Cortical astrocytes develop in a plastic manner at both clonal and cellular levels

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Astrocytes play essential roles in the neural tissue where they form a continuous network, while displaying important local heterogeneity. Here, we performed multiclonal lineage tracing using combinatorial genetic markers together with a new large volume color imaging approach to study astrocyte development in the mouse cortex. We show that cortical astrocyte clones intermix with their neighbors and display extensive variability in terms of spatial organization, number and subtypes of cells generated. Clones develop through 3D spatial dispersion, while at the individual level astrocytes acquire progressively their complex morphology. Furthermore, we find that the astroglial network is supplied both before and after birth by ventricular progenitors that scatter in the neocortex and can give rise to protoplasmic as well as pial astrocyte subtypes. Altogether, these data suggest a model in which astrocyte precursors colonize the neocortex perinatally in a non-ordered manner, with local environment likely determining astrocyte clonal expansion and final morphotype.
Brain functions rely on the efficient cooperation of neurons and glial cells to shape and operate neural circuits. In the cerebral cortex, the center of higher cognitive functions in mammals, numerous studies have deciphered neuronal contributions to cortical circuits, and described how excitatory neurons issued from dorsal progenitors migrate in a stereotyped manner to reach their final location. However, the development of glial cells generated by these same progenitors, in particular astrocytes, remains far less understood, despite their many crucial roles in brain circuit function and formation. Beside their essential function at the blood brain barrier where they regulate nutrient uptake and blood flow, astrocytes also play critical roles in neuronal survival and synaptic function, recycling neurotransmitters and buffering potassium ions. Other studies have also uncovered their active participation in synapse formation and pruning.

Normal resting astrocytes present two paradoxical characteristics: closely apposed to their neighbors, they tile the gray matter and form a seemingly uninterrupted tridimensional array, suggesting that they belong to a homogenous type; at the same time however, it is increasingly recognized that they constitute a heterogeneous population at the morphological, molecular, and functional levels. Considerable divergence exists among astrocyte subtypes such as the fibroblast-like glia limitans of the pial surface and the protoplasmic astrocytes (PrA) of the cortical parenchyma, which themselves display layer-related differences. Former studies have established that cortical astrocytes originate from a fraction of delaminating embryonic progenitors of the dorsal telencephalon (see ref. for review), while a possible additional contribution of the postnatal cortical parenchyma by astrocyte precursors, local proliferation and spatial dispersion progressively abate. Moreover, we uncover their active participation in synapse formation and pruning.

Astrocyte clones show variable and intermixed organization. Tridimensional mapping with ChroMS microscopy revealed a high variability of PrA clones in terms of both their 3D spatial dispersion and number at P7 and P21 stages with near-micrometric resolution, thus giving us access to the structural and tridimensional arrangement of each labeled clone, with all their astroglial cells accounted for.
the volume of individual astrocyte domains, Fig. 2d, e, Supplementary Fig. 2e, f), and there was hence significant intermixing with cells of neighboring clones. The spatial arrangement and volume of the clones were highly variable, at P7 as well as P21 (Fig. 2d, e, Supplementary Fig. 2f, g, also see Supplementary Dataset showing the 3D layout of each clone). Yet at both stages, we found that PrA clones were composed of a similar number of disconnected elements (2.8 ± 0.3 at P7 vs. 2.9 ± 0.3, at P21, Fig. 2f, g), and the proportion of clustered clonal PrA remained stable (78.7% ± 4.5 at P7 and 73.8% ± 4.0 at P21, Fig. 2h). The absence
MM-labeled cortex, and visualization of astrocyte clones spatial arrangement (arising from distinct cortical progenitors. Multiphoton (ChroMS) microscopy relies on the integration of trichromatic two-photon excitation by wavelength mixing with automated blockface imaging. Adapted from the graphics used in Abdelalim et al., Nat. Commun. 2019 Apr 10;10(1):1662, and licensed under a Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/.

