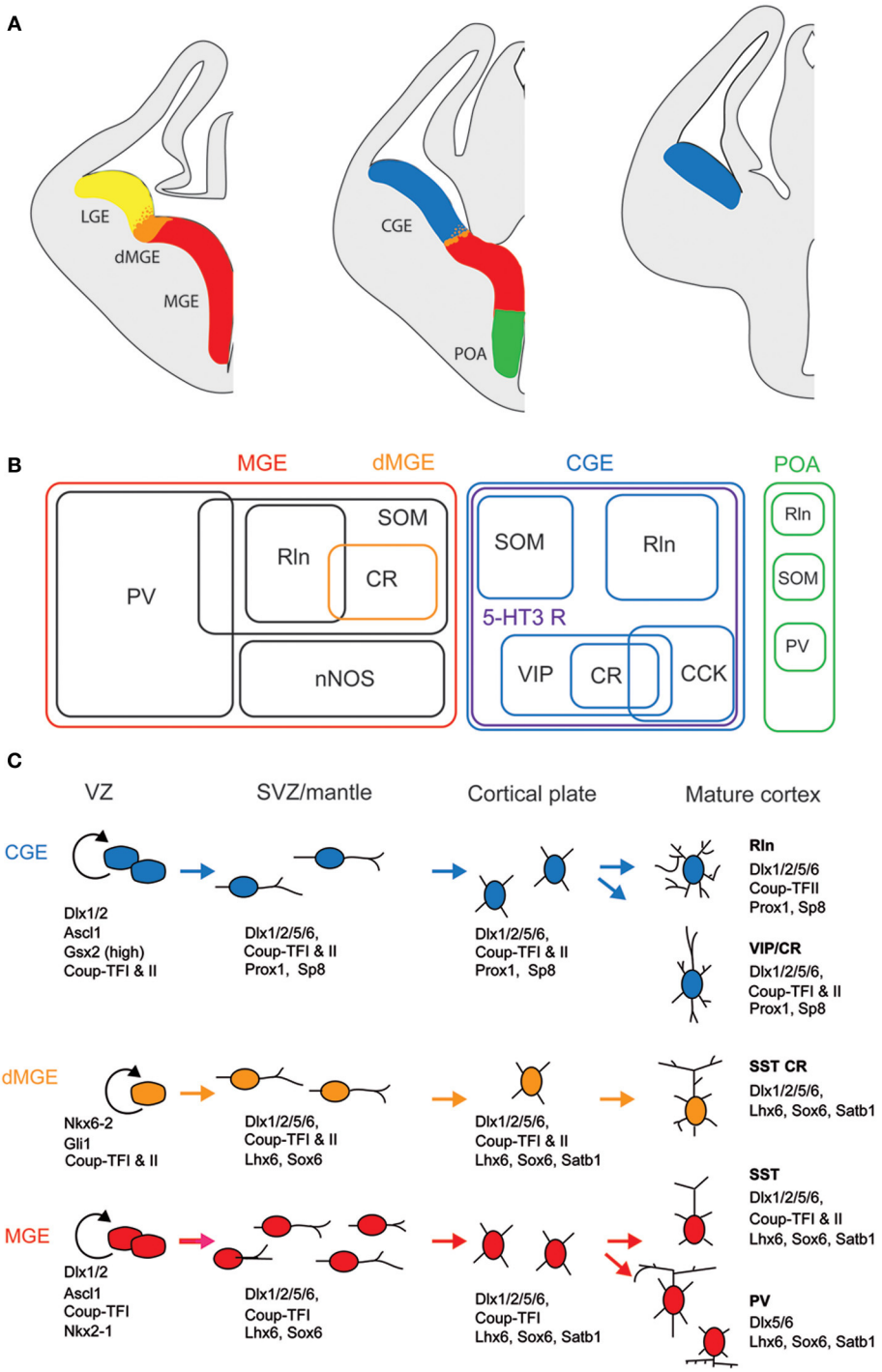

FIGURE 1 | Embryonic origins and genetic factors affecting the mature fate of cortical interneurons. (A) Diagram showing the subdivisions of the embryonic telencephalon. The three regions where cortical and hippocampal interneurons originate are the medial ganglionic eminence (MGE) (including the dorsal MGE-dMGE), the caudal ganglionic eminence (CGE), and the preoptic area (POA). The lateral ganglionic eminence (LGE) gives rise, amongst other, to basal forebrain neurons. **(B)** Ball scheme of the major classes of cortical and hippocampal interneurons identified using neurochemical markers and represented depending of their place of origin in the embryonic telencephalon. The MGE generates 50% of all cortical interneurons and includes mainly parvalbumin (PV)-expressing and somatostatin (SOM)-expressing subtypes. In hippocampus, it also includes a large population expressing the neuronal isoform of nitric oxide synthase (nNOS). CGE-derived reelin (Rln)-expressing interneurons represent the neurogliaform cells. In both cortex and hippocampus, almost all CGE-derived interneurons express the type 3 serotonin receptor (5-HT3 R). In contrast with cortex, hippocampal SOM+ interneuron have a dual origin with a significant subset co-expressing 5-HT3 R. VIP; vasoactive intestinal polypeptide, CR, calretinin; CCK, cholecystokinin. **(C)** Genetic programs controlling neurogenesis, cell commitment, tangential, and radial migration and maturation of cortical interneuron.The subdivision of the neuroepithelium

(Continued)

FIGURE 1 | Continued
can be identified by combinatorial expression of transcription factors involved at different stages of cortical interneuron development. Some of these factors participate broadly in interneuron development such as Dlx and CoupTF gene families. Some transcription factors are unique to specific domains and/or stages of differentiation: Nkx2-1 defines the MGE and activates a cascade of genes including Lhx6, Sox6, and Satb1; Nkx6-2 and GLI1 are enriched in the dMGE. Prox1 and SP8 are expressed in CGE-derived cortical interneurons at all stages of their development (adapted from Kessaris et al., 2014).

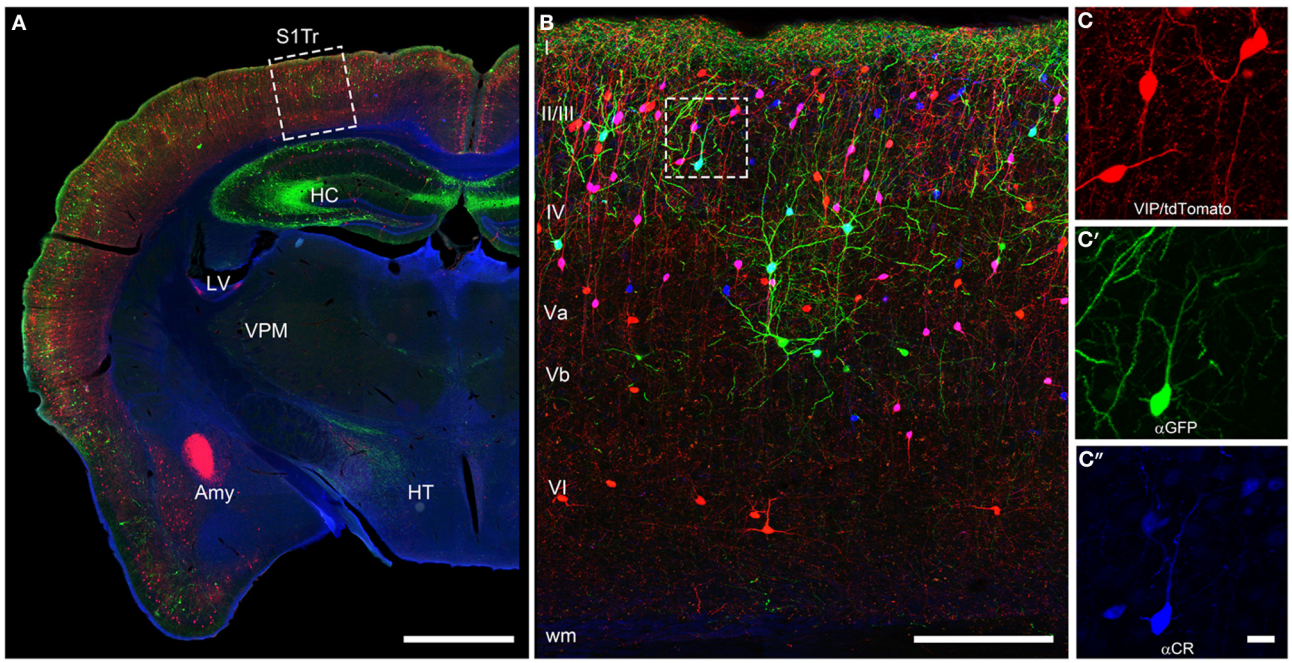

FIGURE 2 | Coronal brain section of a VIPcre/tdTomato/GIN mouse additionally stained for calretinin. Immunostaining of calretinin in a coronal brain section of a VIPcre/tdTomato/GIN mouse shows VIP neurons in red, Martinotti cells in green and calretinin in blue in the primary somatosensory cortex (S1Tr). **(A)** Low magnification of one hemisphere depicting the hippocampus (HC), the lateral ventricle (LV), the amygdala (Amy), the thalamic nucleus ventralis posteromedialis (VPM), and the hypothalamus (HT); the *dashed rectangle* marks the selected area of **(B)**; scale bar: 1000 μm. **(B)** Close-up of the rectangle in **(A)** in a maximum intensity projection; *Roman numerals* indicate cortical layers; *dashed rectangle* marks the selected area of **(C–C″)**; scale bar: 250 μm. Please note that VIP neurons co-localizing CR appear pink whereas Martinotti cells that co-localize CR show a cyan-colored soma. **(C)** Red channel of the inset in **(B)**, showing the tdTomato signal only; **(C′)** green channel of the inset in **(B)**, showing the GFP only; **(C″)** blue channel of the inset in **(B)**, showing the labeled calretinin antibody only; scale bar for **(C–C″)** 20 μm. Please not that nearly all VIP and the single Martinotti cell are co-localizing CR.

over barrel-associated columns (Zilles et al., 1993; Melvin and Dyck, 2003). Such a preference was not obvious for the rat barrel cortex (Bayraktar et al., 2000), although no tangential sections where used, which would have allowed a much better resolution of columnar compartments. In summary, CR+ neurons show a relatively uniform appearance across cortical areas in terms of distribution, numbers and cell types, suggesting that they perform a basic and comparable function in neuronal processing or control of energy supply.

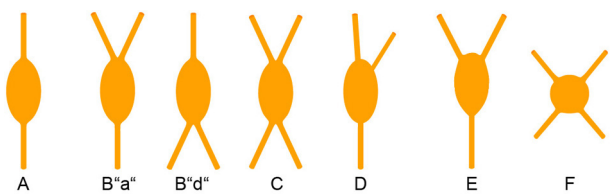

FIGURE 3 | Somatodendritic morphology of CR+ interneurons. For details see text.

CELLULAR MORPHOLOGY

Since the recognition of cortical interneurons as a distinct cell class different from principal (i.e., pyramidal) cells (Jones, 1975; Fairen et al., 1984; Ramón y Cajal, 1995), researchers have been struck by the abundance of morphological features that are already expressed at the somatodendritic level, let alone by the manifold axonal ramification patterns when they later became observable (DeFelipe et al., 2013). To agree upon a common nomenclature on the somatodendritic patterns of GABAergic interneurons, a minimal consensus paper was published (Ascoli et al., 2008). However, we feel that for the appreciation of the full diversity of observable somatodendritic configurations, this terminology has to be extended and refined in the future. In the following, we will elaborate on what we originally proposed for VIP+ neurons in the rat barrel cortex (Bayraktar et al., 2000).

This classification is strictly focused on the *origin* of the primary dendrites at the soma (**Figure 3**). Specific

examples in a histological preparation can be found in **Figure 2**.

A. **Bipolar:** Two dendrites originating from opposite sides of a spindle (to round)-shaped soma. It is not important in this classification how close or far from the soma these dendrites starts to branch into their terminal tufts.
B. **Single tufted:** A single dendrite on one side of the soma and *at least 2* dendrites originating individually from the opposite side. We suggest that acceptable origins from where a dendrite is allowed to emerge for the tufted category are ±20° from the upper or lower pole of the soma. (In case that there are dendritic origins outside these sectors, the neuron belongs to one of the following groups). This group can be further specified as "a" = tuft ascending and "d" = tuft descending.
C. **Bitufted:** Two dendritic tufts as defined above, originating from opposite sides of the soma.
D. **Modified:** Bipolar/single-tufted/bitufted: they possess a third dendrite originating anywhere else than the above defined circumference at the soma.
E. **Tripolar:** This is a difficult term since as such it can be a modified bipolar with a more or less extensive third dendrite (that should also be vertically oriented) or it can be the "smallest" form of a multipolar cell (which then can be oriented to any direction). Whenever this term is used, it should be specified to which of the more general categories of somatodendritic configuration (D or F) the cell is closer.
F. **Multipolar:** At least 4 (or 3, see above) dendrites originating from a round to polygonal soma.
G. **Others:** Any neuron that is so different and rare (i.e., horizontal) that it does not fit in the above categories.

With this in mind, we here shortly summarize that cortical CR+ neurons, on the basis of their somatodendritic properties, were classified by different researchers as bipolar (Jacobowitz and Winsky, 1991; Kawaguchi and Kubota, 1996), bipolar and multipolar (Resibois and Rogers, 1992), or bipolar, bitufted, multipolar and horizontal (Caputi et al., 2009; Barinka and Druga, 2010), respectively. Unfortunately, not too many axonal reconstructions of these cells are available but it is assumed that the bipolar or bitufted dendritic trees are mostly accompanied by a vertical translaminar axonal arbor whereas the multipolar dendritic trees go along with a horizontal transcolumnar ramification of the axon (Caputi et al., 2009).

