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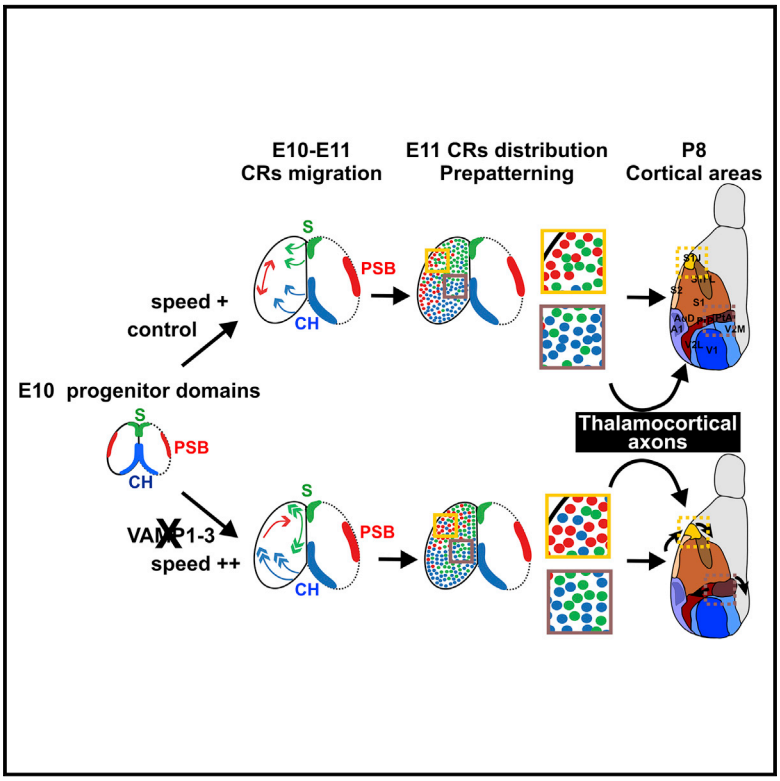
Migration Speed of Cajal-Retzius Cells Modulated by Vesicular Trafficking Controls the Size of Higher-Order Cortical Areas

Graphical Abstract



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In Brief

Barber et al. find that vesicular trafficking modulates the migration speed and cortical distributions of Cajal-Retzius neurons. They show that CR composition influences the size and wiring of postnatal visual, auditory, and somatosensory systems. These findings implicate VAMP3 in CR migration and in the patterning of higher-order cortical areas.

Highlights

- Subtype-specific migration kinetics determine CRs cortical distributions
- Inhibiting VAMP3 increases migration speed, but not directionality, of CR subtypes
- VAMP3-dependent vesicular trafficking regulates CR cortical dispersion
- Altered CRs distribution changes the size and wiring of higher-order cortical areas

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Migration Speed of Cajal-Retzius Cells Modulated by Vesicular Trafficking Controls the Size of Higher-Order Cortical Areas

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SUMMARY

In the neocortex, higher-order areas are essential to integrate sensory-motor information and have expanded in size during evolution. How higher-order areas are specified, however, remains largely unknown. Here, we show that the migration and distribution of early-born neurons, the Cajal-Retzius cells (CRs), controls the size of higher-order areas in the mouse somatosensory, auditory, and visual cortex. Using live imaging, genetics, and in silico modeling, we show that subtype-specific differences in the onset, speed, and directionality of CR migration determine their differential invasion of the developing cortical surface. CR migration speed is cell autonomously modulated by vesicle-associated membrane protein 3 (VAMP3), a classically non-neuronal mediator of endosomal recycling. Increasing CR migration speed alters their distribution in the developing cerebral cortex and leads to an expansion of postnatal higher-order areas and congruent rewiring of thalamo-cortical input. Our findings thus identify novel roles for neuronal migration and VAMP3-dependent vesicular trafficking in cortical wiring.

INTRODUCTION

The neocortex is classically divided into primary areas that process sensory inputs and execute motor commands, and higher-order areas that are essential for the integration of sensory-motor information and cognitive functions. Higher-order areas reside between primary areas and include secondary and association areas that compose most of the human neocortex [1]. These are functionally essential, but their study has been rendered difficult due to their small size in the mouse. While broad patterning of the neocortex into primary areas is regulated by morphogens and gradients of transcription factors at embryonic stages [2, 3], the mechanisms controlling the differentiation of higher-order areas are largely unexplored. Recent studies have revealed that sensory inputs conveyed by thalamocortical axons during postnatal life drive the distinction between primary and higher-order areas [4, 5]. However, it remains unknown whether the delineations between primary and higher-order areas are specified at embryonic stages and, in particular, what determines the final size of higher-order areas.

Cajal-Retzius cells (CRs) are the first born glutamatergic neurons in the embryonic mouse cerebral cortex and occupy the most superficial layer [6–8]. They are best known for their role in regulating cortical lamination through their secretion of Reelin [9–11]. In addition, at embryonic stages CRs have been proposed to regulate the radial glia phenotype [12, 13], the differentiation of upper layers neurons [14], the development of hippocampal connections [15], and the temporal control of interneuron migration [16]. Postnatally, CRs are embedded into immature circuits where they receive GABAergic synaptic inputs and present long-range horizontal projections [17–20] prior to their disappearance around P14. Altogether, these properties suggest that CRs constitute an important component of maturing cortical networks.

Work over the past years has shown that CRs comprise molecularly distinct subtypes that are generated from at least three focal sources at the borders of the developing cortex or pallium: the ventral pallium at the pallial-subpallial boundary (PSB), the pallial septum, and the cortical hem [21–24]. CR subtypes tangentially migrate at a time when the neuroepithelium is regionalized (E10.5–E12.5) and cover the cortical surface by the onset of neurogenesis. CR subtypes invade complementary territories and maintain highly specific distributions in the marginal zone [23–25]. Their early regional distribution coincides with spatial patterning of the cortical primordium, which is required for the formation of functional areas in postnatal mice [2, 3]. We have previously shown by genetic ablation of septum-derived CRs, the role of this subpopulation in regulating the position of the primary motor cortex by signaling to cortical progenitors [25]. This

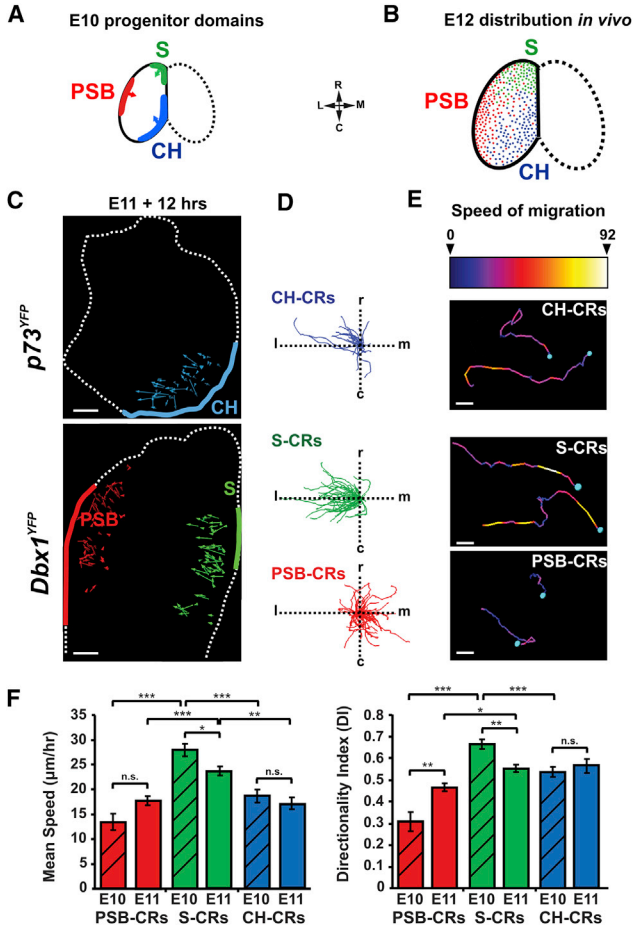


Figure 1. Subtype-Specific Differences in Speed of Migration and Directionality Contribute to the Differential Distribution of CRs in Cortical Territories

(A) CR progenitor domains: the pallial septum (S, green), cortical hem (CH, blue), and ventral pallium at the pallial-subpallial boundary (PSB, red) [22–24].

(B) In vivo complementary distribution of CR subtypes in cortical territories at E12 (taken from [23, 25]): S-CRs (DeltaNp73^{+}Dbx1^{+}, green dots), CH-CRs (DeltaNp73^{+}Dbx1^{-}, blue dots), and PSB-CRs (DeltaNp73^{-}Dbx1^{+}, red dots).