Astrocyte arbor expansion: A time-lapse study. To understand the astrogial anatomical network develops during P7 in absence of proliferation, we analyzed the morphology of individual somatosensory PrA imaged by ChroMS microscopy through semiautomated segmentation (Fig. 2i, Supplementary Fig. 3a, b). We observed that at an individual level, the volume of neural tissue that they covered (termed domain or territorial domain between P7 and P21. These data explain why astrocyte clones at P4 and P7, during and at the end of their proliferative period, showed considerable heterogeneity along both the DV and mediolateral (ML, Supplementary Fig. 4b) axes, with clones that could for instance form tight clusters or spread over most of the cortical wall. In addition, we observed that the relative dispersion of the clones (spread of clones divided by total number of sister cells) did not increase between P4 and P7, showing that astrocyte local proliferation during that period was not accompanied by a wide scattering of clonally related cells. Instead, cells densified over time, as indicated by a reduced relative dispersion (Fig. 3f and Supplementary Fig. 4c). To better understand this process, we analyzed sister cell proliferation within clones. We injected EdU either 48 or 24 h prior to analysis at P4 and P7 (Fig. 3g, h) in order to determine whether recently divided sister astrocytes stay together as pairs of closely apposed cells, long considered features of astrocyte proliferation, or move away from each other over time. We found that sister cells labeled with EdU 48 h prior to sacrifice were located further apart than those marked 24 h in advance, and this both at P4 and P7 (Fig. 3i), showing that their cell bodies moved apart from each other over time. However, markedly smaller spacing (on average inferior to the diameter of astrocyte domains) was observed at P7, indicating that network expansion slowed down as development proceeded (Fig. 3i). Interestingly, we observed that 20% of MM-labeled cells belonged to sister cell “doublets” separated by a distance inferior to the mean size of astrocyte nuclei (6 µm), irrespective of the developmental stage (Fig. 3j, k). In addition, whereas these doublets were not preferentially located in specific cortical layers (Fig. 3l), proliferating sister cells labeled with EdU were mostly found in the upper cortical layers (Fig. 3m). This, added to the drastic drop in the proliferation rate during the first postnatal week (almost null at P21, see ref. 31), and the observation of astrocyte doublets negative for the proliferation marker Ki67 (Fig. 3n), led us to conclude that astrocyte doublets are not a reliable indicator of recent divisions but can result from the stalling of astrocyte expansion in late postnatal development. Altogether, these data suggest that astrocyte clone expansion, after an early phase of non-cohesive proliferation that generates the dispersed patterns observed at P4, evolves towards increasingly cohesive cell divisions, leading to densification of the clones between P4 and P7. Newly generated sister cells move away from their siblings at a distance that diminishes over time and the final...
matrix architecture reflects the progressive stalling of this process, with numerous astrocytes remaining as doublets (Fig. 3o).