NEUROCHEMICAL PROPERTIES: CO-EXPRESSION OF NEUROPEPTIDES AND CLASSICAL NEUROTRANSMITTERS

The degree of co-expression of CR with other markers can strongly vary between species. For instance in rat, a large majority (70–90%) of cortical CR+ cells exhibits VIP immunoreactivity (Rogers, 1992; Kubota et al., 1994) whereas this co-expression drops down to about 35% in mouse (Gonchar et al., 2008; Xu et al., 2010b). This difference probably comes from the presence of a SOM+ neuronal subpopulation accounting for 30–40% of CR+ cells in mouse (Halabisky et al., 2006; Xu et al., 2006; Gonchar et al., 2008), which is virtually absent in rat (Rogers, 1992; Kubota et al., 1994, 2011; Gonchar and Burkhalter, 1997) and human (Gonzalez-Albo et al., 2001). However, such a difference between species is not retrieved at the mRNA level since co-expression of CR and SOM transcripts has been observed using single cell RT-PCR both in mouse (Perrenoud et al., 2013) and rat in which the co-expression level is up to 40% of the CR+ cortical neurons (Cauli et al., 1997, 2000; Wang et al., 2004; Toledo-Rodriguez et al., 2005; Gallopin et al., 2006; Pohlkamp et al., 2013). The absence of co-immunodetection of CR and SOM in rat is probably due to a species-dependent post-transcriptional (Kwan et al., 2012) and/or a post-translational control (Herrero-Mendez et al., 2009). These observations indicate that CR-expressing bipolar cells, but not CR+/SOM+ (often multipolar) cells, are a common cell type in rats and mice.

In addition to VIP (Rogers, 1992; Kubota et al., 1994; Halabisky et al., 2006; Xu et al., 2006; Gonchar et al., 2008), CR+ bipolar interneurons co-express other neuropeptides (**Figure 1**). CRF and the preprotachykinin Neurokinin B (NKB), expressed in about one third of CR+ bipolar neurons, are rather common neuropeptides in superficial layers (Kaneko et al., 1998; Gallopin et al., 2006; Kubota et al., 2011). Enkephalin, an endogeneous opioid, is also expressed in a subpopulation of CR+/VIP+ bipolar interneurons (Taki et al., 2000; Férézou et al., 2007). By contrast, despite its abundance in the cerebral cortex (Beinfeld et al., 1981) and its frequent co-expression with VIP (Cauli et al., 1997, 2000; Kubota and Kawaguchi, 1997; Férézou et al., 2002; Kubota et al., 2011), CCK is only expressed in a minority of CR+ neurons (Cauli et al., 1997; Kubota and Kawaguchi, 1997; Gallopin et al., 2006; Gonchar et al., 2008; Karagiannis et al., 2009; Kubota et al., 2011; Pohlkamp et al., 2013). In addition to neuropeptides, up to 50% of VIP+ bipolar neurons co-express choline acetyl-transferase (ChAT) (Eckenstein and Baughman, 1984; Peters and Harriman, 1988; Chédotal et al., 1994a; Bayraktar et al., 1997; Cauli et al., 1997; Porter et al., 1998; Von Engelhardt et al., 2007; Gonchar et al., 2008; Consonni et al., 2009). Although co-expression of GABA and ChAT has been demonstrated in CR+/VIP+ bipolar interneurons (Kosaka et al., 1988; Bayraktar et al., 1997; Cauli et al., 1997; Porter et al., 1998) (**Figure 4**), the cholinergic nature of bipolar neurons is species-dependent. Indeed, the vesicular acetylcholine transporter, an essential component of the cholinergic system, is expressed in cortical interneurons from rats but not from mice or humans (Schafer et al., 1994, 1995; Gilmor et al., 1996; Weihe et al., 1996; Bhagwandin et al., 2006). Expression of nitric oxide synthase (NOS) is very marginal in bipolar CR+/VIP+ neurons and also appears to occur mainly in mouse (Lee and Jeon, 2005; Gonchar et al., 2008; Tricoire et al., 2010; Magno et al., 2012; Perrenoud et al., 2012a; Pohlkamp et al., 2013).

In summary, CR+/VIP+ bipolar neurons express a large repertoire of neurotransmitters and neuromodulators indicative of their neurochemical diversity. This suggests that they are likely to play multiple roles in cortical physiology.

ELECTROPHYSIOLOGICAL FEATURES

A remarkable passive electrophysiological feature (Ascoli et al., 2008) of CR+/VIP+ bipolar neurons is their relatively high input resistance (Kawaguchi and Kubota, 1996; Cauli et al., 1997,

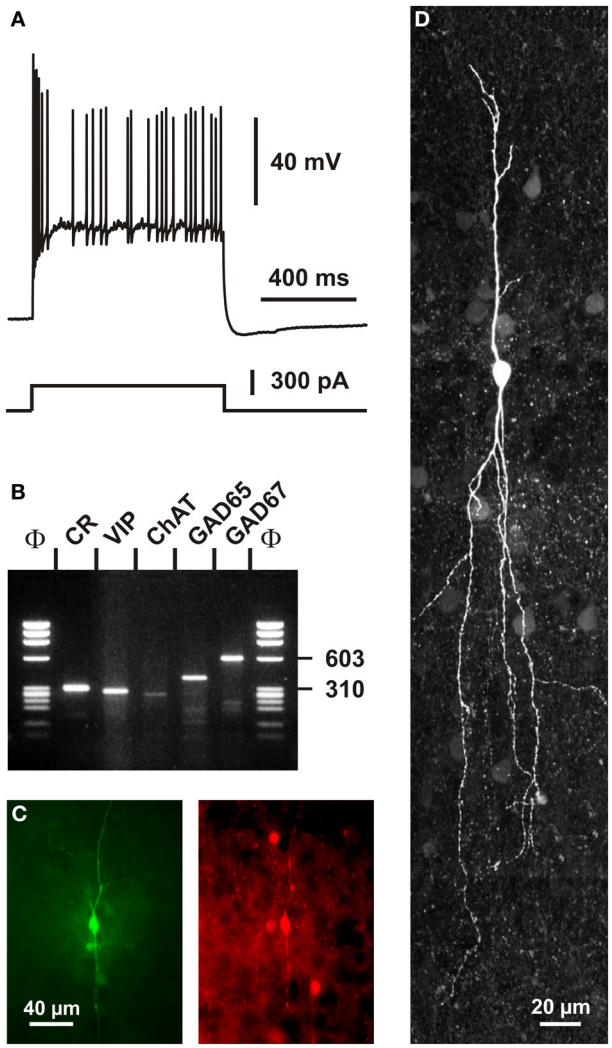

FIGURE 4 | Single cell RT-PCR analysis of a rat CR+ interneuron. (A) Current-clamp recording obtained in response to a depolarizing current pulse (300 pA). Note the initial burst followed by irregularly discharged action potentials. **(B)** Single-cell RT-PCR analysis of the same neuron revealing co-expression of CR, VIP, ChAT, GAD65, and GAD67. **(C)** Comparison of the biocytin labeling of the recorded neuron (left panel) with the immunostaining of the slice with an antibody against CR (right panel). Note the immunoreactivity of the biocytin-labeled cell (scale bar 40 μm, adapted from Cauli et al., 1997). **(D)** Intracellular biocytin labeling of another CR+ bipolar cell analyzed by singe cell RT-PCR. This neuron had a vertically oriented dendritic arborization. Pial surface is upward (scale bar, 20 μm).

2000; Gallopin et al., 2006; Karagiannis et al., 2009; Lee et al., 2010; Vucurovic et al., 2010), contrasting sharply with the low input resistance of fast spiking PV+ neurons (Kawaguchi and Kubota, 1993; Okaty et al., 2009; Battaglia et al., 2013). This property allows CR+/VIP+ neurons to be substantially depolarized by the small excitatory synaptic currents they receive from thalamic inputs (Lee et al., 2010). The presence of a prominent I_H current in SOM+ neurons underlying their distinctive voltage sag induced by hyperpolarization partially explains the slightly lower input resistance and the more depolarized resting membrane potential of SOM+ interneurons compared with those of CR+/VIP+ bipolar neurons (Wang et al., 2004; Halabisky et al., 2006; Ma et al., 2006; Xu et al., 2006; Karagiannis et al., 2009). Alike SOM+ interneurons, VIP+ cells have been reported to exhibit the ability to discharge low-threshold spikes driven by I_T calcium channels (Kawaguchi and Kubota, 1996; Cauli et al., 1997; Porter et al., 1999; Wang et al., 2004). VIP+ bipolar interneurons displayed action potentials of a duration intermediate to those of fast spiking interneurons and pyramidal cells (Kawaguchi and Kubota, 1996; Cauli et al., 1997, 2000; Karagiannis et al., 2009). A complex repolarization phase of their action potentials consisting of a fast after-hyperpolarization (AHP), followed by an after-depolarization and a medium AHP has been frequently observed in both VIP+ and SOM+ interneurons (Wang et al., 2004; Fanselow et al., 2008; Karagiannis et al., 2009), VIP+, but also SOM+ interneurons, characteristically displayed a pronounced frequency adaptation (Kawaguchi and Kubota, 1996; Cauli et al., 1997, 2000; Wang et al., 2004; Halabisky et al., 2006; Ma et al., 2006; Xu et al., 2006) which can result in an irregular firing pattern (**Figure 4**) (Cauli et al., 1997, 2000; Wang et al., 2004) when a slowly inactivating I_D potassium current is prominently present (Porter et al., 1998). Similarly to SOM+ interneurons, CR+ bipolar neurons exhibit backpropagating action potentials accompanied by an intracellular Ca^{2+} increase (Kaiser et al., 2001; Goldberg et al., 2003; Cho et al., 2010), which allows the release of neurotransmitters from dendrites as reported for SOM+ interneurons (Zilberter et al., 1999). Since the extent of action potential backpropagation is modulated by the presence of I_A potassium channels (Goldberg et al., 2003; Cho et al., 2010), this suggests that the dendritic release of CR+ bipolar interneurons (**Figures 5**, **6**) can be finely tuned.

In summary, CR+ bipolar interneurons largely display the electrophysiological features of adapting neurons (Ascoli et al., 2008). Their distinctive intrinsic electrophysiological properties indicate that they have specific integrative properties and therefore are likely to play specific roles in the physiology of cortical circuits, e.g., state-dependently remove the blanket of inhibition from principal neurons (Karnani et al., 2014).

SYNAPTIC INPUTS

CORTICAL

In general, our specific knowledge on connectivity is very restricted. Probably the most precise data for local synaptic inputs to CR+ neurons come from paired recordings in layer II/III of the primary somatosensory cortex in a CR-BAC-transgenic mouse (Caputi et al., 2009). These authors reported that the two types of neurons that they defined as the major cellular components of the CR+ neuronal population receive different types of inputs (and also form different types of output; see below). Bipolar VIP+/CR+ interneurons (BCR) receive functionally depressing inputs from local pyramidal cells with a probability of 18.3%, from fast-spiking interneurons (29.7%), from multipolar SOM+/CR+ interneurons (MCR; 41.1%) as well as facilitating inputs from other BCR (31.8%) (**Figure 5**). By contrast, MCR show facilitating inputs from pyramidal cells (17.4%) and from BCR (76.4%) as well as depressing inputs from fast-spiking cells

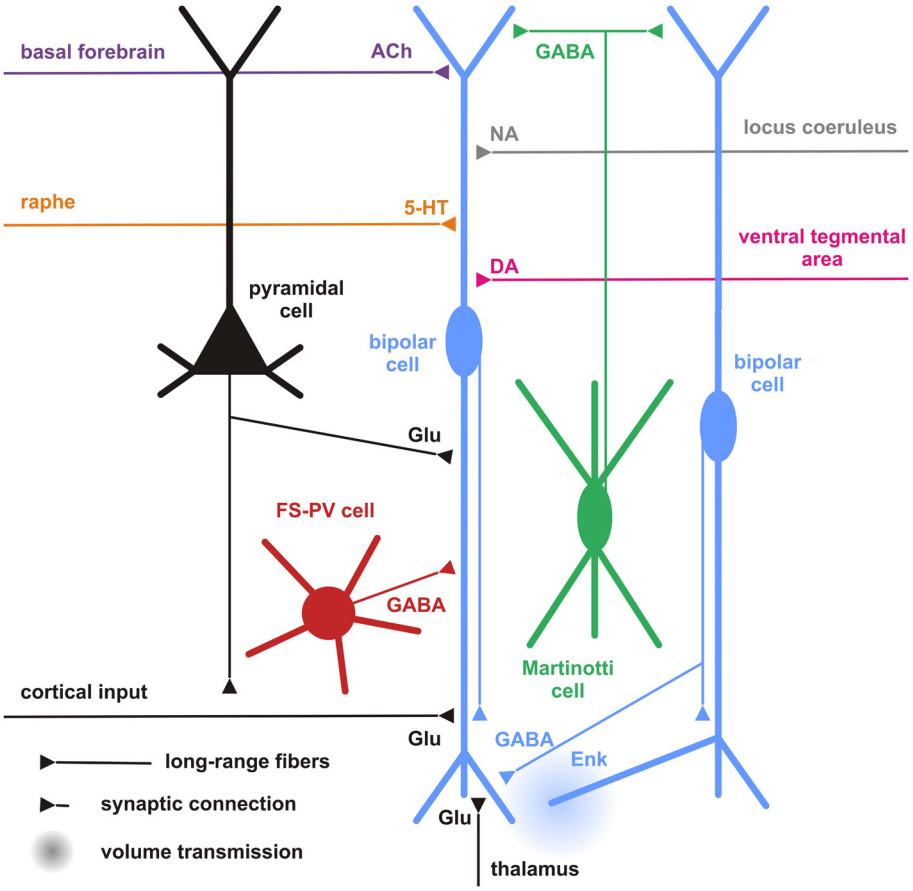

FIGURE 5 | Cortical and subcortical inputs of CR+ bipolar interneurons. Schematic representation summarizing the different local cortical neurons (colored cells) and the long-range cortical and subcortical axon terminals (colored fibers) targeting CR+ bipolar interneurons (centered blue bipolar cell). Dendrites and axons are depicted by thick and thin lines, respectively. Anatomical synaptic connections are represented by triangles and putative volume transmission by spherical gradients. The local neuronal types targeting bipolar CR+ and their respective neurotransmitters are schematized and color-coded; pyramidal cells (black, glutamate), fast spiking (FS)-PV neurons (red, GABA), Martinotti cells (green, GABA) other CR+ bipolar interneurons (blue, GABA and Enk [enkephalin]). Neurotransmitters of the long-range cortical and subcortical fibers are color-coded (Glu [glutamate; Black], 5-HT [serotonin, orange], ACh [acetylcholine, purple], NA [noradrenaline, gray], DA [dopamine, pink]) and their major origins indicated using the same color code.