(C) Displacement vectors of CR cells imaged over 12 hr in E11 *p73^{YFP}* and *Dbx1^{YFP}* whole flattened cortical preparations. CH-CRs (n = 46); S-CRs (n = 58); PSB-CRs (n = 69).

(D) Directionality of trajectories at E11 clustered at a common start point.

(E) Representative tracks of migrating CR subtypes at E10 color coded for speed of migration (blue circle: starting position of tracking) showing that S-CR subtypes spend longer time migrating at ≥50 μm/hr (yellow) and at ≥80 μm/hr (white).

(F) Mean speed of migration and directionality index (DI) (ratio of track displacement length to total path length) of CR subtypes from *Dbx1^{YFP}* (PSB-CRs [red columns] and S-CRs [green columns]) and *p73^{YFP}* (CH-CRs [blue columns]) at E10 (hatched columns) and E11 (solid columns). E10: S-CRs (n = 97), CH-CRs (n = 85), PSB-CRs (n = 18); E11: S-CRs (n = 228), PSB-CRs (n = 147), CH-CRs (n = 81). Values show mean ± SEM; unpaired two-tailed t test *p < 0.05, **p < 0.01, ***p < 0.001.

See also Figure S1.

suggested that the presence and/or composition of CRs at the cortical surface are important for the differentiation of underlying neural circuits. At present, little is known on whether subtype-specific differences in CR migration regulate their cortical distribution and, moreover, whether their specific repartition influences the patterning of higher-order cortical areas.

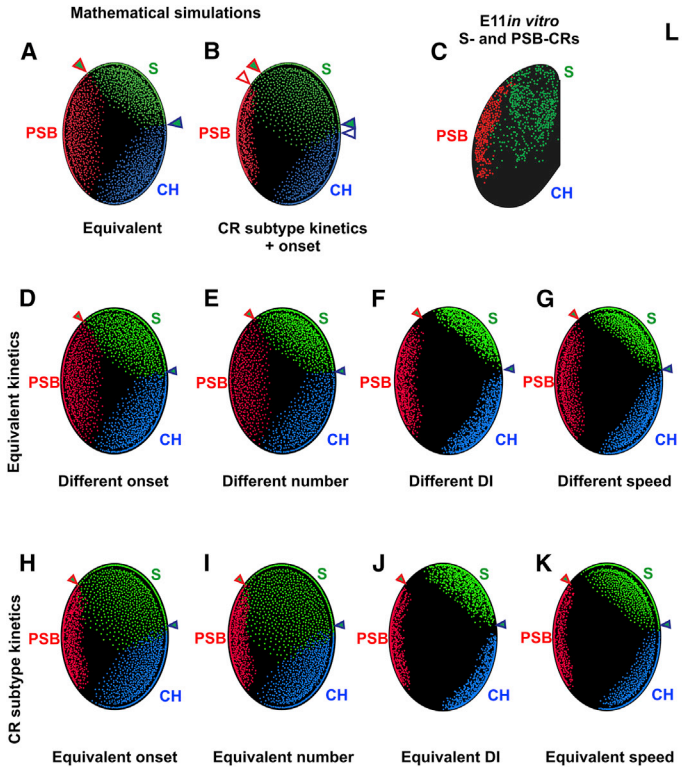
Here, we show that CR subtype-specific differences in migration kinetics determine their specific coverage of the surface of the developing mouse cortex. Furthermore, we reveal that VAMP3-dependent vesicle trafficking cell-autonomously modulates CRs migration speed and their distribution in specific embryonic cortical territories, which, in turn, influences the size and connectivity of higher-order cortical areas in the postnatal cerebral cortex.

RESULTS

Subtype-Specific Differences in Migration Kinetics of CRs Determine Their Differential Invasion of Cortical Territories

Molecularly distinct CR subtypes are generated by three progenitor domains at the borders of the developing mouse cerebral cortex: the pallial septum (S), the cortical hem (CH), and the ventral pallium at the pallial-subpallial boundary (PSB) [22–24] (Figure 1A). Starting at E10, CR subtypes invade the surface of the embryonic cortex by tangential migration. CRs cover the entire cortical sheet at E12 with distinct subpopulations colonizing specific regions: S-CRs (green dots) the rostro-medial, CH-CRs (blue dots) the caudal, and PSB-CRs (red dots) the lateral cortex (Figure 1B) [23, 25].

To investigate how the distribution of CR subtypes is achieved, we genetically traced their migration from their respective sources using a new live-imaging assay on whole flattened cortical vesicles. CR subtypes were labeled with transgenic expression of fluorescent proteins by specific promoters. CH-CRs departing from the caudo-medial (blue) progenitor domain were labeled using *DeltaNp73$^{Cre/+}$;ROSA26$^{YFP/+}$* [26] (hereafter called *p73^{YFP}*), whereas S-CRs and PSB-CRs departing from rostro-medial (green) and lateral (red) progenitor domains, respectively, were labeled with *Dbx1$^{Cre/+}$;ROSA26$^{YFP/+}$* [23, 25] (hereafter called *Dbx1^{YFP}*) (Figures 1C, S1A, and S1D). S-CRs were also traced with the *p73^{YFP}* transgenic line and behaved similarly to S-CRs from the *Dbx1^{YFP}* line (Figures S1A–S1I). Time-lapse analysis from E10 onward showed that S-CRs initiated directed tangential migration before PSB-CRs and CH-CRs (Figures S1A–S1G; Movie S1). By E11, all subtypes migrated extensively from their sources (Figures 1C, S1H, and S1I; Movies S1 and S2). Directed long distance migration originated exclusively from the edges of the cortical explants, whereas YFP^{+} cells in central regions of the explants from both transgenic lines exhibited minimal displacement (Figures S1J and S1K) but actively extended protrusions exploring their environment (Movie S2). This suggested that earlier streams of CRs stopped migrating when reaching the dorsal pallium and streams of later-arriving CRs fill up available space within the expanding dorsal cortex. Clustering of reconstructed cell tracks to a common origin (Figure 1D) showed a clear lateral bias in the directionality of S-CRs and CH-CRs toward the dorsal cortex, in contrast to PSB-CRs, which also migrated along the rostro-caudal axis. Strikingly, from the onset of migration, S-CRs migrated significantly faster than other CRs and along more linear trajectories, as measured by their directionality index (DI) (Figures 1E and 1F). Thus, CR populations differ in migration



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Panel	Onset	Mean speed (μm/hr)	DI	Supply from progenitor domain (Total cell number)
A	=	20	0.51	S-CRs: 720 PSB-CRs: 720 CH-CRs: 720
B	≠	S-CRs: 28.0 PSB-CRs: 13.5 CH-CRs: 18.7	0.67 0.31 0.54	S-CRs: 720 PSB-CRs: 540 CH-CRs: 540
D	≠	20	0.51	S-CRs: 720 PSB-CRs: 720 CH-CRs: 720
E	=	20	0.51	S-CRs: 720 PSB-CRs: 540 CH-CRs: 540
F	=	20	0.255	S-CRs: 720 PSB-CRs: 720 CH-CRs: 720
G	=	10	0.51	S-CRs: 720 PSB-CRs: 720 CH-CRs: 720
H	=	S-CRs: 28.0 PSB-CRs: 13.5 CH-CRs: 18.7	0.67 0.31 0.54	S-CRs: 720 PSB-CRs: 720 CH-CRs: 720
I	≠	S-CRs: 28.0 PSB-CRs: 13.5 CH-CRs: 18.7	0.67 0.31 0.54	S-CRs: 720 PSB-CRs: 720 CH-CRs: 720
J	≠	S-CRs: 28.0 PSB-CRs: 13.5 CH-CRs: 18.7	0.255 0.255 0.255	S-CRs: 720 PSB-CRs: 540 CH-CRs: 540
K	≠	S-CRs: 10 PSB-CRs: 10 CH-CRs: 10	0.67 0.31 0.54	S-CRs: 720 PSB-CRs: 540 CH-CRs: 540

Figure 2. Mathematical Simulations Support the Requirement of CR Subtype-Specific Kinetics of Migration and, in Particular, Speed, to Recapitulate Their Specific Repartition In Vivo

(A and B) Final time frame of mathematical simulations of CR subtypes migrating with equivalent (A) and subtype-specific kinetics (B), corresponding to 24 hr and ~E11.0 in vivo (E10 + 24 hr) (S-CRs, green ○; PSB-CRs, red ○; CH-CRs, blue ○). Green arrowheads outlined in red and blue indicate the rostro-lateral and medial territories where S-CRs/PSB-CRs and S-CRs/CH-CRs come into contact at their migration fronts, respectively. White arrowheads outlined in red and blue indicate shift in regions occupied by S-CRs.

(C) Distribution of Dbx1-derived S-CRs (green ○) and PSB-CRs (red ○) quantified in E11 flattened cortical preparations.