**Single progenitors can produce distinct astrocyte subtypes.** Next, we investigated the subtype composition of astrocyte clones (Fig. 4, Supplementary Fig. 5a, b). Three types of clones could be unambiguously identified based on the distinct morphologies of the cells that they comprised: (i) homogeneous PrA clones (Fig. 4a), (ii) homogeneous pial astrocyte (PiA) clones (Fig. 4b) found at the pial surface, and (iii) heterogeneous clones containing both PrA and PiA (Fig. 4c). The vast majority of labeled clones were composed of only PrA (76%), whereas exclusively PiA clones represented a minority (5%) and 19% contained both PrA and PiA (Fig. 4d), with most PiA (>80%) belonging to heterogeneous clones. This differed from previous work that supported the existence of distinct lineages for these two astrocyte subtypes playing specific roles in the adult brain. Even as
Fig. 2  Clonal organization of astrocytes remains stable from P4 to P21 while their arbor expands and complexifies. (a, b) 3D analysis reveals variable PrA clonal dispersion up to 1.25 and 0.5 mm along the DV and ML/ AP axis, but no difference between P7 and P21 (n = 113). c Example of a 17-cell clone where red represents nuclei and gray the astrocyte territory (left) along with Delaunay Triangulation (DT, middle) and Convex Hull extraction (CH, right). d DT of random PrA clones highlights their distinct shapes. e CH analysis shows high diversity of the volume covered by clones, but stability of its distribution between P7 and P21. f PrA clones were automatically separated into clusters of apposed cells or isolated cells (elements) using their X/Y/Z coordinates and astrocyte mean diameter + s.d. at each stage. g On average PrA clones are composed of 2.8 disconnected elements and this arrangement is stable from P7 to P21. h Totally, 76.2% of PrA belong to clusters and this proportion is stable from P7 to P21. I Segmentation of color-isolated astrocytes from ChroMS images at P7 (left) and P21 (right). j, k Segmented astrocyte territorial volume increases by 65.7% from P7 to P21 (j) and this expansion occurs in the entire cortical parenchyma, here divided in six equivalent bins (Bin 1 = pial surface, k). l High resolution reconstruction of astrocyte arborizations at P7 and P21 reveals a significant increase in branch number (m), total branch length (n), and volume of the model (o). p Close-ups of neighboring PrA labeled with distinct colors (single optical section) show incomplete filling of cortical space by P7 PrA compared to P21 PrA. DV dorsal-ventral axis, AP anteroposterior axis, ML mediolateral axis, VZ ventricular zone. Graph values indicate means ± s.e.m. Kruskal–Wallis associated with Dunn’s multiple comparisons (a) and Mann–Whitney (e, g, h, j, k, m–o) statistical tests have been performed. *, **, *** indicate p value < 0.05, <0.0005, <0.0001, respectively. N = 4 (a, e, g, h, j, k) and 9 (m–o) animals. Scale bars f: 50 (f), 20 (i, l, p) μm
Thus a significant proportion of cortical astrocytes originate from postnatally delaminating cells and like those that emigrate at embryonic stages from the ventricular niche, they disseminate into the cortical parenchyma and give rise to scattered clones that can comprise PrA and more surprisingly PIA, in contradiction with previous work. These data indicate that both embryonically and postnatally delaminated cortical progenitors contribute to the generation of...
Fig. 3 Clonal expansion based on proliferation and spacing of sister astrocytes lessens during development. a PrA clones of various sizes at P4, P7, and P21 after MM IUE at E15. b PrA clones exhibit various sizes at P4, P7, and P21, with clones composed of 1 to 42 cells. c Schematic view of DV, ML, and AP dispersion on serial sagittal sections. e DV dispersion of PrA clones ≥2 cells as a function of clone size is broad and variable. f DV dispersion per cell expressed as % of cortical thickness (DV clonal dispersion/ clone size) decreases from P4 to P7. g P4 and P7 sagittal sections co-labeled by MM IUE at E15 and EdU injection 48 or 24 h prior to analysis. Proliferating PrA are found throughout the entire cortical wall. h Examples of EdU+ PrA astrocyte identified as sister cells with color markers. i Distance between EdU+ paired PrA decreases from 48 to 24 h postinjection and from P4 to P7. j Sister cells closer than astrocyte nucleus mean size (6 µm) form doublets. k Doublets of PrA were found at P4, P7, and P21 in stable proportions (20%). l Doublets relative DV positioning (in % of cortical thickness, see Supplementary Fig. 5e) shows that they occur in the entire cortical parenchyma from P4 to P21 whereas EdU+ astrocyte pairs are located mostly in upper cortical layers (m). n Doublets found at P4–P7 are not all comprised of Ki67+ cycling cells.

Fig. 4 Individual cortical progenitors can generate both protoplasmic and pial astrocytes. a-c Examples of homogeneous clones of PrA (a) or PiA (b) and heterogeneous clones containing both astrocyte types (c). d PrA, PiA, and heterogeneous clones were found in stable proportions (respectively 76%, 5%, and 19% of clones) throughout postnatal development (P4–P7–P21), with a significant majority of homogeneous PrA. e Heterogeneous astrocyte clones comprise significantly larger number of cells than homogeneous PrA clones. f PrA clones whose barycenter is located in the upper half of the cortex (U) are larger than those in lower layers (L) at P4, P7, and P21. Clone barycenter is calculated using the mean of sister cells relative DV positions. g Examples of intermediate morphologies between PiA and PrA observed at P21, presenting both fibroblast-like features at one extremity and bushy ramifications at the other extremity. Graph values indicate means ± s.e.m. Kruskal–Wallis associated with Dunn’s multiple comparisons statistical tests have been performed. **** indicates p value < 0.0001. N = 6 (b), 10 (i), 9 (k) animals. Scale bars: 50 (a), 100 (g), 20 (h, j, n) µm.
both PrA and PiA astrocyte subtypes. These progenitors seed the cortical parenchyma in a scattered manner, in contrast with the orderly cohorts of neurons that form sequential layers during the course of corticogenesis.