(20%) and other MCR (9.8%). Interestingly, it appears that inputs to CR+/VIP+ (BCR) neurons are mainly depressing (Porter et al., 1998; Rozov et al., 2001), whereas all their outputs are facilitating and, vice versa, for CR+/SOM+ (MCR) neurons inputs are often facilitating whereas their outputs show a target cell type-specific variability (Caputi et al., 2009). These data do not agree well with paired recordings from rat barrel cortex, probably due to a mixture of species differences and less defined populations of bipolar vs. multipolar neurons in this study (Reyes et al., 1998).

Another rich source of observations describing the inputs to layer II/III CR+ neurons is provided by the glutamate uncaging studies in different transgenic mouse lines (Xu and Callaway, 2009). They distinguished CR+ and CR- Martinotti cells and (putatively CR+ and VIP+) bipolar cells. CR+ Martinotti cells (as all other types of inhibitory neurons in their study) received their strongest excitatory inputs within layer II/III whereas bipolar cells possessed a second strong origin of inputs located in layer IV.

Going beyond the local cortical circuits, Gonchar and Burkhalter also described long-range cortical inputs to CR+ neurons (**Figure 5**) (Gonchar and Burkhalter, 2003). Here, tracer injections to label connections between primary (V1) and secondary (V2) visual areas disclosed a circuit motif where CR+ neurons (specifically in layer I) are involved in feedback inhibition from V2 to V1, whereas few if any such long range connections are found on layer II/III CR interneurons. In this layer, PV+ interneurons were the main recipients for feedback projections, as they were for V1 to V2-directed feedforward projections.

CR+ bipolar interneurons may integrate these glutamatergic excitatory afferents by expressing ionotropic glutamate receptors of the AMPA subtype exhibiting a low Ca^{2+} permeability (Porter et al., 1998; Rozov et al., 2001) associated with high levels of the GluR2 subunit (Jonas et al., 1994). They, however, distinctly exhibit a low occurrence of GluR3 subunits and mainly express the flop variants of GluR subunits (Lambolez et al., 1996; Angulo et al., 1997; Porter et al., 1998; Cauli et al., 2000). GluR5

and GluR6 are the main kainate receptor subunits expressed in CR+ bipolar neurons (Porter et al., 1998; Cauli et al., 2000). Similarly to other cortical interneurons, CR+ bipolar interneurons express NR2A, B and D subunits of the NMDA receptor, the latter being found at very low levels in pyramidal cells (Flint et al., 1997; Porter et al., 1998; Cauli et al., 2000), indicating that bipolar interneurons can express very slowly inactivating NMDA receptors (Vicini et al., 1998). Similarly to SOM+ interneurons, the activity of CR+ bipolar and can be also modulated by the group I metabotropic glutamate receptors mGluR1 and mGluR5 (Baude et al., 1993; Kerner et al., 1997; Cauli et al., 2000; van Hooft et al., 2000; Ferraguti et al., 2004). The group III metabotropic receptor mGluR7 expressed by CR+/VIP+ bipolar neurons (Cauli et al., 2000) is presynaptically localized onto synapses targeting SOM+ interneurons which suggests a glutamatergic control of disinhibition (Dalezios et al., 2002). In summary, CR+/VIP+ bipolar neurons exhibit a cell-type-specific expression pattern of glutamate receptors allowing them to integrate glutamatergic inputs with distinct temporal and spatial features (**Figure 5**).

In terms of their inhibitory inputs, alike SOM+ cells, CR+ bipolar interneurons sampled from layers II/III, IV, and V, which was quite uncommon for most other cell types studied (Xu and Callaway, 2009). In their study, Gonchar and Burkhalter (1999) corroborated with morphological methods that rat CR+ neurons in layer II/III do innervate each other with numerous GABAergic (symmetric) synapses on the soma as well as proximal and distal dendrites. In addition, many symmetric and asymmetric synapses that were not further identified by their specific origin could be found on the CR+ neurons. This is very similar to what we have described for VIP+ interneurons in rat barrel cortex (Staiger et al., 2004).

Since the expression of GABA receptor subunits by specific cortical neuronal subtypes is still poorly documented (Ruano et al., 1997; Olah et al., 2009) and given the diversity of these subunits (Laurie et al., 1992; Pirker et al., 2000), the specific GABA-A (but also GABA-B) receptor subunits of CR+ bipolar interneurons remain largely undetermined.

Bipolar interneurons also possess receptors for intrinsic cortical neuromodulators including opioids and endocannabinoids. For instance, in the cerebral cortex μ-opoid receptors are mainly expressed by bipolar interneurons which also produce enkephalin, its endogenous agonist (Taki et al., 2000). This autocrine/paracrine μ-opoid transmission has been shown to restrict the inhibitory drive of CR+ bipolar interneurons onto pyramidal cells (Férézou et al., 2007) (**Figure 5**). Similarly, the endocannabinoid transmission has been shown to exert a long-lasting self-inhibition of putative SOM+ neurons (Bacci et al., 2004) by activation of CB1 receptors, which are broadly expressed by cortical neurons (Bodor et al., 2005; Hill et al., 2007). These two neuromodulatory systems, recruited during sustained firing, provide activity-dependent negative feedback to restrict the inhibitory actions of CR+ interneurons.

In summary, CR+ bipolar neurons possess a large integrative capability for excitatory as well as inhibitory inputs across different layers of a cortical column. A lot remains to be done to obtain a conclusive picture of the cortical input connectivity of CR+ bipolar neurons. However, already with the limited information available, it appears that these GABAergic interneurons do not only integrate excitatory and inhibitory inputs from local origin but that they also process long-range inputs from within the cortex and possibly subcortical sources (see next sections).

SUBCORTICAL INPUTS

Serotonergic

Due to the paucity of specific studies on subcortical targeting of cortical bipolar CR+ interneurons, inputs to the partially overlapping populations of VIP+ as well as 5-HT3 R+ interneurons might be considered here, too (Paspalas and Papadopoulos, 2001; Férézou et al., 2002; Cauli et al., 2004; Lee et al., 2010; Vucurovic et al., 2010; Rudy et al., 2011). The median raphe nucleus was found to be the subcortical region that gives rise to serotonergic input to CR+ interneurons in rat hippocampus (Acsady et al., 1993). By using a bridge protein combined with retrograde viral tracers, the paramedian raphe was also found to project to a subpopulation of interneurons, which express ErbB4 in mouse primary somatosensory cortex (Choi and Callaway, 2011). Selectivity of the TVB-NRG1 bridge protein restricted the transfection to the subgroups of interneurons immunopositive for VIP and/or CR as well as ErbB4, including also small numbers of PV+/ErbB4 neurons but not SOM+ cells (Choi et al., 2010; Xu et al., 2010b).

Although the expression of the ionotropic 5-HT3 R is restricted to a subset of cortical interneurons, including CR+ bipolar neurons and neurogliaform cells (Morales and Bloom, 1997; Férézou et al., 2002; Lee et al., 2010; Vucurovic et al., 2010), a subpopulation of hippocampal SOM+ O-LM cells originating from the CGE was recently found to be 5-HT3 R+ (Chittajallu et al., 2013). Cell-type-specific expression patterns of metabotropic 5-HT receptors are by far less documented. Nevertheless, 5-HT1a and 5-HT2a receptors are largely co-expressed in pyramidal cells and in a minority of interneurons positive for PV+ or CB+ (Aznar et al., 2003; Santana et al., 2004), suggesting that CR+ bipolar interneurons barely express these receptors. Similarly, 5HT-2c R is not prominently expressed in CR+ neurons of the rat medial prefrontal cortex (Liu et al., 2007). Given the reported segregation of 5-HT3 and 5-HT2a receptors in the monkey cerebral cortex (Jakab and Goldman-Rakic, 2000), it appears that 5-HT3 R is the major serotonin receptor expressed by CR+ bipolar interneurons. These observations indicate that serotonergic raphe neurons can rapidly activate CR+ bipolar interneurons as shown in the cerebral cortex (Férézou et al., 2002) (**Figure 5**).

Besides the paramedian raphe, additional origins of input were detected (with a decreasing probability) in the thalamus, secondary somatosensory, ipsilateral motor, retrosplenial, and contralateral primary somatosensory cortex as well as the nucleus basalis of Meynert (Choi et al., 2010; Xu et al., 2010b). Amongst the detected brain regions, the thalamus and basal nucleus of Meynert have been mostly investigated.

Thalamic

By anterograde tracing with PHA-L, neurons in the thalamus (mainly ventral posterior nucleus and lateral geniculate nucleus)

were approved to form synaptic terminals onto the cortical VIP+ population of inhibitory interneurons (Staiger et al., 1996; Hajos et al., 1997). These anatomical observations were corroborated by functional evidence showing that 5-HT3 R+ interneurons receive monosynaptic thalamocortical inputs (Lee et al., 2010). By contrast, putative SOM+ interneurons were found to barely receive direct thalamic inputs (Beierlein et al., 2000, 2003).

Cholinergic

The projections from the basal nucleus of Meynert, where most of the cholinergic neurons projecting to the neocortex are located, are probably correlated to the nicotinic responsiveness of VIP+/5-HT3 R+ bipolar interneurons (Porter et al., 1999; Férézou et al., 2002, 2007; Lee et al., 2010; Arroyo et al., 2012; Fu et al., 2014). CR+/VIP+ bipolar neurons express functional high-affinity nicotinic receptors (Porter et al., 1999; Férézou et al., 2002, 2007; Gulledge et al., 2006; Lee et al., 2010; Arroyo et al., 2012; Fu et al., 2014) composed of α4β2 subunits and to a lesser extent α5 subunit, a composition which is developmentally regulated (Winzer-Serhan and Leslie, 2005). Alike SOM+ interneurons, VIP+ bipolar neurons are depolarized by muscarinic agonists (Kawaguchi, 1997; Fanselow et al., 2008). Expression of m2 receptors has been reported in a minority CR+ interneurons of the rat entorhinal cortex (Chaudhuri et al., 2005) but the subtype-specific identity of the muscarinic receptors inducing the depolarization of VIP+ bipolar interneurons remains unknown. This indicates that CR+ interneurons integrate cholinergic afferences from the basal forebrain (**Figure 5**) as recently shown for ChAT+ bipolar interneurons using optogenetics (Arroyo et al., 2012), which might be important for behavioral state-dependent control of cortical circuits (Pi et al., 2013).

Noradrenergic

About 20% of VIP+ interneurons are contacted by noradrenergic fibers (**Figure 5**), however, often with only a single symmetric synapse (Paspalas and Papadopoulos, 1998, 1999; Toussay et al., 2013). Despite the described expression of α- and β-adrenoreceptors in the cerebral cortex (Nicholas et al., 1993a,b; Pieribone et al., 1994; Scheinin et al., 1994; Venkatesan et al., 1996) and reported excitatory effects of α-adrenoreceptors in hippocampal and cortical interneurons (Bergles et al., 1996; Marek and Aghajanian, 1996; Kawaguchi and Shindou, 1998), the expression of adrenoreceptors in CR+ interneurons has not been specifically investigated. However, inhibitory effects of α-adrenoreceptors have been observed in discrete subpopulations of CCK+ and SOM+ interneurons (Kawaguchi and Shindou, 1998), whether or not this inhibitory effect of α-adrenoreceptors is existent in CR+ bipolar interneurons remains unknown. β1- and β2-adrenoreceptors are not as frequently expressed in CR+ interneurons as compared to other types of interneurons (Liu et al., 2014).

Dopaminergic

Dopaminergic terminals have been shown to target interneurons (Sesack et al., 1995b), specifically CR+ interneurons in monkey prefrontal cortex (Sesack et al., 1995a) (**Figure 5**). However, there is no clear segregation in the expression profiles of dopamine receptors, which are frequently observed in pyramidal cells and interneurons of rodent and primate cortex with substantial co-expression (Vincent et al., 1993; Khan et al., 1998, 2000; Ciliax et al., 2000; Wedzony et al., 2000; Rivera et al., 2008; Oda et al., 2010). Nevertheless, regarding the expression of D1-like receptors, D1 receptors predominate in PV+ interneurons of the primate prefrontal cortex whereas it is D5 in CR+ interneurons (Glausier et al., 2009).

At the moment, all that can be stated with certainty is that a lot of work is still needed to get a better idea how cortical CR+ bipolar interneurons are influenced by subcortical sensory or modulatory inputs (**Figure 5**), which receptors are involved and how this modulation specifically affects sensory information processing or more general brain-state activity.