(D–K) Last time frame of simulations of CR subtype migration (S-CRs, green ○; PSB-CRs, red ○; CH-CRs, blue ○).

(A, B, and D–K) Simulations generated using equivalent migration kinetics (A and D–G) and subtype-specific migration kinetics (B and H–K) with equivalent (A and E–H) or different onset of migration 6 hr earlier for S-CRs relative to PSB-CRs and CH-CRs (B, D, and I–K), equivalent (A, D, and F–I), or subtype-specific total number of CRs supplied from each progenitor domain (B, E, J, and K), equivalent (A, D–G, and K), or subtype-specific speed of migration (B and H–J), equivalent (A, D–G, and J), or subtype-specific directionality index (DI) (B, H, I, and K).

(L) Parameters used for mathematical simulations. Homotypic weighting coefficients and number of supplied cells from progenitor domains are indicated (see also the Supplemental Experimental Procedures).

kinetics, and, notably, S-CRs are the first to initiate their migration and migrate faster and along more linear trajectories than other subtypes.

To assess how differences in migration of CR subtypes may impact their distribution in cortical territories, we used mathematical modeling. Specifically, cell migration was modeled by the weighted sum of two components: (1) random walking (RW) and (2) contact repulsion (C-Rep) between cells, as recently reported [27]. Both weighting coefficients were numerically determined so as to reproduce the experimental parameter values for the mean speed and DI of each subtype at E10 (Supplemental Experimental Procedures). A simulation where onset and speed of migration (20 μm/hr) were equivalent for all CR subtypes, as previously suggested [27], resulted in equivalent surface area occupancy of the cortex for each subtype (Figures 2A and 2L; Movie S3, left panel). In contrast, simulations introducing our experimentally observed migration kinetics, including onset, speed, and persistence in directionality (Figures 2B and 2L; Movie S3, right panel), remarkably recapitulated the distribution of CR subtypes observed in vitro (Figure 2C) and in vivo [25] at E11. We further tested how each individual migration parameter influenced CR subtypes distribution in both equivalent and CR subtype-specific kinetics mathematical models. We found that changing exclusively the onset of migration for S-CRs (Figures 2A and 2D) or the number of cells produced by each source (Figures 2A and 2E) did not significantly alter their final repartitioning in cortical territories. Similarly, when differences in onset or number of cells were removed from the model with CR subtype-specific kinetics (Figures 2B, 2H, and 2I), no significant changes were observed. However, when either DI or speed were individually decreased by 2-fold in simulations of equivalent (Figures 2F and 2G) or subtype-specific kinetics (Figures 2J and 2K) CR distribution was clearly altered, indicating that speed and directionality of migration appear to be the primary parameters regulating CRs distribution. Thus, the simulations predict that experimentally observed

differences in migration kinetics of CR subtypes, rather than onset and number of CRs produced at each progenitor domain, are required and sufficient to determine their specific repartition in early cortical territories in vivo.

These results were surprising given that CRs were previously shown to migrate by a common mechanism of contact-mediated repulsion with equivalent speeds and low persistence in directionality on 2D substrates in vitro [27]. We thus studied the migratory behavior of S-CRs and CH-CRs upon cell-cell contact in flattened cortical preparations at E10 and E11 using time-lapse microscopy. We scored three behaviors likely resulting from distinct cellular mechanisms: (1) contact repulsion, (2) contact deviation, and (3) contact steering (Movie S4). At both stages, the most prevalent behavior observed for S-CRs and CH-CR subtypes was contact deviation (Figures S1L–S1N and S1P) with contact repulsion accounting for only a small percentage of behaviors. No significant differences in response to contact were observed between CR subtypes over time although between E10 and E11 S-CRs and CH-CRs differed with respect to speed and directionality. In addition, convergence of migrating CRs was observed at specific rostro-medial levels (Figure S1O; Movie S4). Together, these results suggest that subtype-specific and temporal differences in speed and directionality do not correlate with differences in their contact-repulsive interactions and points to the existence of additional mechanisms regulating these migration parameters.

VAMP3-Dependent Vesicle Trafficking Controls CR Speed of Migration and Redistribution in a Cell-Autonomous Manner

To elucidate the molecular mechanisms underlying CR subtypes differences in migration, we screened a previously reported transcriptomic dataset obtained from FACS-purified S-CRs and PSB-CRs [25]. Surprisingly, vesicle-associated membrane protein 3 (VAMP3), a member of the VAMP1–3 subfamily of recycling endosomal vesicular SNAREs (*N*-ethylmaleimide-sensitive factor attachment protein receptor) [28], which was previously shown to be exclusively expressed in non-neuronal cells in the mature brain [29], was highly expressed in CR cells compared to other VAMPs (Figure S2A). Via its interaction with plasma membrane SNAREs, VAMP3 plays an essential role in the exocytosis of recycling endosomes [30] and promotes the motility of epithelial cells and macrophages through integrin receptor recycling [31–35], raising the possibility that VAMP3 may regulate CR migration. Using VAMP3 immunostaining on E11.5 $Dbx1^{YFP}$ telencephalons, we confirmed the presence of VAMP3-positive vesicles in $YFP^{+}Reln^{+}$ (Reelin) S-CRs, including in their leading process (inset), and in $YFP^{-}Reln^{+}$ CH-CRs (Figure 3A, green and blue arrows, respectively). The microarrays further suggested that S-CRs (DM) expressed VAMP3 at higher levels relative to PSB-CRs (DL), with PSB-CRs reciprocally showing greater enrichment in VAMP2. VAMP2 is best known for mediating neurotransmitter release in the adult nervous system [36]. However, during development, it mediates axon-guidance through the trafficking of Neuropilin1 and PlexinA1 receptors [37], and by asymmetric membrane transport and exocytosis [38], both mechanisms that could underlie the chemotaxis of migrating cells. We therefore quantified the percentage of CR subtypes expressing VAMP2 and VAMP3 in E11 $Dbx1^{YFP}$ and $p73^{YFP}$ cortical regions close to their sources using immunohistochemistry for Reln and Calretinin (Calr) so as to discriminate between the three subtypes (see the Supplemental Experimental Procedures). Counts showed that 88% and 95% of $Dbx1^{YFP+}Reln^{+}$ S-CRs and $Dbx1^{YFP-}Reln^{+}$ CH-CRs, respectively, express VAMP3, in contrast to only 50% of $Dbx1^{YFP+}Reln^{+}$ PSB-CRs (Figure S2B). In addition, 83% of $p73^{YFP-}Calr^{+}$ PSB-CRs expressed VAMP2, compared with only 13% of $p73^{YFP+}$ S-CRs and 19% of $p73^{YFP+}Calr^{+}$ CH-CRs. Further, quantification of the signal intensity of VAMP3 and VAMP2 immunostaining showed significantly higher expression of VAMP3 in S-CRs and CH-CRs relative to PSB-CRs, with PSB-CRs expressing VAMP2 most strongly (Figure S2C), which was consistent with our counts and microarray data. Thus, these data confirm differential expression of VAMP2 and VAMP3 proteins in subsets of migrating CRs and raises the question of their function.

To assess the role of VAMP2 and VAMP3 in CRs migration, we took advantage of the *iBot* transgenic mouse line that allows for Cre recombinase-dependent expression of the light chain of clostridial botulinum neurotoxin B (BoNT/B) (*CAGboNT/B,EGFP*) [39]. This toxin selectively cleaves and inactivates VAMP1–3, which have reported redundant functions [29]. We crossed the *iBot* line with $p73^{Cre}$ mice to specifically target S-CRs (S-*iBot*) and CH-CRs (CH-*iBot*) and allow the simultaneous imaging of their migration. Imaging of E11 $p73^{Cre}$;*iBot* flattened cortices showed a significant increase in CRs mean speed of migration along the entire medial axis relative to control explants (Figures 3B–3H; Movie S5) without changes in their directionality index. These results demonstrate that VAMP1–3-dependent vesicular trafficking, likely the most highly expressed VAMP3, specifically regulates the speed of migration of S-CRs and CH-CRs in a cell-autonomous manner. It also suggests that directionality and speed of migration are controlled by distinct molecular pathways.