**Discussion**

Altogether our data provide a comprehensive view of astrocyte network formation and maturation during mouse cortical development. This complex process, spread out during prenatal...
Fig. 5 Cortical astrocytes arise from Olig2+ seeding units issued from both pre- and postnatal progenitors. a MM electroporated at E15 colocalize with Olig2 marker at P1 (stars in bottom image) but not with S100β (top). b The percentage of Olig2+ cells among MM-labeled cells increases from 5% at E18 to 10% at P0 and is then stable until P1, while no MM+/S100β+ cell is detected at these stages. c IUE of MM in Olig2Cre+ mice at E13 results in labeling of astrocyte-like cells at P7 both at the pial surface and in the cortical parenchyma. d Example of a P7 MM-labeled clone occupying three consecutive serial sections comprising S100β+, Aldh1l1+, and Olig2+ cells, revealed by immunostaining. e IUE of MM in E13 Olig2Cre+ embryos yields sparse recombined cells at E18. Other cells express nuclear EBFP2, indicating efficient targeting of cortical progenitors. f Olig2+/Aldh1l1+ cells are found scattered in the E18 cerebral cortex after E15 IUE of integrative Tol2CAG-mEYFP vector. g P1 co-electroporation of episomal CAG-RFP and integrative Tol2CAG-mEYFP vectors labels few astrocytes expressing RFP and markedly more expressing EYFP at P7, among both protoplasmic and pial subtypes (arrows). h PO electroporation of SeCre plasmid in CAG-Cytoβow mice labels clones of PrA and PiA (arrows) at P7. PrA identity is confirmed by S100β immunostaining (stars). i PO electroporation of integrative Tol2CAG-mEYFP plasmid labels YFP+ cells scattered in the entire cortical thickness at P3, several of which coexpress Olig2 and Aldh1l1. Graph values indicate mean ± s.e.m. A two-tailed Mann-Whitney statistical test has been performed. * indicates p-value < 0.05. N = 11 animals. Scale bars: 50 (a, d), 100 (c, e–i) μm

Fig. 6 Comprehensive model for astrocyte development in the mouse cortex. Until now the accepted model of mouse cortical astrocyte development consisted of a first phase where embryonic progenitors colonize the neocortical wall followed by a second step relying on local proliferation of these first settlers after birth, the contribution of postnatal progenitors being debated. Here, we propose that mouse cortical astrocytes are issued from a dual contribution of delaminated embryonic apical progenitors and early postnatal progenitors that both generate pial (PiA) and protoplasmic (PrA) astrocytes. Furthermore, our data show that during the first postnatal week (P0–P7) both pre- and postnatal progenitors scatter throughout the neocortical wall while proliferating. This dynamic phase is followed by a maturation phase (P7–P21) where the clones stop both expansion and proliferation while individual astrocytes increase their volume and the complexity of their processes.

and postnatal development, can be separated in three successive, partially overlapping steps (Fig. 6): (1) colonization of the neocortical wall by a fraction of embryonic apical progenitors which delaminate from the VZ; (2) expansion during the first postnatal week involving the local proliferation of astrocytes derived from scattered delaminated embryonic progenitors, but also continued, non-ordered colonization of the cortical parenchyma by new precursors that remain capable of generating both protoplasmic and pial astrocytes; (3) a maturation phase where individual astrocytes increase both the volume of their territory and the complexity of their processes, while addition of new astrocytes to the already existing network has ceased.

By combining multiplexed clonal labeling with MM and high-resolution 3D large volume multichannel imaging using ChroMS microscopy, we provide for the first time a detailed view of the clonal architecture of the astroglial tridimensional matrix. We show that the apparent continuity of this anatomical network conceals its disparate clonal composition. Contrary to pyramidal neuron clones, which form relatively stereotyped columnar units typically spanning the entire thickness of the cortex24, the astrocyte matrix is built of a patchwork of clones highly diverse in terms of cell numbers, subtype composition and shape (Figs. 2 and 4 and Supplementary Fig. 2). This clonal variability, characterized here in mammals, parallels that recently reported for the Drosophila neural cord where individual neuroblasts generate fixed sets of neurons but produce variable numbers of neuropil glia that adopt non-stereotyped morphologies33. The average cortical astrocyte clone is comprised of eight cells distributed in two to three separate clusters that are not systematically radially oriented. This suggests the following developmental model, supported by our time-course and birth dating data (Fig. 3): during early stages of clonal expansion, sister astrocytes can disperse in all directions (and not necessarily in strict radially restricted manner) and intermix with neighboring clones, either through non-cohesive divisions or intercalation of newly delaminated precursors. As development proceeds, clonal dispersion...
progressively diminishes, resulting in the formation of cohesive clusters of sister cells within clones (Fig. 3i). Ultimately, as space becomes restricted, newly divided cells cannot separate entirely from each other and form stable doublets with juxtaposed cell bodies (Fig. 3j). As a result of this developmental process, the astroglial matrix presents a high degree of clonal intermixing at any location in the cortex, a feature that we hypothesize could confer robustness against somatic mutations affecting a fraction of clones.