TARGETS AND FUNCTIONS

CORTICAL CIRCUIT

Neuronal targets

Hippocampal CR+ interneurons are considered to be interneuron-specific interneurons (Klausberger and Somogyi, 2008), which preferentially or even exclusively target other interneurons, presumably on their dendritic shafts. In rat hippocampus, post-embedding immunogold studies showed that most of the targets of CR+ and VIP+ boutons (which might be derived from the same bipolar neurons co-localizing CR and VIP) were GABAergic dendrites (Acsady et al., 1996a; Gulyas et al., 1996). In the neocortex, the GABAergic dendrite targeting property of CR+ or VIP+ interneurons is more complex when taking cortical areas and layers into account. In monkey and rat visual cortex, differential modes of innervation by CR+ interneurons exist in different layers. In layers I-III, CR+ interneurons tended to synapse on dendritic shafts of other not further specified GABAergic interneurons. However, in layers IV-VI, GABA-negative spines belonging to pyramidal neurons were primarily innervated (Meskenaite, 1997; Gonchar and Burkhalter, 1999).

Paired recordings in layer II/III of the primary somatosensory cortex of CR-BAC-transgenic mice (Caputi et al., 2009) gave an idea of the functional output of these CR neurons. Bipolar CR+ (BCR) innervate pyramidal cells (11.6%), fast-spiking interneurons (29.7%), other BCR (31.8%) and as already mentioned, preferably MCR (76.4%). It is remarkable that the input with the highest probability in this study represents indirect evidence for the recently disclosed disinhibitory circuit from VIP neurons to Martinotti cells (**Figure 6**), for which several direct (Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013; Fu et al., 2014) and indirect (Staiger et al., 2004; Gentet et al., 2012) lines of evidence are now available.

It has been shown in both, hippocampus and somatosensory cortex of rodents, that calbindin-expressing (CB+) interneurons are the targets of CR+ or VIP+ interneurons (Acsady et al., 1996b; Gulyas et al., 1996; Staiger et al., 2004). CB+ interneurons form a heterogeneous population and can coexpress other neuropeptides or other calcium binding proteins, thus, it is reasonable to further differentiate CB+ interneurons in order to distinguish the CR+ or VIP+ outputs to specific sets of interneurons. The most likely contacts are made with

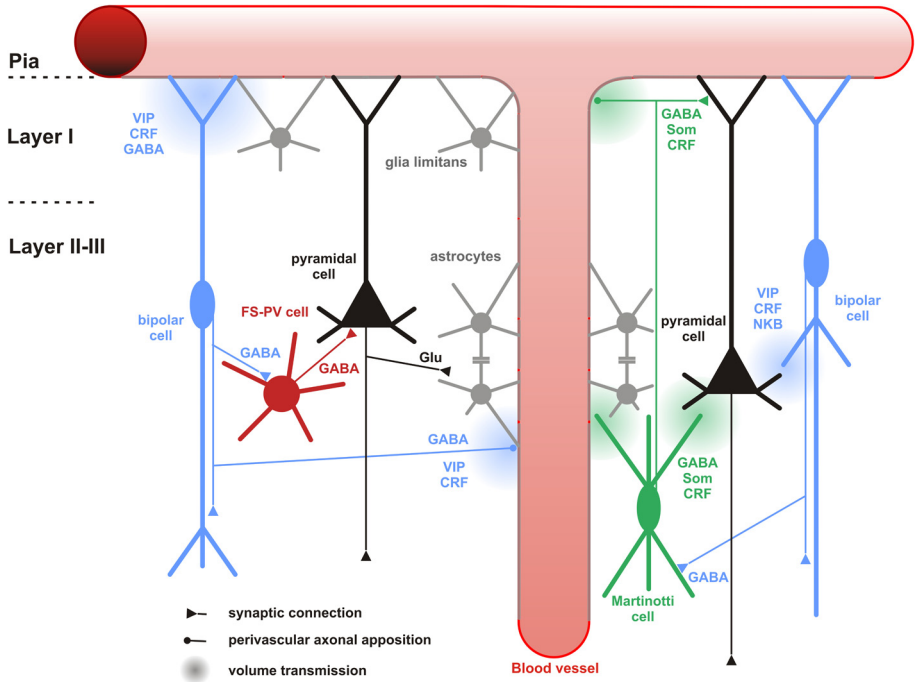

FIGURE 6 | Neuronal and non-neuronal targets of CR+ bipolar cells. Schematic representation summarizing the different neuronal and non-neuronal targets of CR+ bipolar interneurons (blue neurons) and Martinotti cells (green neuron). Dendrites and axons are depicted by thick and thin lines, respectively. Anatomical synaptic connections are represented by triangles, axonal perivascular appositions by dots and putative volume transmission by spherical gradients. The cell types targeted by CR+ neurons are schematized and color-coded; FS-PV neurons (red), Martinotti cells (green), pyramidal cells (black), glial cells (gray). Astro-glial coverage of blood vessels is illustrated by a gray-colored abluminal side. The specific action of the multiple neurotransmitters released by CR+ neurons is depicted by their location near the specific target and their origin color-coded (bipolar cell, blue; Martinotti cells, green). VIP, vasoactive intestinal polypeptide; CRF, corticotrophin releasing factor; NKB, neurokinin B; Som, somatostatin. The disinhibitory action of bipolar cells is represented by disynaptic circuits involving a bipolar interneuron, an intermediary interneuron (left: FS-PV cell; right: Som-Martinotti cell) and a pyramidal cell, respectively.

Martinotti cells which coexpress the markers CB and SOM (Kawaguchi and Kubota, 1996; Wang et al., 2004). Supporting this possible connection, recent functional studies described that in mouse somatosensory, visual, auditory, and prefrontal cortex, SOM+ interneurons were preferentially innervated by local VIP+ interneurons (Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013). Because of the diversity of VIP+ and SOM+ neuronal subgroups, it would be important to know what exact types in terms of morphology contribute to this microcircuit. Some indirect evidence already exists. Bipolar CR+ interneurons (BCRs) were immunopositive for VIP while multipolar CR+ interneurons (MCRs) were not (Caputi et al., 2009) and subgroups of SOM+ interneurons coexpressing CR with up to 95% or more than 50% were found in mouse visual and somatosensory cortex layers I–III, respectively (Xu et al., 2006). Therefore, it is hypothesized that BCRs, which are presumably CR+/VIP+ bipolar interneurons, target MCRs, which are probably CR+/SOM+ Martinotti interneurons.

Beside Martinotti interneurons, subgroups of basket cells such as large basket cells and nest basket cells could be immunopositive for CB, however, it is much more likely that these co-localize PV and not SOM (Wang et al., 2002). Two anatomical studies showed that VIP+ interneurons innervated PV+ interneurons, although it is debated whether there exists a preferential targeting in terms of putative PV+ subgroups (Dávid et al., 2007; Hioki et al., 2013). Behavioral experiments on mouse auditory and prefrontal cortex found that next to SOM+ interneurons, PV+ interneurons were the second major outputs of VIP+ interneurons (Pi et al., 2013) (**Figure 6**), lending further support to a functional role of the above mentioned anatomical connections. In summary, the CR+ bipolar interneurons seem to preferentially but not exclusively target other GABAergic interneurons, the molecular and anatomical properties of which need to be better analyzed.

Neuronal processing

The input-output connectivity pattern described above (Cortical and Neuronal targets) implies that bipolar CR+ interneurons do participate in disinhibitory but also feedforward and feed-back inhibitory circuits (Isaacson and Scanziani, 2011) of any cortical layer, column and area, especially when considering their high degree of colocalization with VIP (Porter et al., 1998; Reyes et al., 1998; Gonchar and Burkhalter, 2003; Caputi et al., 2009; Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013). Whether the content of CR in these circuits is meaningful on its own, like it has recently been suggested for another calcium-binding protein (i.e., parvalbumin in the hippocampus; Donato et al., 2013), is currently unknown (but see Schwaller, 2014 for a recent review). The best understood function is now the disinhibitory action

that is dependent on a unique circuit motif, i.e., targeting of other inhibitory interneurons, mostly SOM- (or CB-)expressing Martinotti cells. In the somatosensory system of mice, Lee et al. (2013) found that a specific projection from the whisker motor cortex excites VIP+ neurons and subsequently inhibits SOM+ neurons in the primary somatosensory cortex. This leads to a disinhibition of the apical dendrites of the pyramidal cells during exploratory behavior (**Figure 6**), probably acting as a "gate-opener" for the paralemniscal pathway (Gentet et al., 2012). A similar gate-opening mechanism might be functional in the primary auditory cortex, when animals learn to associate a tone stimulus with an aversive context (Pi et al., 2013).

Peptidergic neuromodulation

The expression of numerous neuropeptides in CR+ bipolar interneurons (Kubota et al., 1994, 2011; Kaneko et al., 1998; Taki et al., 2000), whose release requires a high level of neuronal activity (Zupanc, 1996; Baraban and Tallent, 2004), could also allow an activity-dependent fine tuning of the cortical network. For instance in the cat visual cortex, exogenously applied VIP had little or no effect on recorded neurons in the absence of visual stimulation, but enhanced their visual responses (Murphy et al., 1993; Fu et al., 2014). Consistently, by activating VPAC1 receptors and cAMP/PKA signaling, VIP reduced the slow AHP current and the tonic potassium current which regulates the excitability of hippocampal and cortical pyramidal neurons (Haug and Storm, 2000; Hu et al., 2011). Interestingly, CRF whose CRF1 receptors are also expressed by pyramidal neurons (Gallopin et al., 2006), induced an even more pronounced increase in cAMP/PKA signaling and modulation of potassium currents than VIP (Haug and Storm, 2000; Hu et al., 2011). Presumably because of the rapid desensitization of CRF1 receptors (Hauger et al., 2000), the action of CRF rapidly declined.

CR+ bipolar interneurons are also likely to enhance glutamatergic activity via NKB signaling (**Figure 6**). Indeed, NK-3 receptor, the most selective receptor for NKB (Shigemoto et al., 1990), is expressed by layer V pyramidal cells (Ding et al., 1996; Shughrue et al., 1996; Gallopin et al., 2006) and its activation depolarizes them (Stacey et al., 2002; Rekling, 2004; Gallopin et al., 2006).

In summary, to be operational, this complex peptidergic neuromodulation of the cortical circuit (**Figure 6**) requires a selective enhancement of CR+ bipolar neurons activity to achieve a substantial release of neuropeptides (Zupanc, 1996; Baraban and Tallent, 2004). This prerequisite could be met during specific brain states such as locomotion (Fu et al., 2014), which associates an increased activity of basal forebrain cholinergic neurons (Lee et al., 2005) and serotonergic neurons of the raphe (Wu et al., 2004) leading to the concomitant activation of excitatory receptors on CR+ bipolar neurons (see Subcortical inputs).

GLIO-VASCULAR NETWORK

Non-neuronal targets

Only very few studies have investigated the association of CR+ interneurons with non-neuronal elements (Consonni et al., 2009). However, perivascular appositions of intrinsic cortical VIP+ and ChAT+ neurons, two markers frequently coexpressed in CR+ bipolar interneurons (**Figure 4**), are well documented in the rat cerebral cortex (Eckenstein and Baughman, 1984; Galea et al., 1991; Chédotal et al., 1994a,b; Paspalas and Papadopoulos, 1998; Fahrenkrug et al., 2000; Cauli et al., 2004). Different vascular compartments including pial and diving arterioles as well as capillaries are targeted by these terminals (**Figure 6**), which can be of conventional axonal or more unconventional dendritic origins. The precise examination of these perivascular appositions at the ultrastructural level revealed that VIP+ and ChAT+ varicosities are enriched in the vicinity of the vascular wall but systematically being separated from it by a thin astrocytic leaflet (**Figure 6**)(Chédotal et al., 1994a,b; Paspalas and Papadopoulos, 1998). These anatomical observations suggest that CR+ bipolar interneurons through glial and vascular interactions are likely to play a role in the control of cortical energy supply (see below).

Neurovascular coupling

VIP is a potent vasodilatory neuropeptide of pial and diving cortical arterioles (Wei et al., 1980; Itakura et al., 1987; Yaksh et al., 1987; Cauli et al., 2004) and the expression of VPAC1 receptors has been described in blood vessels (Martin et al., 1992; Fahrenkrug et al., 2000; Cauli et al., 2004). Furthermore, acetylcholine, but also GABA, induce vasodilatation of cerebral arterioles (Lee et al., 1984; Fergus and Lee, 1997). Numerous studies have reported an intimate association between VIP+/ChAT+ terminals of bipolar interneurons with cortical blood vessels (Eckenstein and Baughman, 1984; Galea et al., 1991; Chédotal et al., 1994a,b; Paspalas and Papadopoulos, 1998; Fahrenkrug et al., 2000; Cauli et al., 2004) (see Non-neuronal targets). Based on these observations, as early as in the 1980s and until recently, VIP+ bipolar neurons have been proposed to be involved in the control of regional cerebral blood flow (Eckenstein and Baughman, 1984; Magistretti, 1990; Buzsaki et al., 2007; Cauli and Hamel, 2010). Although, single cell stimulation of VIP+ bipolar neurons was sufficient to elicit vasodilatation of nearby diving arterioles, the messengers recruited in these vascular responses were undetermined (Cauli et al., 2004). Indeed, the contribution of endogenous VIP to the control of regional cerebral blood flow has been largely restricted by the lack of selective VIP receptors antagonists (Yaksh et al., 1987). The role of VIP in neurovascular coupling was recently questioned in studies showing that vascular responses induced by sensory or pharmacological stimuli efficiently activating VIP+/ChAT+ bipolar neurons were insensitive to VIP receptors blockade (Lecrux et al., 2011; Perrenoud et al., 2012b). It also is unlikely that acetylcholine released by VIP+/ChAT+ bipolar interneurons significantly contributes to the neurovascular coupling induced by sensory stimulation as these responses were insensitive to the blockade of muscarinic receptors (Lecrux et al., 2011) known to be involved in the vasodilatations induced by acetylcholine (Elhusseiny and Hamel, 2000; Kocharyan et al., 2008). GABA is another possible vasoactive messenger produced by bipolar interneurons since the activation of GABA-A receptors can indeed elicit vasodilation (Fergus and Lee, 1997) and their blockade impairs the neurovascular response to sensory stimulation (Lecrux et al., 2011). However, it is difficult to discriminate between a pure vascular effect of GABA from an alteration of the cortical network activity (**Figure 6**).

