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Development of Piriform Cortex Interhemispheric Connections Via the Anterior Commissure: Progressive and Regressive Strategies

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Abstract

The anterior commissure (AC) is a phylogenetically conserved inter-hemispheric connection found among vertebrates with bilateral symmetry. The AC connects predominantly olfactory areas but many aspects of its development and structure are unknown. To fill this gap, we investigated the embryonic and postnatal development of the AC by tracing axons with DiI and the piggyback transposon multicolor system. With this strategy we show that axon growth during establishment of the AC follows a strictly regulated timeline of events that include waiting periods (“regressive strategies”) as well as periods of active axon outgrowth (“progressive strategies”). We also provide evidence that these processes may be regulated in the midline via overexpression of chondroitin sulfate proteoglycans. Additionally, we demonstrate that the ipsi- and contralateral innervation of piriform cortex occurs simultaneously. Morphologically, we found that 20% of axons were myelinated by postnatal day (P) 22, in a process that occurred fundamentally around P14. By immunohistochemistry we described the presence of glial cells and two new subtypes of neurons: one expressing a Calretinin (CR)⁻/MAP2⁺ phenotype, distributed homogeneously inside the AC; and the other expressing a CR⁺/MAP2⁺ phenotype that lies beneath the bed nucleus of the stria terminalis. Our results are consistent with the notion that the AC follows a strictly regulated program during the embryonic and postnatal development similarly to other distal targeting axonal tracts.

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COMPLIANCE WITH ETHICAL STANDARDS

Ethical approval

All animal procedures were approved by Yale University Animal Care and Use Committee.

Informed consent

This manuscript has been approved by all co-authors and agreed with the submission of the manuscript to *Brain Structure and Function*.

Disclosure of potential conflict of interest

The authors declare that they have no conflict of interest.

Keywords

Anterior commissure; piriform cortex; axons growth; embryonic development; versican; myelination; piggyBac

INTRODUCTION

The information generated in the telencephalon of bilaterian animals is integrated by connecting the hemispheres through bundles of axons, the brain commissures. In vertebrates several commissures cross the midline along the rostral-caudal axis that connect functionally distinct areas of the brain, including the pallial and hippocampal (HC) commissures, anterior commissure (AC) and corpus callosum (CC) (Suarez et al. 2014). The AC is the most phylogenetically conserved and the only commissure present in all vertebrates, although the brain areas it connects varies depending on the species. In mice the AC is predominantly paleocortical while in other species (i.e. rats, cats, wallaby and monkeys) it carries neocortical projections mostly from the visual and auditory areas of the ventral temporal lobe (Fitzpatrick and Imig 1980; Gross et al. 1977; Horel and Stelzner 1981; Jouandet 1982; Jouandet and Gazzaniga 1979; Shang et al. 1997). The paleocortical areas projecting through the AC are the olfactory bulb, olfactory cortices (including the anterior olfactory nucleus (AON), piriform cortex (PC) and olfactory tubercle), amygdaloid complex, entorhinal cortex (EC), preoptic area, nuclei of the AC, nuclei of the lateral olfactory tract (nLOT), nuclei of the stria terminalis and nucleus of diagonal band (Brodal 1948; Gurdjian 1925; Haberly and Price 1978a; Haberly and Price 1978b; Ramon y Cajal 1901). Fibers from these areas converge in the three main tracts: the anterior limb (*pars olfactoria or bulbaris*) of the AC (ALAC), the posterior or associational limb (*pars interhemispherica or sphenoidal*) of the AC (PLAC), and the portion communicating bilaterally the bed nuclei of the stria terminalis (*pars ad striam terminalem*) (Jouandet and Hartenstein 1983; Lent and Guimaraes 1991; Ramon y Cajal 1901). Within these tracts, bundles of myelinated and unmyelinated axons expressing different neurotransmitters intermingle with neurons and glial cells (Brunjes 2013; Larriva-Sahd et al. 2002; Misaki et al. 2004; Sturrock 1974a; Sturrock 1974b; Sturrock 1975; Sturrock 1977).