Interestingly, we were not able to link any property of astrocyte clones with their final location in the cortical parenchyma except for their size, clones present in upper cortical layers being significantly larger compared to those in lower layers (Fig. 4f). This may be due to several reasons: first, their parent progenitor may have colonized the upper neocortex early on and have had more time to divide. However, upper layers continue to be supplied by new astrocytes postnatally (Fig. 5g, h), arguing against this idea. Sparse precursor seeding in upper layers (Fig. 5i) may also necessitate larger clonal expansion to establish continuous tiling in these areas. The augmented clonal size could also be related to the presence of mitotic cues in the upper part of the cortex that could for instance be produced by the surface vasculature. In the rest of the cortex, which are the factors that may determine and regulate the size of astrocyte clones? A recent study, based on MADM genetic fate mapping has shown that astrocyte intermediate progenitor proliferation increases in a cell autonomous manner in absence of Lgl1.15 While intrinsic determinants such as Lgl1 may limit astrocyte proliferation, the variability of clonal size and patterns that we observed indicates that additional factors are likely at play. Competition for access to neurons and blood vessels may condition their ability to proliferate. Astrocytes are also likely candidates to regulate their own proliferation through competition for space and homeostatic interactions with their neighbors.33,46, which may sufficient to ensure complete tiling of the neuropil. Astrocyte doublets may result from such interplay. Molecular cues produced by astrocytes, neurons and endothelial cells could also participate in this process. The nature of these instructive signals remains to be established, as well as their source of production. A candidate may be vascular derived HBEGF, found to modulate astrocyte survival in vitro.47 Many other diffusible or membrane-bound candidates could be considered, such as proliferative growth factors that may be secreted by the basal lamina or endothelial cells, or trophic factors and transmembrane proteins produced by neighboring neurons.

The diversity of cortical astrocyte clones that we observed in terms of size and spatial localization argues against the existence of heterogeneous progenitors with distinct and deterministic behaviors. A second observation is also not in favor of such deterministic progenitors: a significant proportion of heterogeneous clones (19%) comprise both PrA and PiA, and most of PiA (>80%) belong to heterogeneous clones, arguing in favor of a common progenitor for these two subtypes. This finding is corroborated by the striking observation of cells displaying an intermediate morphology between PrA and PiA (Fig. 4g). This suggests that these two categories of astrocytes do not represent defined subtypes specified by an intrinsic program, but correspond instead to plastic morphotypes resulting from the adaptation of a single type to its local environment. Indeed each morphotype retains the ability to form close interactions with astrocytes of the other category, ensuring continuous tiling of cortical tissue. This conclusion is at odds with previous clonal studies based on the StarTrack clonal labeling strategy, which reported only a minority (20%) of PiA-containing clones that also included PrA. This led the authors to conclude that these two astrocyte subtypes derive from largely separate lineage branches.

Our discordant conclusions are likely explained by the use of a GFAP promoter to drive StarTrack clonal markers, known to be unequally regulated among astrocytes, with high PiA and low PrA expression.19,20,36 MM label both astrocyte populations more widely, thus identifying a larger proportion of bipotent precursors.