CRF is another putative candidate peptide for neurovascular regulation through its release from CR+ bipolar cells (Cauli and Hamel, 2010) (**Figure 6**). It is a vasodilator of blood vessels (De Michele et al., 2005) and its CRF1 receptors are expressed in blood vessel (Chalmers et al., 1995). To our knowledge, the contribution of CRF in neurovascular coupling has not been investigated and it may represent one of the yet undetermined vasodilatory messengers in the neurovascular response (Leithner et al., 2010; Liu et al., 2012). Interestingly, CRF is also expressed in a subpopulation of SOM+ interneurons (Gallopin et al., 2006; Kubota et al., 2011) but both SOM (Long et al., 1992) and the direct stimulation of SOM+ interneurons were shown to induce vasoconstrictions (Cauli et al., 2004). Given that SOM+ neurons are a major neuronal target of CR+ bipolar interneurons (see chapter 6.1.1), it is likely that CR+/VIP+ bipolar interneurons and CR+/SOM+ interneurons (**Figure 6**), via vascular and/or synaptic interactions, play opposite roles in the control of regional cerebral blood flow (Kleinfeld et al., 2011).

Neurometabolic coupling and gliotransmision

The axonal and dendritic terminals of VIP+/ChAT+ bipolar neurons, enriched in the vicinity of blood vessels, are physically separated from the vascular wall by a thin astrocytic leaflet (**Figure 6**), as already mentioned above (Chédotal et al., 1994a,b). Since astrocytes express VIP receptors (Martin et al., 1992), this suggests that bipolar interneurons may also activate astrocytic functions.

It is well established that astrocytes play a key role in cerebral metabolism, notably by storing blood glucose into glycogen (Cataldo and Broadwell, 1986; Magistretti, 1990; Allaman et al., 2011) but also by stimulating glycolysis and lactate release during neuronal activity (Pellerin and Magistretti, 1994; Ruminot et al., 2011; Choi et al., 2012), with lactate being a major oxidative energy substrate over glucose for neurons (Bouzier-Sore et al., 2003). Besides its role in energy metabolism astrocyte glucose is also the precursor of several gliotransmitters such as D-serine (Ehmsen et al., 2013) and possibly lactate (Barros, 2013) which play a key role in synaptic plasticity and memory formation (Panatier et al., 2006; Suzuki et al., 2011).

Interestingly, VIP, but not GABA, cholinergic agonists, somatostatin, CRF, or enkephalins, were shown to stimulate glycogenolysis in cortical slices (Magistretti et al., 1981, 1984; Magistretti, 1990). This indicates that VIP+/ChAT+ bipolar interneurons can specifically enhance the recruitment of astrocytic glycogen stores (**Figure 6**) via VIP receptors (Martin et al., 1992) and cAMP/PKA signaling (Magistretti and Schorderet, 1984). In addition to this short term effect occurring within minutes, VIP also transcriptionally promotes glycogen re-synthesis within hours (Sorg and Magistretti, 1992). By tightly regulating glycogen content, and therefore astrocytes, glucose metabolism VIP+ bipolar neurons might play essential roles in the regulation of energy metabolism but also memory (Buzsaki et al., 2007; Pi et al., 2013).

CONCLUSIONS

More than 25 years ago, bipolar cells were considered to be enigmatic due to their rare occurrence, unusual synaptology, and unknown functions (Peters and Harriman, 1988). A big step forward in specifically studying the properties and functions of cortical inhibitory interneurons was the generation of specific mouse lines, first BAC-transgenics (Caputi et al., 2009), later Cre-driver lines (Taniguchi et al., 2011). The latter can be used now together with other exciting new methods, to precisely determine their inputs (via cre-dependent Rabies virus tracing; Choi et al., 2010; Fu et al., 2014) or outputs (via Cre-dependent channelrhodopsin-expressing vectors; Yizhar et al., 2011), both, *in vivo* and *in vitro*.

Eagerly awaiting these studies that will provide direct evidence for the functions of CR+ bipolar interneurons, summarizing the available -mostly indirect evidence coming from studies on VIP Cre interneurons that are likely to co-express CR- one can state that probably the bipolar neurons are amongst the most complex cells in the cerebral cortex because they seem to be involved in every major mechanism that has to be active to support successful neuronal computation. At a supportive level, they play a core role in controlling blood flow and energy metabolism, using a multitude of effector molecules and associated receptors. At a neuronal level, it seems that they are able to integrate local and distant cortical excitatory inputs, together with subcortical sensory and modulatory inputs from all major neurochemical systems that regulate functions like arousal, attention, reward, and sleep-wake cycles, to name but a few. All these inputs are integrated and conveyed as a processed output to the still badly characterized target neurons. However, assuming that CR+ interneurons share most of their properties with VIP+ interneurons, the now well-established "disinhibitory circuit motif" might be the core function of this neuron class. This function was so tightly connected to several behavioral correlates that the notion came up that inhibitory interneurons are not only "modulators" of the information processing by pyramidal cell circuits but do compute information themselves (Hangya et al., 2014; Karnani et al., 2014; Munoz and Rudy, 2014).

With all this new information in mind, we think it is safe to conclude that cortical inhibitory bipolar interneurons are not that enigmatic anymore as they appeared roughly 25 years ago.

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The vulnerability of calretinin-containing hippocampal interneurons to temporal lobe epilepsy

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This review focuses on the vulnerability of a special interneuron type—the calretinin (CR)-containing interneurons—in temporal lobe epilepsy (TLE). CR is a calcium-binding protein expressed mainly by GABAergic interneurons in the hippocampus. Despite their morphological heterogeneity, CR-containing interneurons form a distinct subpopulation of inhibitory cells, innervating other interneurons in rodents and to some extent principal cells in the human. Their dendrites are strongly connected by zona adherentiae and presumably by gap junctions both in rats and humans. CR-containing interneurons are suggested to play a key role in the hippocampal inhibitory network, since they can effectively synchronize dendritic inhibitory interneurons. The sensitivity of CR-expressing interneurons to epilepsy was discussed in several reports, both in animal models and in humans. In the sclerotic hippocampus the density of CR-immunopositive cells is decreased significantly. In the non-sclerotic hippocampus, the CR-containing interneurons are preserved, but their dendritic tree is varicose, segmented, and zona-adherentia-type contacts can be less frequently observed among dendrites. Therefore, the dendritic inhibition of pyramidal cells may be less effective in TLE. This can be partially explained by the impairment of the CR-containing interneuron ensemble in the epileptic hippocampus, which may result in an asynchronous and thus less effective dendritic inhibition of the principal cells. This phenomenon, together with the sprouting of excitatory pathway axons and enhanced innervation of principal cells, may be involved in seizure generation. Preventing the loss of CR-positive cells and preserving the integrity of CR-positive dendrite gap junctions may have antiepileptic effects, maintaining proper inhibitory function and helping to protect principal cells in epilepsy.

Keywords: calretinin, interneuron, dendritic inhibition, synchronization, epilepsy

INTRODUCTION

Calretinin (CR) is a calcium-bindig protein, belonging to the calmodulin superfamily, which was shown to be present in many brain regions (Rogers, 1987; Faas et al., 2007).

In the rodent hippocampus the majority of the CR-positive cells seem to be GABAergic interneurons (Jacobowitz and Winsky, 1991; Miettinen et al., 1992; Liu et al., 1996).

They represent a distinct subpopulation of interneurons (Gulyás et al., 1992; Liu et al., 1996) with a negligible overlap with other calcium binding protein-containing interneurons—parvalbumin and calbindin—in rat and monkey (Miettinen et al., 1992; Rogers and Resibois, 1992; Seress et al., 1993b).

Interneurons of the hippocampus can be divided into three main functional groups according to their role in the neuronal network (Freund and Buzsáki, 1996). Perisomatic inhibitory cells innervate the soma, axon initial segment or proximal dendrites of principal cells (basket and axo-axonic cells) (Handelmann et al., 1981; Emson et al., 1982; Somogyi et al., 1983; Kosaka et al., 1985, 1987; Katsumaru et al., 1988; Seress et al., 1991, 1993a; Li et al., 1992; Ribak et al., 1993; Halasy et al., 1996) and control the output of principal cells (Arai et al., 1995; Freund and Buzsáki, 1996; Miles et al., 1996; Holmes and Levy, 1997). Dendritic inhibitory cells innervate the distal dendrites of principal cells (Kawaguchi and Hama, 1988; Gulyás et al., 1993; Han et al., 1993; Buhl et al., 1994; Sik et al., 1994, 1995, 1997; Buckmaster and Schwartzkroin, 1995; Halasy et al., 1996) and control the generation of dendritic calcium spikes and synaptic plasticity (Freund and Buzsáki, 1996; Miles et al., 1996). The interneuron-selective inhibitory cells innervate other interneurons, and thus have a role in the synchronization of dendritic inhibition (Acsády et al., 1996; Gulyás et al., 1996; Hajos et al., 1996; Urbán et al., 2002).

The different vulnerability of interneurons in temporal lobe epilepsy (TLE) was shown in numerous animal models and human patients (Babb et al., 1989; Houser, 1991; Sloviter, 1999; Ben-Ari and Cossart, 2000; Bouilleret et al., 2000; André et al., 2001; Ben-Ari, 2001; Arellano et al., 2004; Ben-Ari and Holmes,

Abbreviations: CA 1, 2, 3, regions of the Cornu Ammonis according to Lorente de No; CR, calretinin; TLE, temporal lobe epilepsy.

2005; Kuruba et al., 2011; Marx et al., 2013). In most studies, the relative preservation of the inhibitory input to the perisomatic domain of principal cells was described, whereas dendritic inhibition was found to be decreased (Cossart et al., 2001; Sundstrom et al., 2001; Wittner et al., 2001, 2005; Maglóczky and Freund, 2005; Tyan et al., 2014). The third functional type of interneurons are specialized to innervate other interneurons (Freund and Buzsáki, 1996; Gulyás et al., 1996) and contain VIP or CR (Acsády et al., 1996; Gulyás et al., 1996). These neurons are in an ideal position to regulate dendritic inhibition (Gulyás et al., 1996; Hajos et al., 1996; Chamberland et al., 2010; Tyan et al., 2014) and compensate the synchronizing effect of perisomatic inhibition (Cossart et al., 2001; Maglóczky and Freund, 2005), and therefore they may have a critic role in maintaining the normal network activity and may prevent synchronous discharges leading to epileptic seizures.

In this review we focus on the fate of this special interneuron type—the CR-containing interneurons—in TLE.

CR-CONTAINING CELLS IN THE RODENT HIPPOCAMPUS

Two types of CR-positive cells were found in the rat hippocampus (Gulyás et al., 1992), spiny and spine-free dendritic cells. The spine-free type can be observed in all subregions, and have a small cell body with smooth dendrites running through several layers. Their dendrites are often attached to each other over long segments. At the electron microscopic level, several puncta adherentiae were observed among contacting CR-positive dendrites (Gulyás et al., 1992, 1996). The other, spiny type is found exclusively in the hilus of the dentate gyrus and in the stratum lucidum of the CA3 region. Their dendrites run horizontally and are covered with spines. They receive the majority of their inputs from mossy fibers (Gulyás et al., 1992). Miettinen and colleagues have shown that the majority of the former type belonged to GABAergic interneurons, whereas the latter, spiny type was mainly GABA-negative (Miettinen et al., 1992).

Hippocampal CR-positive cells of the mouse are similar to the rat. However, there are species-specific differences among human/rat and mouse hippocampal areas, i.e., young granule cells and mossy cells are CR-positive in mice, whereas they are CR-negative in rats and humans (Liu et al., 1996; Blasco-Ibáñez and Freund, 1997; Fujise et al., 1998; Murakawa and Kosaka, 1999; Mátyás et al., 2004; Seress et al., 2008). However, the main interneuron types, e.g., the bipolar or bitufted, rarely spiny cells with dendro-dendritic connections, are present both in rodents and humans in hippocampal areas (Gulyás et al., 1992; Nitsch and Ohm, 1995; Urbán et al., 2002). This cell type is thought to be responsible for the inhibition of other interneurons (Gulyás et al., 1996). It is well preserved throughout evolution, and therefore its role can be studied in rodent models of epilepsy.

CR-CONTAINING CELLS IN THE HUMAN HIPPOCAMPUS

In the adult human hippocampus, CR is expressed by non-principal cells (Nitsch and Ohm, 1995; Urbán et al., 2002). Besides the interneurons, there are a few remaining Cajal-Retzius cells at the border of stratum moleculare and stratum lacunosum-moleculare that also show CR-immunoreactivity (Abraham and Meyer, 2003). Unlike in mice and to some extent in non-human primates, mossy cells of the human dentate gyrus are negative for CR-immunostaining (Maglóczky et al., 2000; Seress et al., 2008).