Reflecting the complexity of axonal types traveling through it, the embryonic development of the AC is a highly regulated process involving many different families of molecules. Among them are proteoglycans and tenascins (Pires-Neto et al. 1998), ephrins and eph receptor (Henkemeyer et al. 1996; Kudo et al. 2005; Kullander et al. 2001; Robichaux et al. 2016), molecules of the semaphorin-plexin superfamily (Falk et al. 2005; Sahay et al. 2003; Suto et al. 2005), Robo family receptors (Andrews et al. 2006; Calloni et al. 2017), immunoglobulin-like receptors (Abudureyimu et al. 2018), cytoskeleton associated proteins (Deuel et al. 2006; Klingler et al. 2015; Koizumi et al. 2006; Shen et al. 2002), cell adhesion molecules (Lustig et al. 2001; Munakata et al. 2003; Tissir et al. 2005; Zhou et al. 2008), small GTPases (Brouns et al. 2000; Chen et al. 2007; Kassai et al. 2008), FGFs family of growth factors (Tole et al. 2006), Frizzled receptors (Hua et al. 2014; Wang et al. 2002), and autophagy related proteins (Wang et al. 2017; Yamaguchi et al. 2018). Postnatally the

development of the AC is simplified to processes of axonal maturation and myelination (Chebat et al. 2006; Collins et al. 2018; Sturrock 1975; Sturrock 1976).

Although knowledge regarding the molecules involved in the development of the AC is rapidly expanding, less attention has been paid to the dynamics of axonal extension and elongation during AC pathfinding. To date, it seems clear that the onset of AC axogenesis in mice is around embryonic day 13 (E13) (Kallen 1954; Klingler et al. 2015; Silver et al. 1982), although data about how and when they reach and cross the midline (ML) is less clear (Klingler et al. 2015; Schneider et al. 2011; Wahlsten 1981). Since anomalies in the AC development are associated with the appearance of neurological disorders such as schizophrenia, bipolar disorder and autism (Adler et al. 2004; Doderio et al. 2013; Hulshoff Pol et al. 2004; Kikinis et al. 2015; Saxena et al. 2012), precise understanding of mechanisms regulating the establishment of the AC will improve our understanding of disorders of axon pathfinding.

To further our understanding of axon pathfinding we characterized axon elongation during development of the AC, their arborization in the contralateral side, the myelination process at postnatal stages and the phenotype of cells residing in the AC following maturation. To study embryonic development we used DiI injections and labeling strategies based on the piggyBac transposon (Martin-Lopez et al. 2017). Interestingly, we found that AC formation follows a sequential process of “pioneer axon” extension which precede the subsequent “follower axons” using both “progressive” (extension and elongation) and “regressive” (pauses and waiting periods) mechanisms. The first appearance of AC axons was at E13.5, simultaneous with the beginning of the PC neuroblast migration through the lateral cortical stream (LCS) (Kallen 1954; Klingler et al. 2015; Silver et al. 1982). For the first time, we suggest a role of chondroitin sulfate proteoglycans (CSPG) in the sorting of AC axons while crossing the ML. Postnatally, we studied the myelination process of the AC using electron microscopy (EM) and staining for the myelin-associated enzyme CNPase. Our CNPase staining showed that myelination of AC progressed rapidly and uniformly after postnatal day 14 (P14), without increasing until P21. The EM analysis revealed that myelination rate of the AC was 20% around weaning day (P22) with an average axon diameter of 0.52 μ m. Using specific markers, we characterized two new subpopulations of neurons inside the AC: one expressing the marker MAP2 and distributed throughout the AC, and another expressing both MAP2 and Calretinin (CR) that is located exclusively in the ML infiltrating from the bed nucleus of the stria terminalis (BDST). In brief, our observations provide the first evidence of pioneer axons initiating the development of the AC which then proceeds with periods of both progressive axon extension and accompanying periods of quiescence which we characterize as “regression.” Our observations of the timeline of myelination within the AC are largely in agreement with prior reports, while our additional immunohistochemical analyses demonstrate the presence of previously unrecognized populations of neurons as well as a spatially restricted distribution of CSPGs that may be important determinants of AC boundaries.

MATERIALS AND METHODS

Animals

All experiments were conducted on CD1 embryos and postnatal mice (males and females), obtained from Charles River Laboratories (Wilmington, MA), and housed on a 12-hour light cycle with access to standard chow *ad libitum* at Yale University *vivarium*.