Beyond astrocyte lineage, multicolor labeling grants access to fine cellular morphology in dense labeling conditions, which allowed us to assess the morphological changes that astrocytes undertake during corticogenesis. Recently, Stogsdill and collaborators have shown an increase in the territory and volume infiltrated by V1 astrocytes between P7 and P21. Detailed reconstructions enabled by our semiautomated segmentation pipeline provide access to astrocyte arborization at these stages, showing how its complexity increases over time in situ. Our data also indicate that cortical astrocyte territorial expansion can be at least partially accounted for by the filling of gaps between astrocyte main branches (Fig. 2l, p). In addition, local adaptation of astrocytes to their substrate likely plays an important role in shaping their territory. Indeed in a recent study, Lajjakornsripan et al.15 have found layer differences in astrocyte territory that are altered in reeler mutant mice presenting inverted cortical layers. Yet, to which extent variations in astrocyte territory may be explained by modifications in the underlying neuropil remains to be examined.

The availability of the Olig2 marker also enabled us to follow the morphology of early invading glial cells. During embryogenesis, as well as at P4, these cells exhibited mainly a bipolar morphology, but contrary to neurons of the same developmental stage, they presented ramified extremities that were not strictly radially oriented (Fig. 5a, e). Thus, the morphology of young cortical astrocytes becomes gradually more complex over time, apparently independent of the timing of their arrival in the cortex or their proliferation status, but rather correlated to their environment. Indeed, at any given stage, all astrocytes appear to present a same degree of morphological complexity that progressively increases from embryonic to mature stages, independent of their mitotic status.

Tracking the earliest steps of astrocyte development has been difficult due to a lack of marker. Our findings reveal that the transcription factor Olig2, classically associated with oligodendrocyte lineage, also allows the tracing of the entire cortical gray matter astroglial lineage, extending previous findings.41–44 Although not specific to astrocytes, this marker enabled us to follow the population of young glial cells that includes astrocyte precursors as they colonize the neocortex. With this tool, we did not observe an organized pattern of migration like that of pyramidal neurons which radially migrate in cohorts related to their birth date.1,2 Non-ordered colonization of the cortex by astrocytes was confirmed by labeling postnatal progenitors, which showed the same nonrestricted potentialities as those targeted during embryogenesis to invade all cortical layers and, contrary to previous reports, to generate distinct astrocyte subtypes. We thus reconcile previous observations about the source of cortical astrocytes, delaminated embryonic26,31 versus postnatal progenitors,28–30 by showing that both contribute to astroglial development. Active division of the latter ones explains why postnatal electroporation of episomal reporter vector fails to label more than a few astrocytes.31,38 These postnatal progenitors are accessible to postnatal ventricular electroporation and could thus comprise either radial glia cells that remain present during the perinatal period, but also subependymal zone progenitors. Both delaminated embryonic and postnatal-derived astrocyte precursors colonize the neocortex seemingly at random, forming scattered seeding units at the basis of the clonal architecture described above.
In conclusion, our work argues in favor of the existence of nonspecific astrocyte progenitors generating plastic, intermixed clones and whose daughter cells may adopt different morphotypes through interactions with their environment.

**Methods**

**Transgenes.** MM (CAG-CytoB and T oligo:CAG-Nucbow transposons) and vectors expressing self-excitable Cre recombinase, PB and T2 transposons from a CAG promoter are described in ref. 34. The T oligo:CAG-MEYFP integrative vector was produced by cloning mEYFP 10 under a CAG promoter in a custom-designed plasmid bearing 5' and 3' T oligo transposition sequences. A CAG-driven vector expressing mRFP1 was also used in some experiments. All plasmid concentrations are indicated in Supplementary Table 1. Detailed maps and sequences of the vectors are available upon request.

**Animals.** Mice were housed in a 12 h light/12 h dark cycle with free access to food, and animal procedures were carried out in accordance with institutional guidelines. Animal protocols were approved by the Charles Darwin animal experimentation ethical board (CEEACID/201505). Transgenic CAG-CytoB mice were generated by pronuclear injection of the corresponding transgene linearized with HindIII and MluI. Oligo Z080 (Olig2tm2(TVA;cre)Rhi) mice were kindly provided by A. Chédotal.