To study how faster migration speed of S-*iBot* and CH-*iBot* CRs impacts their distribution in the embryonic cortex, we generated mathematical simulations using data obtained from our live-imaging experiments and analyzed the distribution of CR cells in vivo. Simulations predicted a precocious invasion of S-*iBot* and CH-*iBot* in the dorsal cortex (Figure 3I, dashed area; Movie S5) and increased mixing of CR subtypes in the rostro-lateral and caudo-dorsal regions (Figure 3I). We next analyzed the in vivo distribution of CR subtypes using immunohistochemistry for Reln and Calr (goat antibodies (Swant)) [23], which allows to discriminate S-CRs ($Reln^{+}Calr^{-}$) from both PSB-CRs and CH-CRs ($Reln^{+}Calr^{+}$), and for GFP, which allows to label both S-CRs and CH-CRs (Figures 4A–4G and S3). We found an ectopic accumulation of CH-CRs ($GFP^{+}Reln^{+}Calr^{+}$) in the rostro-dorsal cortex, and, consistent with the simulations, of S-CRs ($GFP^{+}Reln^{+}Calr^{-}$) in the caudo-dorsal cortex in *iBot* mutants. No changes in the total number of $Reln^{+}$ cells were observed, indicating that CRs were correctly generated and specified. The ectopic accumulation of CH-CRs and S-CRs in the dorsal cortex at rostral and caudal levels, respectively, in *iBot* embryos was consistent with their increased speed of migration observed by time-lapse imaging resulting in their invasion and accumulation in more distant territories (blue and green arrows in Figure 4H). This also led to a change in the relative number of S-CRs and CH-CRs in the caudal cortex (green and

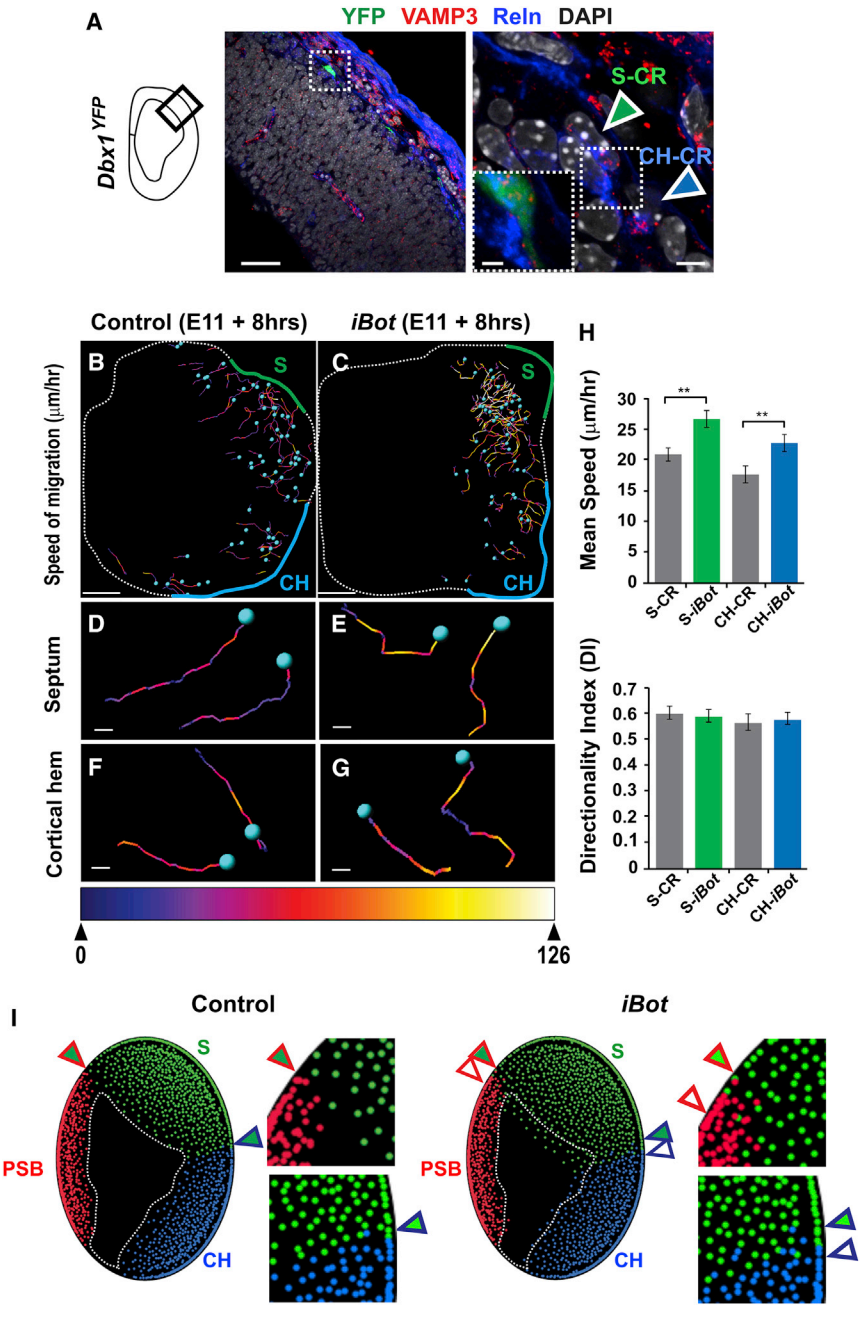

Figure 3. CRs Express VAMP3 and Cleavage of Vamp1–3 Increases Their Speed of Migration in a Cell-Autonomous Manner

(A) Confocal images of the dorso-lateral cortex from E11.5 *Dbx1*^{YFP} embryos immunostained for VAMP3 (red), Reln (blue), GFP (green), and nuclear counterstain with DAPI (gray). High magnification (right) shows VAMP3 vesicular-like staining in migrating Dbx1-derived YFP^{+}Reln^{+} CRs (green arrow) and YFP^{-}Reln^{+} CH-CRs (blue arrow). Inset is a high magnification of the leading process enriched in VAMP3. VAMP3 staining is also observed in cells surrounding CRs and meninges.

(B–G) Cell tracks from 8-hr live imaging of S-CRs and CH-CRs in flattened cortices of *p73*^{YFP} control and BoNT/B-expressing *p73;iBot* at E11. Tracks are color coded for speed (high magnification of representative cell tracks in D–G).

(H) Mean speed of migration and DI of S-CRs and CH-CRs in control (gray) and *p73;iBot* (green and blue columns, respectively). S-CRs (n = 134 cells), S-*iBot* (n = 129 cells); CH-CRs (n = 81 cells), CH-*iBot* (n = 77 cells for mean speed and n = 75 cells for DI). Values: mean ± SEM; two-tailed unpaired t test **p < 0.01.

(I) Final time frame of mathematical simulations generated from experimental data of tracked CRs in E11 control (*p73*^{YFP}) and mutant embryos (*p73;iBot*) showing predicted repartition of S-CR (green ○), PSB-CR (red ○), and CH-CR (blue ○). Green arrowheads outlined in red and blue indicate where S-CRs/PSB-CRs and S-CRs/CH-CRs come into contact at their migration fronts, respectively, in the rostro-lateral and medial territories (shown at high magnification in the right-hand images). White arrowheads outlined in red and blue show shift in regional occupancy of S-CRs in *iBot* mutants.

Scale bars, 50 μm (A, left); 5 μm (A, right); 2.5 μm (right, inset); 200 μm (B and C); 30 μm (D–G). See also Figure S2.

blue ○ in Figure 4H), as predicted by the simulation (Figure 3I). Furthermore, an ectopic accumulation of PSB-CRs (GFP^{-}Reln^{+} Calr^{+}) was observed in the dorsal and lateral cortex (Figures 4H and S3) at rostral levels, showing a non-cell autonomous rostro-medial redistribution of PSB-CRs in *iBot* mutants (red arrows in Figure 4H), likely preventing S-CRs from invading the rostral dorso-lateral cortex. Analysis of the distribution of GFP^{+} p73-derived CRs (S-CRs and CH-CRs) in *iBot* mutants at E17.5 showed their increased accumulation in the dorsal cortex at rostral levels, with no changes in their total number, consistent with their enhanced dispersion resulting from their faster migration speeds at earlier stages (Figures S3F–S3H). As VAMP3 was the most highly expressed member in S-CRs and CH-CRs, we further assessed whether it was specifically involved in CRs migration by analyzing the distribution of CR subtypes in *Vamp3* mutant embryos (Figure S3I). We found that VAMP3 inactivation altered the distribution of CR subtypes, but not the total number of Reln^{+} cells, in the dorsal and lateral cortex indicating that the cell-autonomous activity of VAMPs in CR cell migration likely involves VAMP3. Together, these results show that cell-autonomous inactivation of VAMP3 in S-CRs and CH-CRs increases their migration speed resulting in their redistribution to more distant locations and enhances mixing of CR subtypes specifically in the dorso-lateral cortex at rostral levels and the dorsal cortex at mid-caudal levels in vivo.