DiI injections

A stock solution of 1 mg/mL DiI (Molecular Probes) was prepared in dimethylformamide (DMF, Sigma-Aldrich), then diluted 40:1 in Pluronic F127 (Sigma-Aldrich) and kept in aliquots at -20°C until use. Embryos were obtained from pregnant females euthanized with CO_2 at the embryonic stages of E13, 14, 15, 17 (N=3). Tissues were fixed by immersion in 4%PFA-PBS. Postnatal tissues were obtained from pups at the newborn day 0 (N=3) and the postnatal day 21 (P21) (N=3), euthanized with an overdose of Euthasol (Virbac) before perfusing transcardially with a saline solution followed by 1 mL/g of body weight of ice-cooled 4%PFA-PBS. All tissues were postfixed for 7 days before the DiI injections. The day of injection, borosilicate micropipettes were pulled to obtain tips with an outer diameter of 50 μm and then loaded with the DiI solution. The areas of brain covering both the anterior PC (aPC) and posterior PC (pPC) were injected unilaterally with $\sim 50\text{--}300$ nL of DiI using a Picospritzer (Parker). Next, all brains were incubated at 37°C in PBS for 1 to 3 months. Brains were subsequently cryoprotected in 30% sucrose-PBS, embedded in OCT compound (Fisher Scientific), and sectioned in a Reichert Frigocut (E-2800) cryostat to collect 50 μm -thick sections in the horizontal plane (Schambra et al. 1992). Immediately before imaging, sections were rehydrated with PBS and slides mounted with Mowiol 4–88 (Sigma-Aldrich).

In utero injections and electroporations (IUE)

We labeled the PC progenitor cells using the piggyBac transposon system expressing either one single or multiple fluorophores as we previously described (Martin-Lopez et al. 2017). Briefly, pregnant females at gestational day 11 (E11) were deeply anesthetized with 100 mg/Kg Ketamine (Ketaset, Pfizer) + 10 mg/Kg Xylazine (AnaSed, Lloyd), an incision was made in the midline of the abdominal wall through the alba line, and embryos were pulled out from the abdomen for IUE. Two sets of animals were injected with 0.2–0.5 L of plasmids solution at 1 mg/mL: (1) one set was injected with a mix of three plasmids expressing the fluorophores EGFP (pPB-CAG-EGFP), tdTomato (pPB-CAG-tdT) and iRFP670 (pPB-CAG-iRFP670), together with the transposase-expressing plasmid (pCAG-PBase), and used to study the growing and formation of AC (E13-P0); (2) another set was injected with only pPB-CAG-tdT + pCAG-PBase and used for the analysis of ipsi- and contralateral PC projections (E17, P0, P1, P3 and P7) and colocalization with CSPG (E16). Plasmids were injected into the cerebral ventricle of embryos using a Picospritzer, targeting the lateral ganglionic eminence from which PC projection neurons originate. Electroporations were performed by delivering 5 pulses of 35 V using a pair of gold tweezers (Genepaddles-542, Harvard Apparatus) connected to an ECM 830 electroporator (BTX Harvard Apparatus). Following electroporation embryos were rehydrated with pre-warmed saline solution (Hospira) and introduced back into the abdominal cavity. The peritoneal membrane and skin were closed using 5/0 PGA absorbable (AD Surgical) and a

5/0 silk braided (Ethicon) sutures, respectively. Postsurgical care was applied for 5 consecutive days by close monitoring of animals and by injecting 4 mg/Kg of the analgesic Meloxicam (Eloxiject, Henry Schein).

Tissues that received IUE were studied at embryonic and postnatal stages to track axonal navigation. Embryonic tissues were collected at consecutive stages of E13 to E18 (N=4/each) and fixed by immersion in 4% PFA-PBS (see above), whereas postnatal tissues were obtained from P0-P3 (N=2), P7 (N=2) and P21 (N=3) pups. Postnatal tissues were fixed by transcardial perfusion with saline solution followed by 4% PFA-PBS at 1 mL/g of body weight (see above). Both embryonic and postnatal brains were postfixed overnight at 4°C and then cryoprotected in 30%-sucrose-PBS prior to sectioning in a cryostat (Reichert Frigocut). Animals electroporated with the multicolor method were sectioned at 50 μ m in the horizontal plane (Schambra et al. 1992), while animals electroporated exclusively with a plasmid expressing just one fluorophore were sectioned at 20 μ m in the horizontal plane. Sections from multicolor electroporations were rehydrated with PBS and mounted with Mowiol 4–88 before imaging, whereas one fluorophore-expressing tissues were kept at –80°C until use for immunostaining.

Processing of non-electroporated tissues

A group of non-electroporated animals were used for the immunohistochemistry analysis. Tissues from embryos of E12 to E18 stages (N=3/each) and postnatal pups of P7 and P21 (N=3/each) were collected and processed equally to the electroporated animals (see above), with the exception that P21 animals were sectioned at 20 μ m in the coronal plane.