The distribution of the CR-positive elements shows the typical distribution of a non-perisomatic inhibitory interneuron type. They are abundant in the stratum radiatum and at the border of lacunosum-moleculare in the Cornu Ammonis and in the hilus of the dentate gyrus (**Figures 1**, **2**; Nitsch and Ohm, 1995; Maglóczky et al., 2000; Urbán et al., 2002).

The human CR-containing interneurons form a morphologically heterogeneous cell population: (i) multipolar or fusiform cells in the hilus, with dendrites restricted mainly in this subregion; (ii) fusiform cells in the strata moleculare and oriens with horizontal dendrites; (iii) multipolar cells in all layers; and (iv) a group of small cells with a few short dendrites in the dentate gyrus (Nitsch and Ohm, 1995; Maglóczky et al., 2000; Urbán et al., 2002; Tóth et al., 2010).

The human CR-positive interneuron population differs somewhat from those in the rat and mouse (Murakawa and Kosaka, 1999; Mátyás et al., 2004). In human, there is an abundant group of multipolar CR-immunoreactive interneurons at the border of the CA1 stratum lacunosum-moleculare, and a group of small CR-positive cells in the dentate gyrus (Nitsch and Ohm, 1995), which are absent in the rat. In addition, the characteristic spiny CR-positive cells of the rat CA3 region (Gulyás et al., 1992) are missing in the human (Nitsch and Ohm, 1995; Urbán et al., 2002).

The dendrites of the human CR-positive interneurons are smooth or rarely spiny. Similarly to rats, long segments of CR-positive dendrites of different cells are often attached to each other, especially in the CA1 region (**Figure 3**; Urbán et al., 2002). Zona- or puncta adherentia-type contacts were observed between these dendrites at the electron microscopic level (Urbán et al., 2002; Tóth et al., 2010).

EXTRINSIC CR-CONTAINING NETWORK

Besides the intrinsic hippocampal GABAergic CR-system deriving from the local CR-containing inhibitory interneurons, there are extrinsic glutamatergic inputs that also contain CR. In rats, monkeys and humans, a dense CR-positive fiber network can be observed at the top of the granule cell layer in the inner third of the stratum moleculare (Gulyás et al., 1992; Nitsch and Léránth, 1993; Nitsch and Ohm, 1995). The vast majority of the CR-positive terminals here are putative excitatory terminals with a thick postsynaptic density (Gulyás et al., 1992; Maglóczky et al., 2000). This excitatory pathway was shown to be originating from the supramammillary nucleus both in rats and monkeys (Nitsch and Léránth, 1993; Maglóczky et al., 1994; Borhegyi and Leranth, 1997) and, besides the stratum moleculare, it also innervates the pyramidal layer of the CA2 region (Nitsch and Léránth, 1993; Maglóczky et al., 1994; Nitsch and Ohm, 1995). Finally, a dense CR-positive axonal network can be seen at the border of strata radiatum and lacunosum-moleculare in the CA1 region, with numerous presumably excitatory CR-positive axon terminals

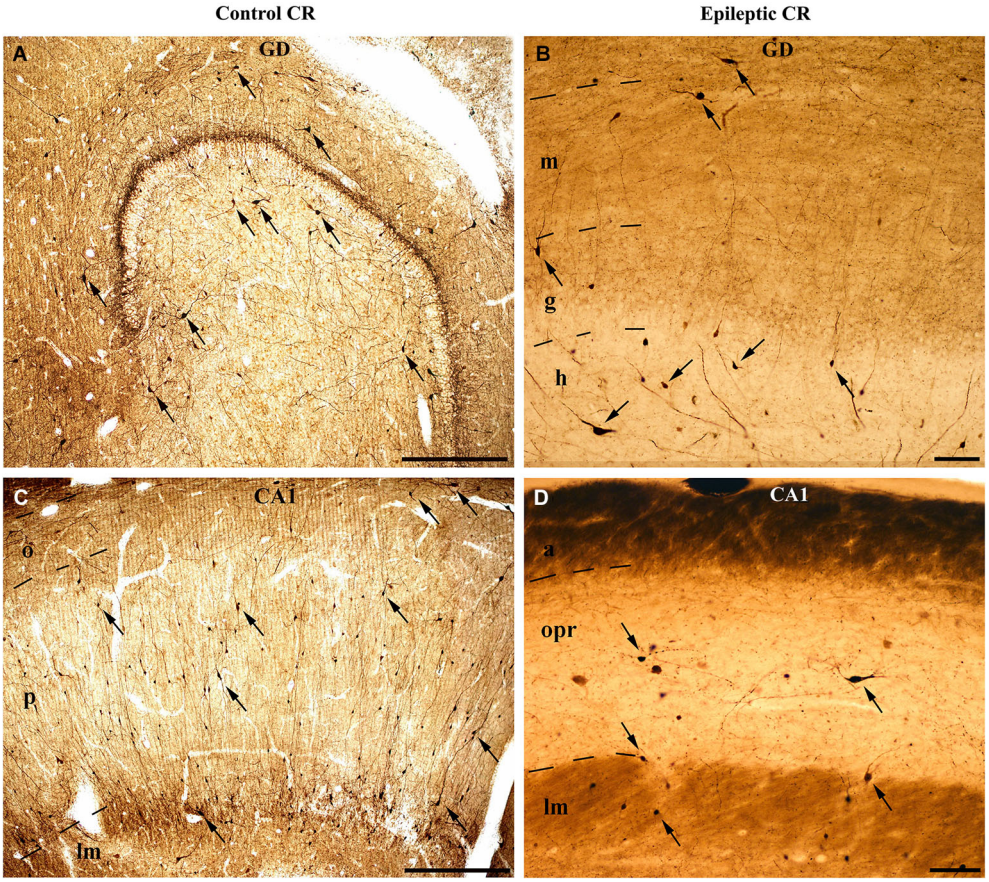

FIGURE 1 | Light micrographs show the distribution and density of CR-containing interneurons in the control (A, C) and epileptic human hippocampus (B, D). CR-immunopositive cells can be observed in the entire hippocampus. **(A)** They are present in large numbers in the control dentate gyrus, especially in the hilus. The immunopositive cells in the outer part of the molecular layer near the hippocampal fissure are presumably Cajal-Retzius cells. **(B)** The epileptic dentate gyrus contains less immunopositive cells. **(C)** Many CR-positive interneurons can be seen in the control CA1 region, scattered throughout all layers. The largest amount of cells is present in the stratum radiatum and at the border of stratum lacunosum-moleculare. **(D)** The sclerotic epileptic CA1 region contains only a few CR-immunopositive interneurons with short and distorted, often segmented dendrites. GD: dentate gyrus; m: stratum moleculare; g: stratum granulosum; h: hilus; CA1: Cornu Ammonis 1; o: stratum oriens; p: stratum pyramidale; lm: stratum lacunosum-moleculare; opr: strata oriens-pyramidale-radiatum. Scales: 500 μm in **(A)** and **(C)**; 100 μm in **(B)** and **(D).**

giving asymmetric synapses with thick postsynaptic densities (Urbán et al., 2002). In rats and monkeys, this pathway was shown to originate from the thalamic reunions nucleus (Amaral and Cowan, 1980; Fortin et al., 1996; Bokor et al., 2002; Drexel et al., 2011).

FUNCTION OF CR-CONTAINING INTERNEURONS IN THE HIPPOCAMPUS

The CR-positive interneurons are interneuron-selective inhibitory cells in the CA1 region of the rat hippocampus (Gulyás et al., 1996). Meskenaite have shown that the postsynaptic targets were partially GABAergic local circuit neurons in the monkey neocortex (Meskenaite, 1997). According to Urbán et al. a large proportion of these cells also belong to this functional type of interneuron in the human hippocampal CA1 region, innervating other CR-containing dendrites or unstained interneuron dendrites (Urbán et al., 2002). Additionally, in humans, there is a population which innervates the dendrites of the pyramidal cells (dendritic inhibitory interneurons) (Urbán et al., 2002).

Interneuron-selective cells were suggested to be important in the synchronization of dendritic inhibitory cells (Gulyás et al., 1996; Maglóczky and Freund, 2005; Chamberland et al., 2010; Tyan et al., 2014). This is supported by the fact that: (i) their dendrites are strongly connected by zona adherentiae and possibly by gap junctions in rats (Gulyás et al., 1992, 1993), which allows the synchronous activation of the connected cell population (Galarreta and Hestrin, 1999; Gibson et al., 1999); (ii) their dendrites often run parallel with each other in the human CA1 region, forming close contacts; additionally, zona adherentiae were identified in these segments at the electron microscopic level (Urbán et al., 2002), which is a characteristic structure to support the development of gap junctions (Kosaka and Hama, 1985); and (iii) the calbindin-containing dendritic inhibitory

interneurons are innervated by CR-positive inhibitory cells in both rats and humans (**Figure 4**; Gulyás et al., 1996; Tóth et al., 2010).

Dendritic inhibitory interneurons innervate the distal dendritic tree of pyramidal cells. The synchronization of dendritic inhibitory cells is a crucial process to provide an effective inhibitory control of excitatory synaptic input of pyramidal cell dendrites (Miles et al., 1996; Chamberland et al., 2010; Tyan et al., 2014).

These observations suggest that CR-containing cells form a unique inhibitory cell population in the hippocampus. Despite their low number, they play a key role in hippocampal inhibitory circuits. By synchronizing the dendritic inhibitory interneurons, they can control the efficacy of excitatory inputs to pyramidal cells (Maglóczky and Freund, 2005; Tyan et al., 2014). Therefore, studying their fate in different brain disorders, especially in epilepsy, is of special interest.

CHANGES IN THE NUMBER AND DISTRIBUTION OF CR-POSITIVE INTERNEURONS IN THE HUMAN EPILEPTIC HIPPOCAMPUS

The sensitivity of CR-expressing cells to epilepsy was discussed in several reports, both in animal models and in humans (Maglóczky and Freund, 2005; Barinka and Druga, 2010; Tóth et al., 2010). CR-containing cells were also found to be sensitive in focal cortical dysplasias (Barinka et al., 2010).

However, the published data on the sensitivity of human CR-containing cells in epilepsy is controversial. According to Blümcke et al. the CR-containing cells are preserved in epilepsy (Blümcke et al., 1996); moreover, the number of CR-positive Cajal-Retzius cells is even increased (Blümcke et al., 1999; Thom et al., 2002). However, our group observed increased vulnerability of CR-positive cells in human TLE, including the CR-positive Cajal-Retzius cells (**Figure 1**; Maglóczky et al., 2000; Tóth et al., 2010).

The contradiction possibly emerged from the sensitivity of CR-immunostaining to the length of the post mortem delay of the applied control samples (**Figure 2**). The post mortem delay in the studies of Blümcke et al. varied between 6 h and up to 3 days (Blümcke et al., 1996, 1999). In our studies, post mortem delays for control samples were between 2–4 h (Maglóczky et al., 2000; Tóth et al., 2010). According to Urbán et al. the age of the subject, the post mortem delay and the fixation procedure has an extreme impact on the quality and quantity of CR-immunostaining (Urbán et al., 2002). The preservation of the immersion-fixed control samples with short post mortem delays was comparable to the perfusion-fixed animal tissue and immediately fixed epileptic samples (Tóth et al., 2010).

According to our results, the number of CR-containing cells decreased significantly, both in the dentate gyrus (especially in the hilus) (Maglóczky et al., 2000; Tóth et al., 2010) and in the sclerotic CA1 region (**Figures 1**, **2**; Tóth et al., 2010). The density of the presumably persisting CR-positive Cajal-Retzius cells (Abraham and Meyer, 2003) at the border of the stratum moleculare and hippocampal fissure was also significantly reduced (Haas et al., 2002; Tóth et al., 2010), even in the non-sclerotic samples (Tóth et al., 2010).

In addition, the preserved cells had an altered morphology, suggesting the degeneration of their dendritic tree (Maglóczky et al., 2000; Tóth et al., 2010; **Figure 3**). In the non-sclerotic epileptic CA1 region, the number of CR-positive cells was unchanged. However, their dendrites were varicose, and contacts between the CR-positive dendrites were less frequently seen (Tóth et al., 2010; **Figure 3**). Consistently, zona adherentia-type contacts were rarely observed between CR-positive dendritic profiles at the electron microscopic level (Tóth et al., 2010), decreasing the possibility of the establishment and maintenance of gap junctions (Fukuda and Kosaka, 2000).

Thus, the synchronous activation of these interneuron-selective inhibitory cells is possibly impaired in the human epileptic hippocampus. As part of the CR-containing cells are dendritic inhibitory interneurons in humans, this means that by losing their zona adherentia-type dendro-dendritic contacts, the CR-positive population of dendritic inhibitory cells also loses the ability to function synchronously.

SYNAPTIC REORGANIZATION OF CR-POSITIVE INTERNEURONS IN THE HUMAN EPILEPTIC DG AND CA1

Electron microscopic examinations revealed that the CR-containing interneuronal (inhibitory) synaptic network is also changed in the epileptic human samples.

Despite the sensitivity and loss of CR-containing interneurons in the DG (Maglóczky et al., 2000; Tóth et al., 2010), an increased frequency of CR-positive interneuronal terminals was found in epilepsy (Maglóczky et al., 2000). Dentate granule cells receive an excess excitation in epilepsy due to mossy fiber sprouting (Davenport et al., 1990b; Houser et al., 1990; Represa et al., 1990; Sloviter, 1994; Mathern et al., 1995; Zhang et al., 2009). Although the increase of the frequency of CR-positive inhibitory terminals was low, regarding the severe cell loss, this might mean a sprouting of the remaining CR-containing inhibitory cells as a compensatory mechanism to offset the enhanced excitatory input on granule cell dendrites.