**In vivo experiments.** In utero electroporation (UE): timed-pregnant females were anesthetized with Ketamine/Xylazine, injected with Buprenorphine (Bupreacre) to allow pain relief, shaved and placed on a heating pad. A midline laparotomy was performed and uterine horns were exposed under oblique illumination to allow embryo visualization. One microliter of DNA combined with sterile Fast green dye (Sigma) was injected with a Femtotjet microinjектор (Eppendorf) into the lateral ventricle of each embryo with a glass capillary (FHC, 10-10-L). A CUY21EDT electroporator (NepaGene) was used to induce 4 (resp. 3) 50 ms pulses of 80 V with 5 mm diameter Tweezertrodes (Sonidel Limited) positioned to target the dorsal wall of the lateral ventricle. Following surgery, the incision site was sutured (4-0, Ethicon) and mice were allowed to wake up in a warming chamber. Postnatal electroporation: neonatal animals were anesthetized on ice for 1 min and 2 µl of DNA combined with sterile Fast green dye (Sigma) was injected with a glass capillary (FHC, 10-10-L) into the lateral ventricle by mouth pipetting. A CUY21EDT electroporator (NepaGene) was used to induce five 50 ms pulses of 80 V with 5 mm diameter Tweezertrodes (Sonidel Limited). Depending on the experiment, recombination was induced either by ScCre DNA electroporation or Cre-expressing mouse lines. Mice were euthanized by cervical dislocation at the required developmental stage to harvest embryonic brains. Pups and adults were anesthetized with Dolethal (Vetoquinol) and perfused intracardially with 4% paraformaldehyde (Antigene, Diapath) for postnatal dissection. Dissected brains were postfixed for 4 h (embryos) or O/N (pups and adults) at 4°C under agitation in Antigene before several washes in phosphate-buffered saline 1X (PBS). Electroporation and analysis stages, DNA concentrations, mouse lines and associated genetic background used in each figure are listed in Supplementary Table 1.

**Histology and immunostaining.** Totally, 80 (embryos and pups) or 60 (adults) µm mouse brain sections were cut with a vibrating-blade microtome (VT1000S, Leica) to induce lateral ventricle by mouth pipetting. A CUY21EDIT electroporator (NepaGene) was used to induce four (resp. three) 50 ms pulses of 35 nm laser lines to separately excite EBFP2, mCerulean/mTurquoise2, EYFP, tdTomato respectively. Immunostaining was performed and uterine horns were exposed under oblique illumination to allow detection of fluorescent cells. The glass capillary (FHC, 10-10-L) was used to inject Green dye (Sigma) into the ventricle of each embryo with a glass capillary (FHC, 10-10-L). A CUY21EDIT electroporator (NepaGene) was used to induce four (resp. three) 50 ms pulses of 80 V with 5 mm diameter Tweezertrodes (Sonidel Limited). Depending on the experiment, recombination was induced either by ScCre DNA electroporation or Cre-expressing mouse lines. Mice were euthanized by cervical dislocation at the required developmental stage to harvest embryonic brains. Pups and adults were anesthetized with Dolethal (Vetoquinol) and perfused intracardially with 4% paraformaldehyde (Antigene, Diapath) for postnatal dissection. Dissected brains were postfixed for 4 h (embryos) or O/N (pups and adults) at 4°C under agitation in Antigene before several washes in phosphate-buffered saline 1X (PBS). Electroporation and analysis stages, DNA concentrations, mouse lines and associated genetic background used in each figure are listed in Supplementary Table 1.

**ChroMS microscopy.** ChroMS imaging was performed on a lab-built laser scanning confocal microscope equipped with a vibrating-blade microtome 10 using a water-immersion objective (25× 1.05 NA, XLPLN25XWMP2, Olympus) and the wavelength mixing method described in ref. 35. Imaging depth was set from 117 to 160 µm and slicing from 80 to 120 µm, with a XYZ spacing of 0.4–0.6× 0.4–0.6 × 1.5 µm.

**Image processing.** In all figures, tdTomato/mCherry/mRFP1/Alexa Fluor 594, EYFP, mCerulean/mTurquoise and EBFP2/Alexa Fluor 647 are respectively rendered in red (or magenta in Fig. 5g, i), green, blue and gray. Stitching and image contrast adjustments were performed using Fiji 145 or Fluoview (Olympus) and Photoshop (Adobe). 3D renderings were generated using Imaris (Bitplane, Switzerland).