Electron microscopy

Animals at postnatal day 22 (P22) were euthanized with an overdose of Euthazol (Virbac) and immediately perfused transcardially with 1 mL/g of body weight of saline solution followed by ice-cooled 4% paraformaldehyde (PFA, JT Baker) and 0.2% glutaraldehyde (Electron Microscopy Sciences) in phosphate buffered saline (PBS). Brains were postfixed overnight at 4°C, and then 50 μ m coronal sections were collected with a vibratome (Pelco 101). Lipids were fixed by treating sections with 4% osmium (Electron Microscopy Sciences) for 1 h, dehydrated through graded alcohols, and treated with uranyl acetate (Electron Microscopy Sciences) and lead citrate (Electron Microscopy Sciences) to increase the contrast of tissue ultrastructure. Finally, sections were polymerized in EPON 812 (Electron Microscopy Sciences) and the AC was microdissected for sectioning at 0.07 μ m with an ultramicrotome (UltraCut-E). Sections were mounted on formvar coated slot grids (Electron Microscopy Sciences) before imaging.

Immunohistochemistry

Immunohistochemically characterization of the AC was made in the following groups: postnatal tissues at P21; embryonic tissues from E12 to E18; and embryonic tissues at E16 from animals electroporated only with the tdTomato-expressing plasmid to detect CSPG. Sections were thawed in a slide warmer at 60°C, and then rehydrated on PBS. All tissues were treated for antigen unmasking by incubating the slides in 0.01M citrate buffer (for CNPase staining, this buffer was supplemented with 0.05% Tween 20 (Sigma-Aldrich)),

pH6.0 for 35 min at 80°C. After cooling down to room temperature (RT), sections were washed with PBS and, exclusively on sections to be stained with the versican antibody, tissues were digested with 0.1 U/mL of protease-free chondroitinase ABC (from *Proteus vulgaris*, Sigma-Aldrich) in 50 mM Tris pH 8.0 (American Bioanalytical), 60 mM sodium acetate (JT Baker) and 0.02 % bovine serum albumin (BSA, Sigma-Aldrich), for 2 h at 37°C. Enzyme was heat inactivated at 70°C for 5 min., and sections were washed with PBS containing 0.1% Triton X100 (Sigma- Aldrich) (PBST). Nonspecific antibody binding was blocked by incubating the slides with PBST supplemented with 5% Normal Goat Serum (NGS, Accurate Chemicals) + 0.1% Bovine Serum Albumin (BSA, Sigma-Aldrich) at RT for 1 h. Next, sections were incubated with specific primary antibodies (Table 1) overnight in blocking solution at 4°C, washed three times with PBST, and incubated with specific secondary antibodies (Table 1) diluted in PBST containing 1 g/mL of DAPI (Invitrogen) and 5 M DRAQ5 (BD Pharmingen) for 2 h at RT. Finally, sections were washed with PBST and mounted with Mowiol 4–88.

Imaging and statistical analysis

Ultrastructural micrographs were taken in a JEOL Electron Microscope (JEM-1200 EX II) in the ALAC, at the level at which a small tail of axons from the PLAC were seen. To quantify the percentage of myelinated axons, the area of the AC was subdivided in six symmetric fields (Fig. 7A), and random images were taken from each of these fields at 10,000X primary magnification. Between 2000–3000 axons were counted per animal (N=3) for statistical analysis. To calculate the diameter of axons, we measured the area of at least 40 axons on each of the random images taken per animal, and the diameters were calculated on the assumption of an axon being circular in transverse section. The number and diameters of myelinated vs. unmyelinated axons were compared using Student's t-test (Statistica 7.0).

To automatically quantify CNPase staining, we first isolated the AC on the ML region on a 16-bit maximum projection of a 20 m image with a freehand drawing tool. The area of this drawn ROI was measured to later determine the density of stained objects. To reduce the noise in the image and correct for uneven illumination, a Difference of Gaussians was applied by subtracting a Gaussian Blur of the image with sigma 25 from a Gaussian Blur of the image with sigma 1. To then segment the CNPase stained pixels from the rest of the image we used the Fiji automatic Otsu threshold function; Otsu's method identifies a threshold pixel value that minimizes the interclass pixel intensity variance between the two-pixel classes ('on' or 'off) within the image and created a binary image by replacing all values above this globally determined threshold with 1s and setting all other values to 0s. After making a mask of this image, quantification of the area of CNPase stained pixels was completed using the Fiji Analyze Particles function.