VAMP3 Controls the Size of Higher-Order Areas in the Postnatal Cerebral Cortex

We have previously shown that loss of S-CRs influence the early regionalization of the cerebral cortex and the size of the primary motor cortex [22]. To investigate whether changes in CRs'

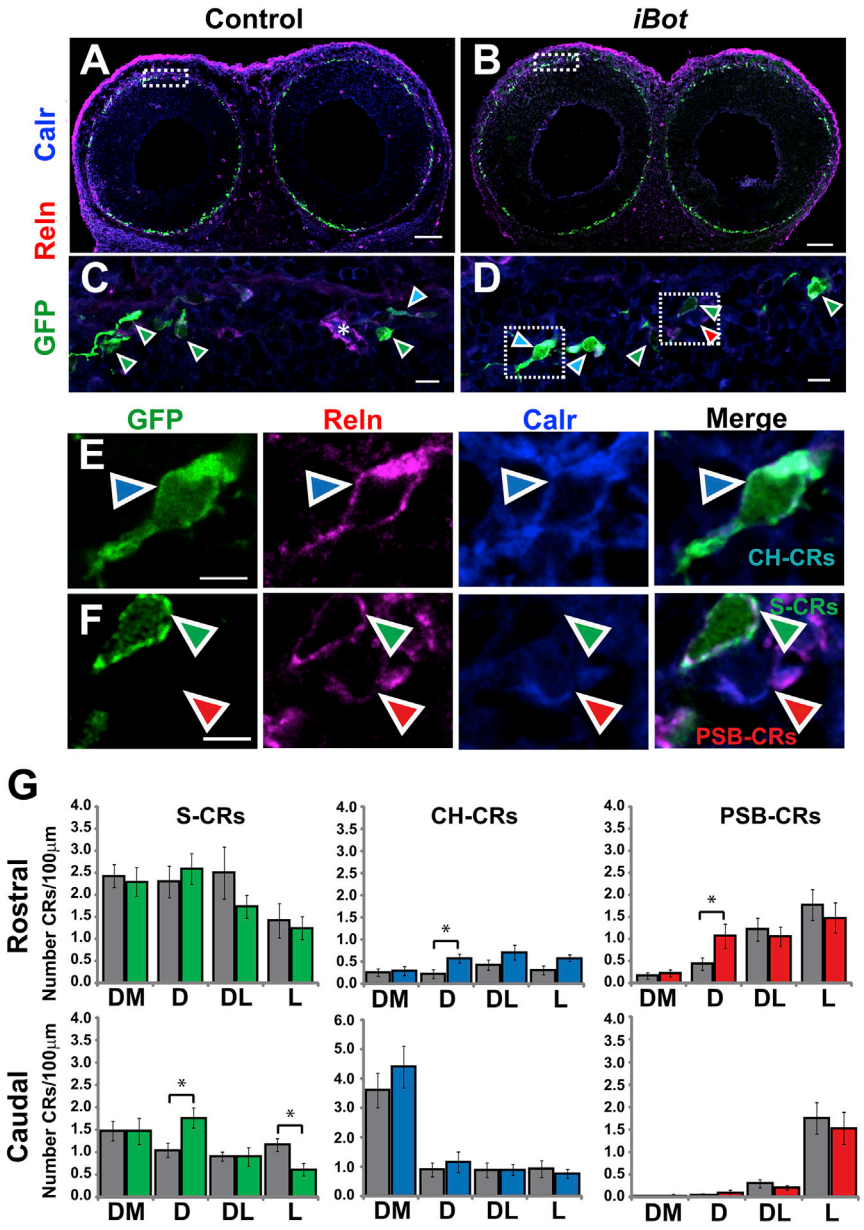

Figure 4. Redistribution of CR Subtypes in the Developing Cerebral Cortex of *iBot* Mutants at E11

(A–F) Confocal images of coronal sections through the rostral telencephalon from E11 control $p73^{YFP}$ and *p73;iBot* embryos immunostained for GFP (green), Reln (red), and Calr (blue). (C and D) High magnifications of the dorsal pallium corresponding to boxed regions in (A) and (B). Asterisk denotes blood vessel. (E and F) High magnifications of boxed regions in (C) and (D). Molecular characterization of S-CRs (GFP^+Reln^+, green arrowheads), CH-CRs ($GFP^+Reln^+gCalr^+$, blue arrowheads), and PSB-CRs ($Reln^+gCalr^+$, red arrowheads) used for quantification in (G).

(G) Quantifications at rostral (L1 in Figure S3) and caudal levels (L4 in Figure S3) along the rostral-caudal axis of the telencephalon. Gray and colored columns show counts in control (Ctrl) and *p73;iBot* mutants, respectively: S-CRs (GFP^+Reln^+, green), CH-CRs ($GFP^+Reln^+gCalr^+$ and single GFP^+ [later born CH-CRs with delayed expression of Reln and Calr, blue]), PSB-CRs ($GFP^-Reln^+gCalr^+$, red). DM, dorsal-medial; D, dorsal; DL, dorsal-lateral; L, lateral (see also Figure S3 for subdivisions along the medial-lateral axis). Graphical representation of normalized cell counts (cells/100 μm ± SEM) (n = 3 brains for each genotype). Student's t test; $^*p \leq 0.05$.

(H) Schema summarizing the distribution and inferred migration pathways of CR subtypes along the rostro-caudal axis in E11 controls and *iBot* mutant telencephalons. Quantifications of CR subtypes (Figure S3) suggested that faster migrating CH-CRs preferentially follow a lateral route to redistribute in rostral territories in *iBot* mutants (blue arrows). High magnifications of yellow and brown boxes highlight the distribution in rostro-lateral and caudo-medial territories, respectively. Double-headed arrows indicate faster migrating CRs.

Scale bars, 100 μm (A and B), 10 μm (C and D), and 5 μm (E and F). See also Figure S3.

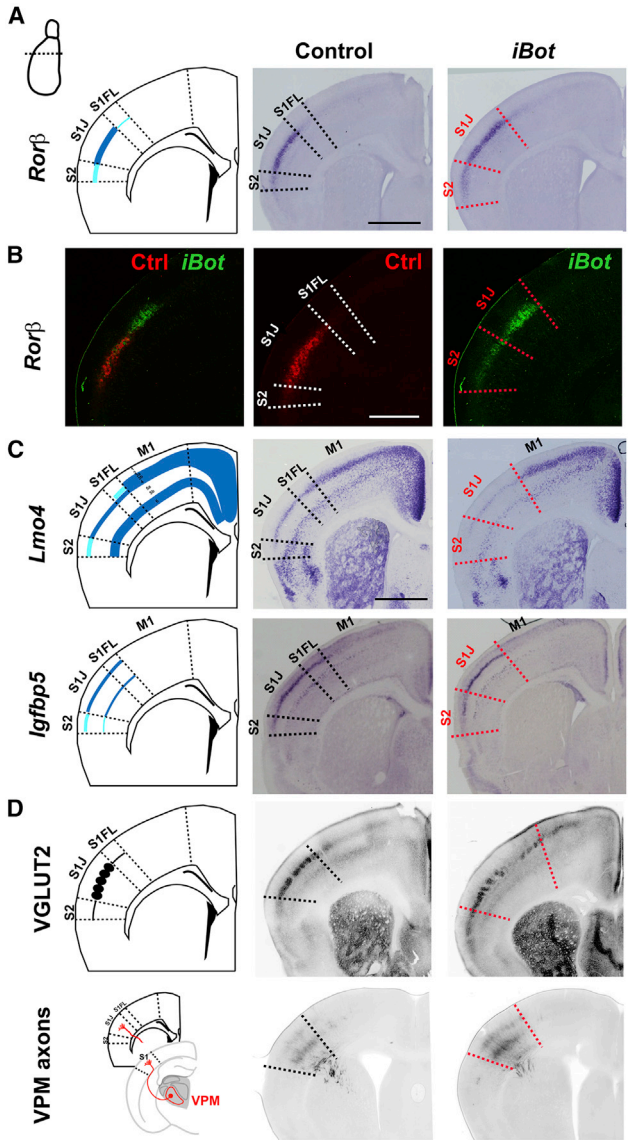

Figure 5. VAMP1–3 Regulates the Position, Size, and Connectivity of Rostro-lateral Cortical Areas

(A–C) In situ hybridization on coronal sections of P8 control and *p73;iBot* mutant brains using mRNA probes for *Rorβ* (n = 3) (A and B), *Lmo4* (n = 5), and *Igfbp5* (n = 3) (C) at rostral levels. (A) shows a dorsal displacement of S1J at the expense of S1FL and an enlargement of S2. (B) *Rorβ* mRNA localization shown in artificial colors for control (red) and *iBot* mutant (green) brains. The left-hand panel shows merged false-color images highlighting the dorsal shift of S1J in mutants. (C) shows the M1/S1 border, demarcated by *Igfbp5* staining of S1, is not altered.

(D) Immunohistochemistry for VGLUT2 and anterograde labeling of thalamocortical axons from the VPM on coronal sections of P8 control and *p73;iBot* mutant at rostral levels showing re-targeting of afferents to the dorsally displaced S1J.