In controls, the majority of the CR-positive inhibitory axon terminals contacted CR-positive interneurons and pyramidal cells in the CA1 region (**Figure 5**; Tóth et al., 2010). In the epileptic samples, the proportion of CR-positive targets was significantly reduced. The decreased innervation of other CR-positive dendrites may reflect the impairment of the CR-containing interneuronal network. The ratio of unstained interneuron dendrites increased among the targets, whereas pyramidal cells were less frequently innervated, even in those patients where the pyramidal cells were present (non-sclerotic patients) (**Figure 5**; Tóth et al., 2010). The frequency of CR-positive terminals giving symmetric synapses was decreased, whereas those giving asymmetric synapses was increased in the epileptic CA1 region (Tóth et al., 2010).

Taken together, these results suggest that both synaptic and dendro-dendritic contacts of CR-positive interneurons are impaired even in the non-sclerotic epileptic human hippocampi, in the absence of any major principal cell- and CR-containing interneuron loss (Tóth et al., 2010).

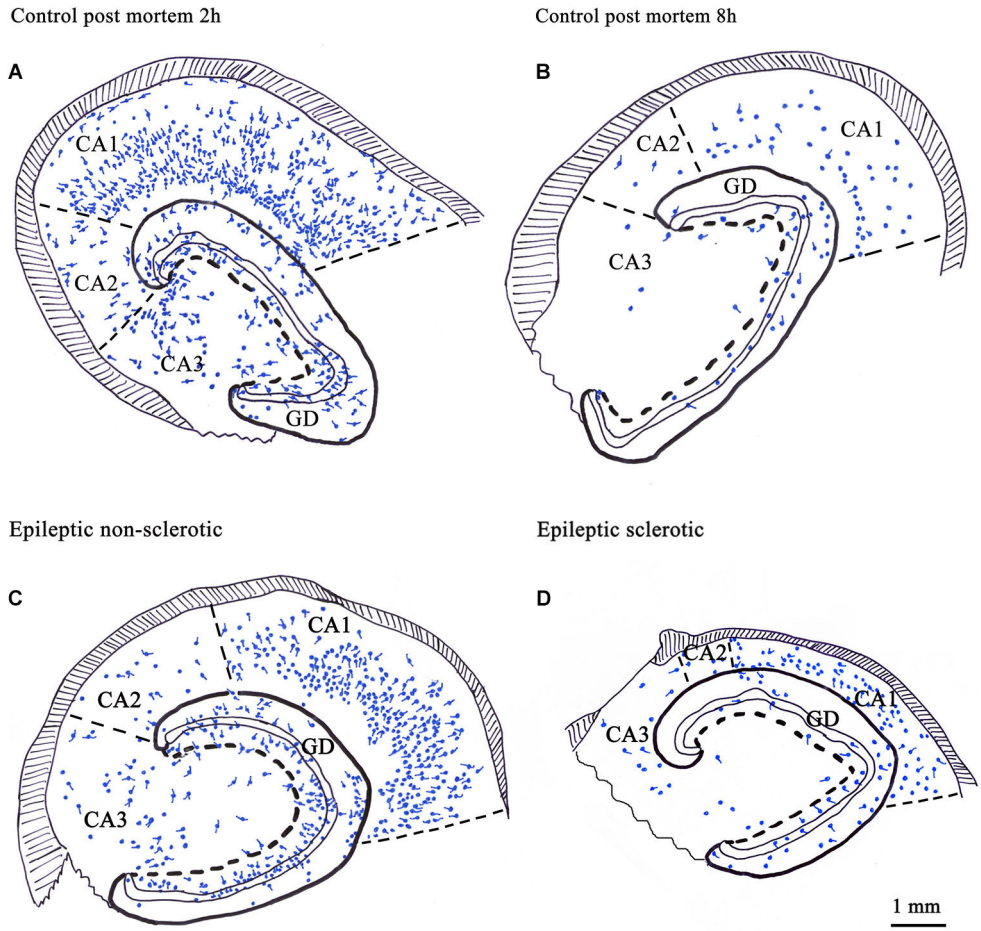

FIGURE 2 | Camera lucida drawings show the number and distribution of CR-immunoreactive cells in control human hippocampi with different post mortem delay (A, B) and in epileptic human hippocampi with different degrees of principal cell death (C, D). CR-containing cells were present in all regions of the hippocampus **(A)**. Their number was significantly reduced in control samples with long post mortem delay **(B)** and in the sclerotic epileptic cases **(D)**. In the non-sclerotic tissues **(C)** the number and distribution of CR-immunoreactive cells were similar to the control, with short post mortem delay **(A)**. The volume of the sclerotic hippocampi is reduced because of the shrinkage of the CA1 region due to the loss of CA1 pyramidal cells **(D)**. GD: dentate gyrus; Scale: 1 mm.

CHANGES TO THE EXTRINSIC CR-CONTAINING SYSTEM IN HUMAN EPILEPTIC HIPPOCAMPUS

Besides the changes affecting the intrinsic CR-containing system, the extrinsic CR-expressing pathways also show alterations. In the epileptic dentate gyrus, the CR-containing excitatory pathway—originating from the supramammillary nucleus—is expanded to the outer two thirds of the molecular layer (**Figure 3**; Maglóczky et al., 2000). This observation was confirmed at the electron microscopic level: whereas in controls the majority of the CR-positive excitatory terminals were located in the inner molecular layer, the frequency of terminals was similar in the inner and outer moleculare in the epileptic cases (Maglóczky et al., 2000). The extension of the supramammillary pathway was independent from the granule cell dispersion, as it occurred in all the subjects examined. The relative increase of the frequency of CR-positive terminals giving asymmetric synapses in the CA1 region could also reflect the sprouting of an excitatory pathway originating presumably from the thalamus (Amaral and Cowan, 1980; Bokor et al., 2002).

EPILEPSY MODELS AND THE FATE OF CR-CONTAINING INTERNEURONS

Numerous models of epilepsy were developed to study the mechanisms of seizures and epilepsy including genetic models, *in vitro* slice models, *in vivo* induced seizures, acquired focal models etc. (Pitkanen et al., 2006). In most cases rodents were used for *in vivo* chronic models, and the cortical and hippocampal areas were especially studied.

The pathological changes of the brain were classified and the sensitivity of neurons was monitored in most models. It was shown that GABAergic cells are more preserved than principal cells (Babb et al., 1989; Davenport et al., 1990a; Houser, 1991; Ben-Ari, 2001; Ben-Ari and Holmes, 2005). However, certain neurochemically identified groups of interneurons proved to be

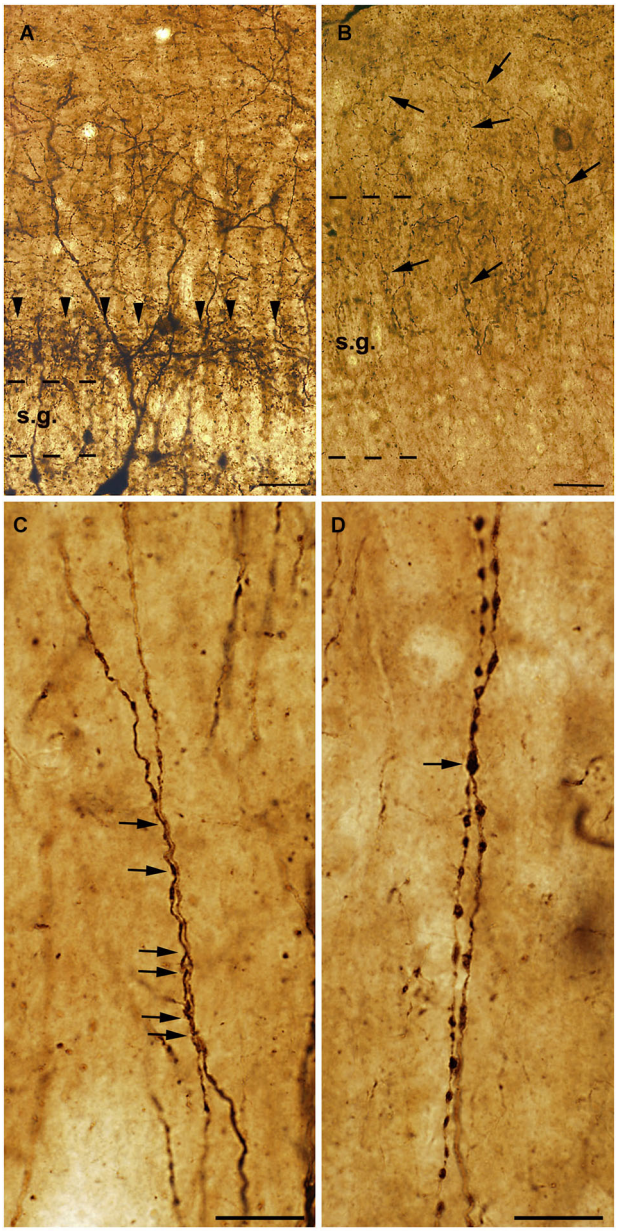

FIGURE 3 | High power light micrographs from control and epileptic samples showing the morphology of CR-positive interneuron dendrites. Many CR-positive interneurons are located in the hilus of the control dentate gyrus **(A)**. They usually have long and smooth dendrites arborizing densely in the stratum moleculare. In the inner molecular layer a dense network of CR-positive axon terminals can be observed, which presumably originate from the supramammillary nucleus (**A**, arrowheads). In the sclerotic epileptic gyrus dentatus **(B)** most of the CR cells disappear, and the CR-positive supramammillary input extends to the entire stratum moleculare (**B**, arrows). The dispersion of the granule cell layer can also be observed in many epileptic cases. In the control CA1 region **(C)**, dendrites of CR-positive interneurons are usually long, smooth and vertically oriented. They are often juxtaposed and run together over short segments (**C**, arrows). However, in the non-sclerotic samples **(D)** the dendrites are varicose and the contacts between CR-positive interneuron dendrites are less frequent than in controls (**D**, arrow). s.g.: stratum granulosum; Scales: **(A, B)**: 50 μm; **(C, D)**: 20 μm.

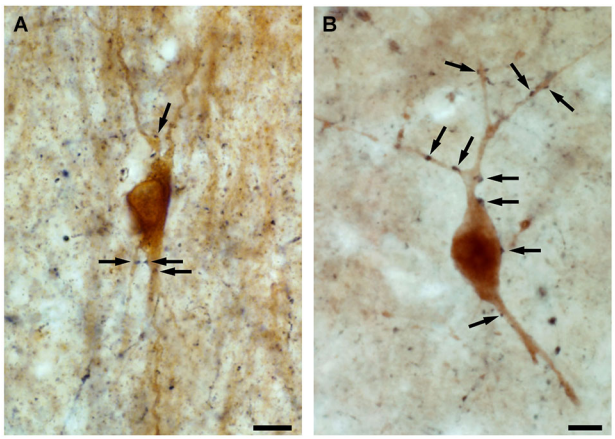

FIGURE 4 | High power light micrographs showing the innervation of calbindin-containing interneurons by CR-positive fibres. By double immunolabeling experiments, applying DAB and DAB-Ni as chromogens, we show that both in the control (A) and in the epileptic (B) human CA1 region, the axon terminals of the CR-containing interneurons (black reaction product) innervate the calbindin-containing interneurons (brown reaction product) (arrows). Scale: 10 μm.

sensitive to epileptic injury, like somatostatin cells in TLE (de Lanerolle et al., 1989; Mitchell et al., 1995; Sundstrom et al., 2001). Interestingly, as compared to other GABAergic interneurons, the fate of CR-containing interneurons was less examined in epilepsy.

Hippocampal CR-positive cells were shown to be vulnerable to excitotoxic cell damage in ischaemia (Freund and Maglóczky, 1993) and in various models of epilepsy (**Figure 6**; Magloczky and Freund, 1993; Maglóczky and Freund, 1995; Ben-Ari and Cossart, 2000; Bouilleret et al., 2000; André et al., 2001; Domínguez et al., 2003; Slézia et al., 2004; van Vliet et al., 2004; Cobos et al., 2005; Tang et al., 2006; Wu et al., 2012; Huusko et al., 2013).

SIMILARITY TO HUMAN TLE

For a detailed comparison to human TLE, data from chronic models of TLE concerning the CR-positive interneurons is summarized here. These models usually show remarkable similarity to human hippocampal pathology in TLE (Sloviter, 1996; Bernard et al., 1998; Ben-Ari, 2001; Sharma et al., 2007; Curia et al., 2008; Tang and Loke, 2010; Carriero et al., 2012), including sclerosis, loss of pyramidal cells in the CA1 and CA3c regions, hilar interneuronal loss, and the preservation of calbindin-containing interneurons (Sloviter et al., 1991; Wittner et al., 2002).

Reduction in the number of CR-containing cells was found in the hippocampus after electrical induction of status epilepticus (van Vliet et al., 2004), in traumatic brain injury (Huusko et al., 2013), in a kainate model of TLE (**Figure 6;** Magloczky and Freund, 1993; Maglóczky and Freund, 1995) and in the pilocarpine model of epilepsy (André et al., 2001; Tang et al., 2006; Zhang et al., 2009; Wu et al., 2012). CR-positive cells showed sensitivity for single status epilepticus without recurrent seizure (Fabene et al., 2001).