**Clonal analysis.** The Fiji TrakEM2 plugin 36 was used to manually point labeled astrocytes and obtain their XYZ coordinates from threedimensional ChroMS datasets (Figs. 1f–k, 2a–k, Supplementary Fig. 2) or from maximal intensity projections of serial 80 µm sagittal sections imaged by tiled confocal microscopy (Figs. 1d–e, 3–5, Supplementary Fig. 1a, c, f, 4a–c, 5, and Z coordinate corresponding to section number). Clones were defined as groups of cells expressing the same rare combination of cytoplasmic/nuclear markers sharing the same color display in these two compartments and located less than 600 µm apart, a threshold determined from observations of very rare color combinations only found in a unique animal among those analyzed (Supplementary Fig. 1c–h). Relative positions within the cerebral cortex thickness on the Y-axis were obtained automatically from XYZ cell coordinates and manually drawn masks of the cortical upper and lower limits using Matlab. For tridimensional analysis (Figs. 1f–k, 2a–k, Supplementary Fig. 2), clonal棉 was obtained using the delaunayTriangulation Matlab function from manually pointed astrocyte XYZ coordinates and by measuring automatically all segments formed by the triangulation. The convex Hull function was used to calculate the volume of the clones. The main axis of each clone was computed by fitting the 3D coordinates of clonally related cells with a line that follows the function: M = r + vT, where r is the average of all X, Y, and Z coordinates, and r is obtained through singular value decomposition of the coordinate variance-covariance matrix. The angle of this line fit to the radial orientation and its radial projection were evaluated and compared to a randomized dataset. The latter was comprised of the original linear fits axes that were randomly rotated in 3D, resulting in an equal spacing mimicking the properties of a sphere. Connectivity within clones was analyzed by automatically sorting clusters or isolated cells using their XYZ coordinates and astrocyte mean diameter + s.d. at each developmental stage: sister cells separated by distances greater than the mean diameter + s.d. were considered disconnected (Fig. 2g, h). Analysis of astrocyte proliferation (Fig. 3g–l) was carried out in a similar way to that of clonal patterns, and by measuring the distance between EdU + astrocyte pairs in 3D using XYZ cell coordinates obtained from image stack.

**Astrocyte domain segmentation.** 3D segmentation of individual astrocyte domains was performed using Imaris (Bitplane, Switzerland) on 3D crops from the ChroMS datasets showing isolated or color-separated astrocytes. Semi-automatic iso-intensity contours were drawn in all slices of the corresponding z-stack. Contours were manually drawn when the iso-intensity contour tool did not provide accurate segmentation. The obtained surfaces allowed the software to automatically calculate astrocyte domain volumes. Segmented astrocytes were then distributed into six equivalent bins according to the relative position of the centroids within the cerebral cortex thickness. Distances from cells to pial surface and VZ were manually measured to calculate the cells relative positions.

**Reconstruction of astrocyte arbors.** High NA diffraction-limited confocal microscopy images of isolated astrocytes were analyzed using Fiji, Vaa3D 37, Matlab (MathWorks, USA), and Autoquant X3.1 (Media Cybernetics, USA). Astrocyte arbors were reconstructed using the Vaa3D-Neuron2 Auto Tracing Based on AP2 plugin and their structure was sorted using the sort neuron SWC plugin. SWC were exported to Matlab using the TREES toolbox and the model was resized to the real dimensions from the initial acquisition. Finally, information from the model was extracted using L-measure 38.

**Statistics.** Graphs were obtained with GraphPad Prism (USA) or Matlab (3D graphs). Statistics were done with GraphPad Prism: 2 groups and ≥3 groups comparisons were carried out using the nonparametric Mann–Whitney test, or the nonparametric Kruskal–Wallis test associated with Dunn’s multiple comparisons test, respectively. Statistics results, tests, p values and n associated to all figures are summarized in Supplementary Table 2.

**Data availability.** The data that support the findings of this study are included in the paper. The 3D layout of each astrocyte clone is available as Supplementary Resource. All
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Author contributions
K.L., J.L. and S.C. designed the experiments and wrote the paper. All authors reviewed and edited the paper. S.C. performed the experiments, acquired all confocal images, analyzed data, and made the figures. L.A. and E.B. designed and built the ChroMS set-up and L.A. acquired and processed the images. E.H. performed high-resolution astrocyte reconstruction with the contribution of G.B. for morphological analysis. R.B. assembled the transgene constructs. S.C., D.N. and SH.I. performed the analysis. J.D. genotyped animals and provided assistance for image processing and quantifications.

Competing interests
The authors declare no competing interests.

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