Cartoons in (A) and (C) display the layer-specific molecular code for each area and in (D) the projections from the VPM thalamic nucleus. Dashed lines delimit each cortical area in controls (black) and changes in mutants (red). Low expression levels, light blue; high levels, dark blue. S1J, primary somatosensory cortex jaw region; S1FL, primary somatosensory cortex forelimb region; S2, secondary somatosensory cortex; M1, primary motor cortex; VPM, ventral posterior medial nucleus. Scale bars, 500 μm (A–C and apply to all other panels). See also Figures S4 and S5.

distribution alter cortical patterning, we first assessed whether the regionalization of the cortex was altered in E11.5 $p73^{Cre}$;*iBot* and control brains by in situ hybridization. This showed no changes in the expression of genes that specify rostral (*Fgf8)* and caudal (*Wnt3a)* signaling centers or in *Pax6*, *Sp8*, *Emx2*, and *COUP-TF1* corresponding to transcription factors whose graded expression is involved in early cortical regionalization (Figure S4) [3]. Moreover, we found the regionalized expression of *Wnt8b* and *Fgf17*, which, respectively, delineate the medial and rostral subdivisions of the cortex [40, 41] were comparable in $p73^{Cre}$;*iBot* and control brains. Together, these results show that inactivation of VAMP2 and VAMP3 in S-CRs and CH-CRs does not alter signaling centers or early cortical patterning. We next examined area formation in $p73^{Cre}$;*iBot* brains at postnatal day 8 (P8) by in situ hybridization on coronal sections using area and layer-specific markers *Rorβ*, *Lmo4*, and *Igfbp5* (Figures 5, 6, and S5). No changes in the border between S1 and primary motor cortex (M1) were detected, as shown by the extent of high *Igfbp5* and *Lmo4* expression (labeling S1 and M1, respectively, at this level) (Figure 5C). Intriguingly, however, we detected changes in subregions of S1 at rostral levels, namely, the replacement of somatosensory forelimb fields (S1FL) by somatosensory jaw territories (S1J) (as labeled by low *Rorβ* expression in layer 4 of S1FL and high of S1J) in *iBot* mutants compared to controls (Figures 5A, 5B, 6C, and S5A), although the overall size of S1 was unchanged. This was associated with an enlargement of secondary somatosensory cortex (S2) (low *Rorβ*, *Lmo4*, and *Igfbp5* expression). Notably, immunocytochemistry against VGLUT2, which is expressed specifically by the presynaptic terminals of thalamocortical synapses [5] and anterograde labeling of thalamo-cortical axons from the ventral posterior medial (VPM) nucleus, which conveys input from the snout and jaw, also showed a dorsal shift of layer 4 afferents (Figure 5D) and confirmed a re-routing of sensory connections. Furthermore, at mid-caudal levels medial visual secondary cortex (V2ML/MM) was replaced by parietal association cortex (LPtA/MPtA) as shown by *Lmo4* mRNA expression restricted to layers 4 and 6 and low levels of *Rorβ* in layer 4 characteristic of LPtA/MPtA in *iBot* mutants compared to controls (V2ML/MM expressing *Lmo4* in layers 3, 5, and 6 and no *Rorβ*) (Figures 6A and 6C). Analysis along the entire rostro-caudal axis indicated a 400-μm caudal expansion of parietal association cortex at the expense of visual secondary areas (Figure S5B). An increase in the size of primary somatosensory (S1)/parietal posterior association (PtPR/PtPD) (S1/PtP) cortex and a reduction of auditory secondary areas (AuD) were also observed at this level. Similar phenotypes were detected in *Vamp3* mutants. In particular, V2ML/MM was replaced by LPtA/MPtA, S1/PtP was increased at the expense of AuD (Figure S6A), and the lateral visual secondary cortex (V2L) was replaced by visual primary cortex (V1) (Figures 6B, 6C, and S6A). In contrast, no differences in the size of S1 and S2 areas were detected at rostral levels in *Vamp3* mutants (Figure S6B). Thus, VAMP3 inactivation results in the expansion of parietal association cortex and V1 at the expense of visual and auditory secondary areas. Collectively, our data indicate that recycling endosomal VAMPs, most likely primarily VAMP3, modulate the speed of S-CRs and CH-CRs migration in a cell-autonomous manner and their redistribution in embryonic cortical territories. It further shows that CR repartition is involved

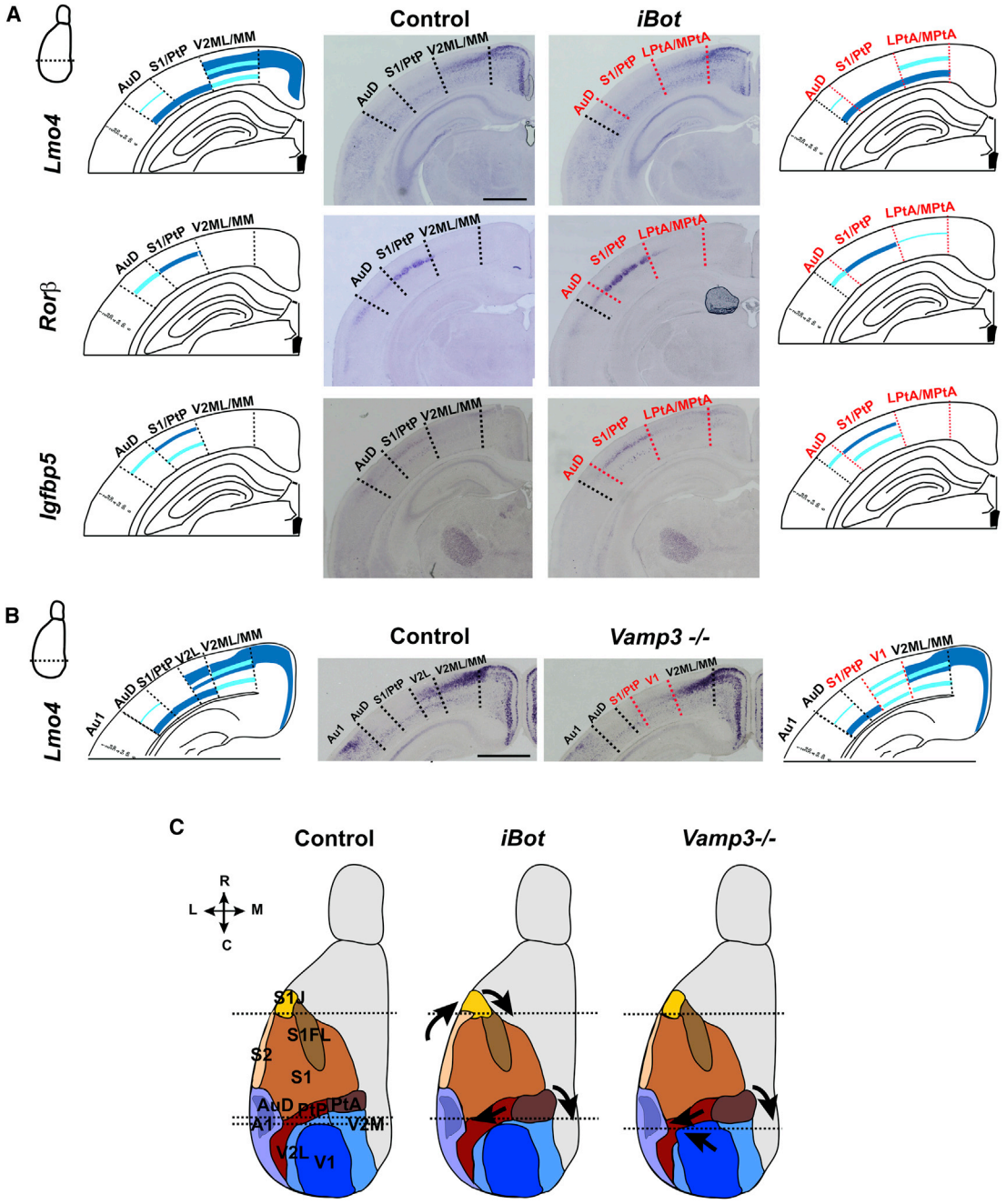

Figure 6. VAMP3 Regulates the Size of Higher-Order Cortical Areas in the Caudal Neocortex

(A) In situ hybridization on coronal sections of P8 control and *p73;iBot* mutant brains using mRNA probes for *Lmo4* (n = 5), *Rorβ* (n = 3), and *Igfbp5* (n = 3) at mid-caudal levels showing a replacement of V2ML/MM by LPtA/MPtA and an enlargement of S1/PtP at the expense of AuD.

(B) In situ hybridization on coronal sections of P8 *Vamp3*$^{+/-}$ (control) and *Vamp3*$^{-/-}$ mutant brains at caudal levels using an *Lmo4* mRNA probe showing V2L is replaced by V1 (n = 3 for each genotype).