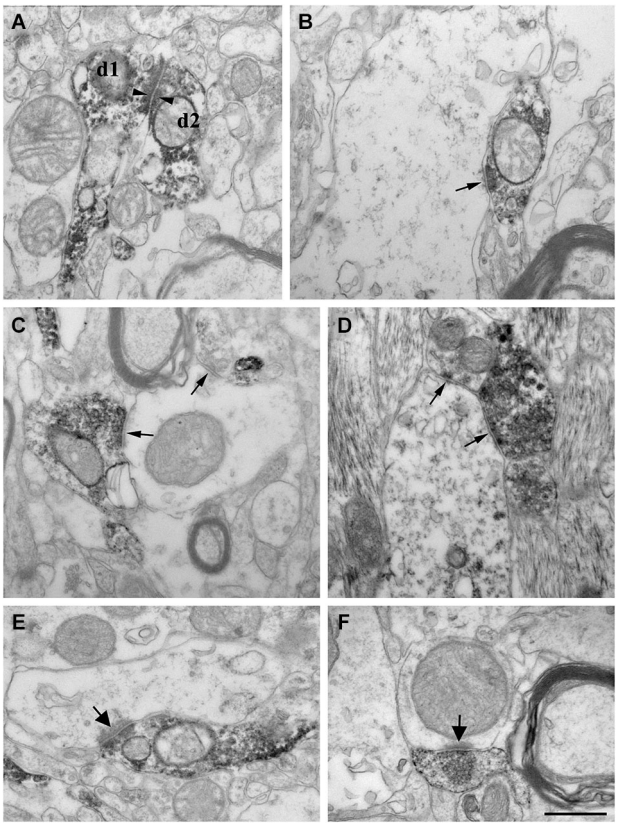

FIGURE 5 | High power electron micrographs from control (A, B, E) and epileptic CA1 region (C, F-non sclerotic, D-sclerotic). In the control samples, dendrites of CR-positive interneurons are often attached to each other by zona adherentia-type contacts (d1 and d2 in **A**, arrowheads). Terminals of CR-positive interneurons establish symmetric synaptic contacts (**B**, **C**, **D**, arrows). In the control CA1 region they frequently terminate on pyramidal cell dendrites **(B)**. In the non-sclerotic epileptic samples, unlabeled interneuron dendrites are more often targeted **(C)**. Almost all of the targets belonged to interneuron dendrites in the sclerotic CA1 region **(D)**. In addition, CR-positive terminals giving asymmetric synaptic contacts (presumably excitatory) were also frequently observed both in control (**E**, arrow) and epileptic samples (**F**, arrow). Most of them were located in the stratum lacunosum-moleculare. Scale: 500 nm.

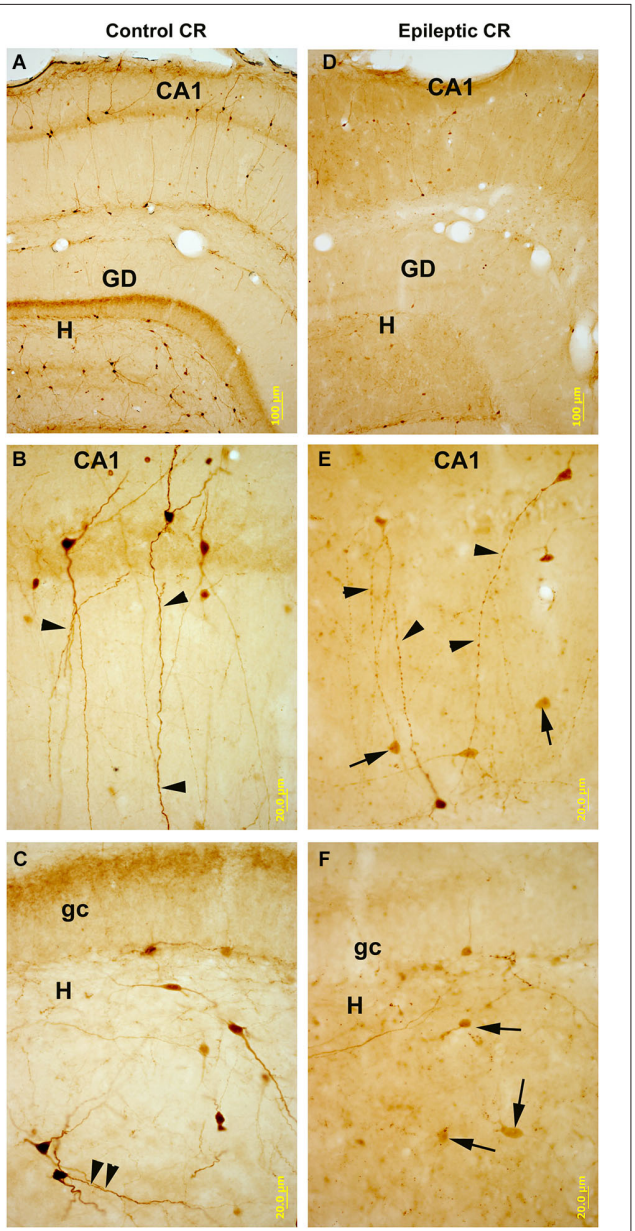

FIGURE 6 | CR-immunostained sections of hippocampal subfields in control and kainate-treated epileptic rats after one weak survival time. In control **(A–C)** CR-containing interneurons are numerous in all subfields of the hippocampus. They are heterogeneous in shape and localization (panel **A**), they posses smooth or sparsely spiny dendrites. In the CA1 region bipolar cells are dominant with long, parallel running dendrites (panel **B**). Arrowheads point to the dendritic contacts. In the hilus (H) of the dentate gyrus (DG, panel **C**) they are mostly multipolar cells, and parallel running dendrites are also present here (arrowheads). In the hippocampi of the kainate-treated rats **(D–F)** the number of CR-containing cells is profoundly decreased in all subfields. In CA1 (panel **E**) the surviving cells have segmented, varicose dendrites (arrowheads). In the hilus the decrease in the number of cells is more severe than in the CA1. Arrows point to the dying, faint cells with reduced/absent dendritic tree. DG: dentate gyrus; gc: granule cell layer; H: hilus. Scales: **(A,D)**: 100 um; **(B–F)**: 20 um.

Although CR-immonopositive interneurons are morphologically and functionally different (Gulyás et al., 1992), no selective loss of a certain morphological type was found in the models of epilepsy. The spiny CR-positive cells in the stratum lucidum of CA3 regions showed profound sensitivity, as they die both in ischaemia (Freund and Maglóczky, 1993) and epilepsy (Magloczky and Freund, 1993; Maglóczky and Freund, 1995; André et al., 2001; Domínguez et al., 2003; Zhang et al., 2009) presumably due to their strong mossy fiber input, which is further enhanced in epilepsy. The bipolar cells—presumably responsible for interneuron specific inhibition—are also strongly decreased in number, and their dendritic tree is reduced in size and shows segmentation in epileptic hippocampi (**Figure 6;** Magloczky and Freund, 1993; Slézia et al., 2004; Zhang et al., 2009), similarly to that found in human TLE (Tóth et al., 2010). Besides synaptic contacts, CR-immunopositive cells are also connected by their dendritic tree (**Figure 6;** Gulyás et al., 1992), and therefore the disruption of the dendritic

connections may cause dysfunction of these cells in rodent models.

Interestingly, even a small dose of kainate (Maglóczky and Freund, 1995) may cause reduction in the number of CR-containing cells, especially in the hilus, suggesting that hilar CR-positive interneurons may be more sensitive than neurons in other subfields (**Figure 6**).

Analyses of the time course of the cell loss showed that it begins soon after excitotoxic insults (Freund and Maglóczky, 1993; Maglóczky and Freund, 1995). After few hours, a significant decrease can be seen in the hilus and CA3 of the rat.

However, granule cells and mossy cells also contain CR in mice (Liu et al., 1996; Fujise et al., 1998; Mátyás et al., 2004), especially in young animals (Blasco-Ibáñez and Freund, 1997; Brandt et al., 2003). In older animals the CR-staining of the principal cells is weaker. In addition, induction of status epilepticus may transiently accelerate the production of newly formed neurons (Kralic et al., 2005), which are mostly granule cells (Hester and Danzer, 2013). Therefore, more numerous CR-positive cells might be seen in the epileptic mice after pilocarpine-induced epilepsy, their number is decreased at the chronic phase with the decreasing tendency of neuron production (Kralic et al., 2005) and weakening of the CR-immunopositivity of principal cells with time (Brandt et al., 2003).

MECHANISM OF DEATH OF CR-CONTAINING NEURONS

The exact mechanism of death of CR-containing cells is not known. According to the morphological signs (Martin, 2001), CR-containing cells die by a necrotic type of excitotoxic degeneration, not by apoptosis. Their electron microscopic examination shows degenerating cytoplasm, decaying mitochondria and numerous phagocytic vacuoles, suggesting the overproduction of abnormal proteins and energy failure (Maglóczky et al., 2000; Tóth et al., 2010).

Although these cells contain a calcium binding protein, they proved to be extremely sensitive for epileptic and ischemic conditions with large calcium ion influx. One explanation of their extreme sensitivity can be their strong connectivity with each other, as they may react to the insults as a network (Gulyás et al., 1992; Tóth et al., 2010). On the other hand, they contain thin cytoplasm and they are poor in organelles. Therefore we can assume that their energy supply is weaker and makes them vulnerable to excitotoxic stress (Hipólito-Reis et al., 2013). The increased excitability of CR-positive cells in epilepsy, caused by the upregulation of a voltage-gated Na channel (Kim et al., 2008) may also contribute to their vulnerability to excitotoxic insults.

CONCLUSION—CONSEQUENCES OF THE VULNERABILITY OF CR-POSITIVE INTERNEURONS

The alterations and/or sensitivity of different calcium-binding protein-containing interneurons in human TLE were discussed in several reports (Sloviter et al., 1991; Maglóczky et al., 2000; Wittner et al., 2001, 2002; van Vliet et al., 2004; Tóth et al., 2010). Calbindin-containing interneurons seem to be preserved and enlarged, and display a sprouted axonal arbor. The parvalbumin-containing cells are also preserved, although many of them most likely loose immunoreactivity for parvalbumin due to calcium-overload (Johansen et al., 1990). In contrast, CR-containing interneurons are highly sensitive. The different vulnerability of these calcium-binding protein containing interneurons probably depends on the distinct input-output properties of these cells and the intrinsic enzymatic properties, number of mithochondria etc., rather than the type of the calcium-binding protein they express. CR-containing interneurons were shown to be vulnerable in epilepsy in most published studies. Decreased cell number (Magloczky and Freund, 1993; Maglóczky and Freund, 1995; André et al., 2001; Slézia et al., 2004; van Vliet et al., 2004; Tang et al., 2006; Muzzi et al., 2009), altered dendritic tree, decreased amount of dendritic contacts between cells (Tóth et al., 2010) and a synaptic reorganization of CR-positive inhibitory terminals were described (Maglóczky et al., 2000; Tóth et al., 2010).

These results are of special interest, since dendritic inhibition was shown to be impaired in epilepsy, together with an intact perisomatic inhibition (Cossart et al., 2001). This can be only partially explained by the sensitivity of somatostatin and neuropeptide Y-containing dendritic inhibitory interneurons (de Lanerolle et al., 1988; Mitchell et al., 1995; Sundstrom et al., 2001), since the axons of these cells show a remarkable sprouting (de Lanerolle et al., 1989; Sperk et al., 1992). In addition, the well preserved (Sloviter et al., 1991) calbindin-containing dendritic inhibitory cells also show an axonal sprouting (Wittner et al., 2002). However, despite of the sprouting of dendritic inhibitory cells, dendritic inhibition of pyramidal cells was found to be ineffective in TLE (Cossart et al., 2001; Ben-Ari and Dudek, 2010).

Taken together, these findings suggest that the sensitivity of CR containing interneurons plays an important role in the impairment of dendritic inhibition in epilepsy:

1. the synchronous activation of these interneuron-selective inhibitory cells is possibly impaired, leading to an asynchronous and less effective dendritic inhibition
2. the CR-containing population of dendritic inhibitory cells are also impaired, further decreasing the efficacy of dendritic inhibition
3. due to the changes in their synaptic target distribution, they innervate principal cell dendrites less frequently in epileptic human hippocampus

Since the impaired dendritic inhibition may cause a less effective control of the efficacy and plasticity of excitatory inputs to principal cells, and subsequently to the formation of principal cell assemblies connected by abnormally potentiated synapses, the impairment of CR-positive cells can be involved in epileptogenesis and seizure generation (Maglóczky and Freund, 2005; El-Hassar et al., 2007; Tóth et al., 2010). Furthermore, these excitatory inputs were shown to undergo sprouting both in animal models (Perez et al., 1996; Esclapez et al., 1999; Ben-Ari, 2001; Lehmann et al., 2001) and humans (Lehmann et al., 2000; Magloczky, 2010), partly explaining why severe intractable seizures can occur even in non-sclerotic patients, where the majority of principal and non-principal cells are preserved (Maglóczky and Freund, 2005; Magloczky, 2010; Tóth et al., 2010). The excessive reorganization of the hippocampal inhibitory network, the sprouting of either intrinsic or afferent excitatory pathways, together with the intact

output from the CA1 region, may render the non-sclerotic hippocampus a potent epileptogenic region (Maglóczky and Freund, 2005; Magloczky, 2010).

The sensitivity of CR-expressing interneurons for excitotoxic insults was also shown in animal models (Magloczky and Freund, 1993; Maglóczky and Freund, 1995; André et al., 2001; Domínguez et al., 2003; Zhang et al., 2009). Since the early loss of these cells can be observed in the acute and latent phase of epileptogenesis (Zhao et al., 2008), we can hypothesize that recurrent seizure generation might be associated with a loss of a certain amount of CR-positive cells, among other factors.

Prevention of the loss of CR-positive cells and preserving the integrity of the attached CR-positive dendrites may have antiepileptic effects, protecting the proper inhibitory function and helping to spare principal cells in epilepsy (Tóth et al., 2010). Since they die by degeneration after excitotoxic insults in the acute-to-latent phases, one therapeutic possibility may be to promote their survival during the initial phase of epilepsy.

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