(C) Cartoon of arealization defects in *iBot* and *Vamp3* mutant brains. Dashed lines show levels along the anterior-posterior axis corresponding to coronal sections in (A) and (B) and Figure 5 for rostral levels.

Cartoons in (A) and (B) display the layer-specific molecular code for each area. Dashed lines delimit each cortical area in controls (black) and changes in mutants (red). Low expression levels, light blue; high levels, dark blue. S1J, primary somatosensory cortex jaw region; S1FL, primary somatosensory cortex forelimb region; S2, secondary somatosensory cortex; M1, primary motor cortex; V2ML/MM (V2M in C), secondary visual cortex mediolateral/mediomedial; LPtA/MPtA (PtA in C), lateral parietal association/medial parietal association cortex; S1/PtP (PtP in C), primary somatosensory/parietal association cortex posterior rostral and dorsal; AuD, auditory secondary cortex dorsal (AuD). Scale bars, 1 mm (A and B). See also Figure S6.

in controlling the size of higher-order areas in the postnatal cerebral cortex and somatotopic targeting of sensory afferents from the thalamus (Figure S6C), without affecting signaling centers.

DISCUSSION

Our findings reveal that in the cerebral cortex, the migration kinetics of early-born signaling neurons regulates the size of higher-order areas, whose expansion is a hallmark of cortical evolution. We show that speed of migration is controlled cell-autonomously in CR subtypes by VAMP3-dependent vesicular trafficking mechanisms, which determines their distribution and, likely, their spatial-temporal signaling activities in cortical territories, to ultimately influence the size and connectivity of higher-order cortical areas. Mechanistically, our results demonstrate for the first time a role for VAMP3 in postmitotic neurons (CRs) and in early brain development. Previously, this v-SNARE was thought to be expressed exclusively in non-neuronal cells in the mature brain and was considered to have a redundant role with VAMP1 and VAMP2 when expressed in the same cells [29, 42]. Our observations reveal a key role for this v-SNARE in modulating the migration speed of an important neuronal population during cortical development and in regulating the size and wiring of higher-order areas in the auditory, visual, and somatosensory systems.

Mechanisms of CR Migration and Vesicular Trafficking

Our results show that the specific repartition of CR subtypes in the developing cortex is crucial for cortical wiring. This repartition is determined by subtype-specific migration kinetics and, in particular, speed and directionality. Several molecular pathways have been recently shown to regulate CR migration, namely, Ebf2/3, Cxcl12/Cxcr4, Eph/Ephrins, and Sema3E/PlexinD1 [27, 43–45]. However, these studies focused on migration from the cortical hem and little was known regarding the migration of CRs from other sources. Indeed, it was suggested that common mechanisms govern the movement of all subtypes on a 2D substrate in vitro [27]. Here, we reveal a more complex regulatory mechanism in which CR subtypes differ in their onset, kinetics, and direction of migration. Using mathematical models, we predict that differences in migration speed and DI are primary parameters that regulate CR subtypes differential invasion and occupancy of cortical territories. We confirm these predictions by identifying a novel VAMP3-dependent mechanism that cell-autonomously modulates the migration speed of two CR subtypes, S-CRs and CH-CRs, and show that impairing VAMP3 activity increases their migration speed and dispersion thereby altering the composition of CRs in specific cortical territories in vivo. Contact repulsion was previously shown to be a mechanism by which CRs disperse and reciprocally prevent their invasion of adjacent cortical territories [23, 25, 27, 44]. Notably, abrogation of Ephrin/Eph signaling indicated that, while this perturbed contact-repulsive interactions between CRs and increased their directionality index in vitro, it did not affect their migration speed and general motility [27]. We reciprocally show that impairing VAMP3 activity in migrating CRs resulted in faster migration speeds but did not alter their directionality index. Moreover, we observed no significant differences in migratory behavior in response to contact between CR subtypes or overtime although they differed with respect to speed and directionality. Together, these results suggest that directionality and speed of migration are independently controlled by two distinct molecular pathways, likely involving Ephrin/Eph-mediated contact repulsion and VAMP3-mediated vesicular traffic, respectively.

Our data identify a novel molecular pathway that negatively modulates CRs migration speed. Recently, Sema3E/PlexinD1 was shown to decrease CH-CRs motility by negatively modulating ADF/Cofilin signaling downstream of PlexinD1, the former being required to initiate actin polymerization and thus regulate leading process morphology and chemotaxis [45]. Similar to *Vamp3* and *iBot* mutants, an increased dispersion of CH-CRs to more distant cortical territories was reported in *PlexinD1*-null mice in vivo. Notably, VAMP2-dependent vesicular trafficking of PlexinA1/Neuropilin1 receptors has been shown to underlie Sema3A-dependent repulsion during axon guidance [37]. This raises the possibility that defects in VAMP3-dependent recycling of PlexinD1 receptors could impair CH-CRs response to Sema3E and mediate the faster migration speed and redistribution in *Vamp3* and *iBot* mutants.

Our data indicate that VAMP3 is the most important of the VAMP1–3 subfamily of recycling endosome members implicated in this process based on transcriptomics data, immunohistochemical analysis, and the similar cortical phenotype of *iBot* mutants with abrogation of VAMPs1–3 specifically in CRs and *Vamp3* mutants with a ubiquitous invalidation of the gene. Moreover, we find CR subtypes differentially express VAMP proteins with S-CRs and CH-CRs enriched in VAMP3 and PSB-CRs in VAMP2, which could underlie their distinct behaviors. VAMP2 has been shown to regulate axonal repulsion and growth cone attraction by asymmetric membrane trafficking and exocytosis [38, 46]. Growth cone steering in navigating axons and in the leading process of migrating neurons share common mechanisms, with the former suggested to result from an imbalance of localized exocytosis and endocytosis [46]. It remains to be shown whether VAMP2 and VAMP3 differentially regulate endocytosis and exocytosis at leading edge of migrating CR subtypes and give rise to differences in their motility and directionality.

A role for polarized exocytosis has also been shown to underlie autocrine modes of migration through the secretion of chemokinetic gradients, which promote cell motility [47]. Interestingly, inactivation of VAMP3 in epithelial cells has been reported to inhibit migration and integrin trafficking in cultured cells [31] rather than promoting it, suggesting that VAMP3 may regulate the motility of CRs by different mechanisms or that the context of brain slices and developing brains in situ (this study) may differ from in vitro cultures, the latter of which has been shown to favor amoeboid migration [48]. Notably, autocrine stimulation of cell motility through the secretion of EGF modulates migration speed more efficiently than exogenous stimulation [49]. While the molecular partners underlying this mechanism remain to be fully explored, our results further suggest that VAMP3-recycling endosomes may secrete molecules regulating CR migration. Among possible VAMP3-dependent CR secreted molecules, Reelin appears as a good candidate given its established role in the radial migration of glutamatergic neurons [50], the dispersal of progenitors in the rostral migratory stream [51], and the migration of epithelial cells [52]. Recently, the *Drosophila*

ER/Golgi SNARE member Ykt6 was shown to mediate the trafficking of Wnt morphogens through endosomal compartments and their secretion in exosomes [53]. Given that CRs are also enriched in morphogens [25], it is possible that VAMP3-dependent secretion of several molecules including Reelin and morphogens by CR cells may generate an autocrine feedback loop to control their speed and, therefore, their signaling activity in a spatiotemporal manner. This autocrine feedback loop would operate as an intrinsic speed rheostat for CR migration. The simultaneous coupling of CRs motility with potential morphogen secretion would exert a strict spatiotemporal regulation of CRs' signaling activities in cortical territories.

CRs Distribution and Cortical Area Patterning

We have previously shown that CRs are enriched in morphogens and that the ablation of S-CRs between E10.5 and E11.5 results in an invasion of the depleted territories by the other CR subtypes by E12.5 [25]. This influences early patterning gradients of transcription factors and the position and size of the primary motor cortex. Here, we show that impairing VAMP1–3 activity increases S-CRs and CH-CRs migration speed and results in their ectopic redistribution in more distant territories thereby altering the composition of CRs in specific cortical territories in vivo. In particular, CR subtypes specifically redistribute in the dorso-lateral and dorsal cortex at rostral and caudal levels, respectively, without changes in the total number of CRs in the cortex. Intriguingly, this leads to non-cell-autonomous changes in the size of higher-order cortical areas in the somatosensory, auditory, and visual cortices, without altering that of territories allocated to primary cortical function and in the absence of defects in signaling centers or early cortical patterning. Recently, the size of primary cortical areas has also been reported to be altered in hem-ablated mutants, which present a total loss of hem-derived CRs in addition to the cortical hem itself [54]. Together, these results suggest that the total number of CRs is crucial in controlling the size of primary cortical areas, whereas the specific redistribution of CR subtypes in the absence of CR loss is involved in regulating the size of areas that form at regions interspersed between primary areas, namely, secondary and associative territories.

Our mathematical simulations highlight how small changes in migration kinetics affect the distribution of CRs in specific embryonic territories. In addition to an ectopic invasion of the dorsal cortex, these predict a change in composition and increased mixing in the dorso-lateral and dorso-medial cortex at rostral and mid-caudal levels, respectively. These territories are located where gradients of expression of transcription factors, involved in early cortical regionalization [3], are at their lowest levels and specifically at the intersection of opposing patterning gradients. These embryonic territories may correspond to regions interspersed between primary areas in the adult animal where secondary and association areas form and that are of foremost importance for higher level processing in primates and humans. Our time-lapse studies show that the first streams of CRs leaving the progenitor domains at E10 stop migrating when reaching the dorsal cortex while subsequent streams continue migrating from these sources and bypass the previously positioned CRs to fill up available space. Our analysis of *iBot* mutants at E17.5 also show that CRs preferential distributions are still observed at late stages of development. These results strongly argue that the first streams of CRs are anchored to specific cortical territories and that their preferential distributions are established and, at least in part, maintained from the earliest stages of corticogenesis. Consistent with this, it has been shown that CRs are misplaced in layer 1 in the absence of radial glia [55] suggesting that this interaction is involved in maintaining the position and distribution of CRs. This would ensure a temporal and spatial control of their signaling activity and a stable point-to-point link between early cortical regions and areal borders at later stages. Our work, thus, suggests a novel mechanism by which signaling through a precise composition of CR subtypes is required to pre-pattern higher-order cortical territories by delineating the size of these territories. In addition, this also influences the targeting of thalamo-cortical axons, which will later refine patterning by experience-driven inputs [4, 5] (Figure S6C). This is consistent with previous reports that showed that the borders of the secondary visual cortex surrounding primary visual area are not influenced by sensory inputs [4] but is within a uniform visual territory that the distinction between primary and secondary areas occurs upon the arrival of thalamo-cortical inputs at postnatal stages [5]. CRs have previously been shown to guide entorhinal-hippocampal axons [15] and to further integrate in early cortical circuits [17], thus raising the possibility that CRs could additionally influence the differentiation of higher-order areas through promoting the maturation of cortical columns. Pre-patterning through VAMP3-mediated secretion regulating migration and distribution of CR subtypes in prospective higher-order cortical territories would, thus, ensure a bifunctional mechanism allowing for robustness of patterning by setting the size of these territories at embryonic stages and plasticity by later refinement driven by sensory experience. Notably, narrow areas both at the occipital-temporal and parietal-temporal borders have been described in rodents, in addition to primates, which contain a mixture of modality-specific neuronal types as well as multisensory neurons [56]. These multisensory populations in these border regions represent a vast proportion of the neurons suggesting that these transitional zones may integrate both cross-modal and multisensory information processing. The territories, whose size we find to be influenced by VAMP3-mediated redistribution of CR subtypes, comprise secondary and association areas of the somatosensory, auditory, and visual systems and are all located at these borders between uni-modal primary areas and are characterized by a mixed cytoarchitecture [57]. This strongly supports the view that they are territories likely responding to cross-modal and/or multisensory inputs. The relative size of cortical territories dedicated to higher-order areas have particularly evolved in humans. Our results open the intriguing possibility that pre-patterning through a tight control of kinetics of migration of signaling CR subtypes might have contributed to the evolution of cortical connectivity.

EXPERIMENTAL PROCEDURES

Ethics Statement

All animals were handled in strict accordance with good animal practice as defined by the national animal welfare bodies, and all mouse work was approved by the Veterinary Services of Paris (authorization number: 75-1454) and approved by the Animal Experimentation Ethical Committee Buffon (CEEA-40) (reference: CEB-34-2012).

Animals

Dbx1^{iresCre} [23, 25] and *DeltaNp73*$^{CreiresEGFP/+}$ [26] were crossed with *ROSA26*$^{loxP\text{-}STOP\text{-}loxP\text{-}YFP}$ reporter line to permanently trace CR subtypes of the *Dbx1* (septum and PSB) and *DeltaNp73* (septum and cortical hem) lineages. Mice in which Botulinum neurotoxin B is expressed upon Cre recombination (*iBot* line; Tg[CAG-boNT/B,-EGFP]U75-56Fwp/J) [39] were crossed with *DeltaNp73*$^{CreiresEGFP/+}$ mice to specifically cleave VAMP 1,2,3 in migrating CR cells. Global inactivation of VAMP3 was analyzed in *Vamp3*-null mice [42].

Whole Flattened Cerebral Cortical Preparations

Flattened whole cerebral cortices were dissected from E10-E12 *Dbx1*$^{Cre/+}$;*ROSA26*$^{YFP/+}$ (*Dbx1*^{YFP}), *DeltaNp73*$^{Cre/+}$;*ROSA26*$^{YFP/+}$ (*p73*^{YFP}), and *DeltaNp73*$^{Cre/+}$;*iBot:GFP* (*p73;iBot*) embryos, ensuring the meninges were preserved and cultured for 4 hr on Millicell (Millipore) permeable membrane inserts in phenol-red-free high glucose DMEM (Sigma) with B27 serum (Gibco) and 100 μg/ml penicillin/streptomycin antibiotics (Sigma) at 37°C with 5% CO_2 prior to live imaging. Immediately before imaging, the flattened cortical explants were transferred with the underlying Millicell membranes and inverted onto a bespoke glass-bottom microscope imaging chamber with the pial surface directly adjacent to the glass, and secured by adding 100 μl of purified bovine collagen (Advanced Biomatrix), which was polymerized at 37°C and 5% CO_2. Inverted confocal microscopy (Leica 710 and Leica 780) was used for live-image acquisition over 4–14 hr at 37°C and 5% CO_2 in a regulated chamber. Time lapses were analyzed with the spot cell-tracking module of Imaris software.

Mathematical Simulations of the Dynamics of CR Cell Distribution

Mathematical simulations were generated according to the experimentally obtained parameters of (1) onset of migration, (2) mean speed of each CR subtype, and (3) persistence in directionality (directionality index [DI]) (see the Supplemental Experimental Procedures). Cell migration was modeled by the weighted sum of two components: (1) random motion (walking) and (2) contact repulsion between cells. Mathematically, it is given by the following stochastic differential equations:

$$dx_i = w_1 dw_x - w_2 \left(\frac{\partial}{\partial x_i} \sum_j U(r_{i,j}) \right) dt \quad (1)$$

$$dy_i = w_1 dw_y - w_2 \left(\frac{\partial}{\partial y_i} \sum_j U(r_{i,j}) \right) dt, \quad (2)$$

where (x_i, y_i) is the position of the i-th cell, and w_j $(j = 1, 2)$ is the weight of each component of movement. The first term of the right-hand side represents Brownian motion where dw_x and dw_y are the increments obeying Gaussian distribution $N(0, dt)$, respectively. In the second term, U is a potential energy for generating the force of contact repulsion, and, in particular, we used the following quadratic function:

$$U(r_{i,j}) = \begin{cases} \left(\frac{r_{i,j}}{L} - 1\right)^2 & (0 < r_{i,j} \leq L) \\ 0 & (L < r_{i,j}) \end{cases},$$

where $r_{i,j}$ is the center-to-center distance between cell i and j. The contact repulsion between cells is assumed to occur when the intercellular distance is within L, and its effect is stronger when the distance is smaller, which was implemented not as an "all or none" response but as a response showing an inverse linear relationship with intercellular distance. The value of L was determined by the sum of mean sizes of cell body and filopodia (specifically, we used L = 55 (μm) in the simulations) (see also the Supplemental Experimental Procedures).

For experimental procedures on tissue preparation, in situ hybridization, immunohistochemistry, anterograde labeling, and quantifications, see the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.08.028.

AUTHOR CONTRIBUTIONS

Y.A. and Y.M. contributed equally to this work. M.B. and A.P. conceived the study. M.B. carried out most of the experiments. Y.A. and Y.M. designed and performed the mathematical simulations, L.V. the arealization analysis, F.C. early regionalization analysis and VAMP2 immunostaining, U.B. microarrays and MATLAB analysis, E.C. and F.L. analysis of late CR distribution, S.G. and D.J. anterograde labeling and vGlut2 staining and analysis, and V.C. participated in the analysis with the Imaris software. V.P.-G. and T.G. provided expertise on VAMPs and membrane trafficking, *Vamp3* mutant mice and helped designing the experiments. F.W.P. provided the B6;FVB-Tg(CAGboNT/B,EGFP and F.T. the *DeltaNp73* mouse lines. M.B., Y.M., Y.A., V.P.-G., T.G., D.J., and A.P. wrote the manuscript. A.P. supervised the project. All authors discussed and edited the manuscript.

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