Quantification of DAPI stained nuclei was performed as above but with additional steps using Fiji (ImageJ) software with the MorphoLibJ integrated library and plugin (Legland et al. 2016). Following segmentation with an Otsu threshold we applied a closing morphological filter of a 0.8-pixel radius disk to close the staining of nuclei. Finally, the stained nuclei were isolated by applying a gray scale attribute opening filter of area minimum of 150 pixels and connectivity of 8 before quantifying nuclei number with the

Analyze Particles function. Comparisons of stained CNPase area fractions and number of DAPI nuclei between P0-P21 postnatal ages was made by applying a one-way ANOVA followed by a post-hoc Tukey test (Statistica 7.0).

For DiI staining, sections were imaged using an Olympus BX51 epifluorescence microscope coupled to a digital camera Olympus HKH027241. Immunostained sections and tissue from electroporated animals were imaged using a Leica TCS-SL confocal equipped with the following laser lines: 488 nm used to capture EGFP and Alexa 488 signals; 543 nm used to capture tdTomato and Alexa 546 signals; and 633 nm used to detect iRFP670 and Draq5 signals (both emitting in far-red but shown in blue). Since AC axons elongated from neurons migrate ventrally from the LGE during the development, we manually photomerged images taken in different consecutive sections, along the dorsal-ventral axis, to illustrate the embryonic development in both the ipsi- and contralateral sides (Fig. 2).

RESULTS

Developmental dynamics of the anterior commissure studied with DiI and IUE

Our initial approach was injecting DiI along the anterior-posterior axis of PC at different embryonic and postnatal stages to study the development of the AC (Fig. 1). At E13 the dye was covering the extension of the PC but no axon was distinguishable (Fig. 1A, A'). The first commissural growth cones were observed at E14 forming small bundles of pioneer axons extending from the PC and approaching the ML (Fig. 1B, B' arrowhead); they reached the ML at E15 (Fig. 1C, C' arrowhead). The first evidence of AC axons crossing the ML was observed at E17 (Fig. 1D), forming a delineated and engrossed bundle of axons at the ML at P0 (Fig. 1E). At P21, the AC displayed a mature morphology with the presence of both ALAC and PLAC (Fig. 1F). Although we could trace a rough map of AC axons growth development (Fig. 1G), the contralateral innervation and a clear the visualization of axonal navigation through the brain parenchyma was not possible due to technical issues DiI diffusion through axons.

To properly track AC axon navigation and improve the visualization of growth cones, we expressed different fluorescent reporter proteins in PC neuroblasts of the dorsal LGE at E11, which is the area from which the PC projection neurons originate and migrate ventrally through the lateral cortical stream (LCS) (Garcia-Moreno et al. 2008; Huilgol and Tole 2016; Martin-Lopez et al. 2017), by using a multicolor piggyBac-transposon system (Martin-Lopez et al. 2017). This method was shown to specifically label AC axon outgrowth and decussation during embryonic development (E13–17), as well as their projections and arborizations to the contralateral PC at postnatal stages (Fig. 2 and 3).

In contrast with the observed after DiI injections, we first showed that PC neuroblasts begin extending commissural axons at E13, while these cells were still proximal to the LGE (Fig. 2A-E). These cells continue migrating ventrally through the LCS until E15, where they were no longer visible next to the LGE (Fig. 2J, K). However, after E15 many other PC neuroblasts were still being produced in the LGE and migrating through the LCS (Martin-Lopez et al. 2017),-suggesting that commissural PC neurons are produced at restricted times during the development. Similar behavior has been reported for other commissural tracts

such as the CC (Lent et al. 1990). At E13 the AC axons were defasciculated and they seemed to establish the pathway for later axons, and therefore we identified them as the group of “AC pioneer axons” and the neuroblasts extending them as “AC pioneer neurons” (Pires-Neto and Lent 1991; Silver et al. 1982). As described, the elongation of AC axons occurred while the AC pioneer neurons continued migrating to areas nearby the prospective PC (Fig. 2A, B; F, G; J, K); we concluded that the emission and extension of AC axons is a dynamic and intrinsic process occurring independently of the PC neuronal activity. Although the onset of AC axons at E13 supported previous descriptions in mice (Kallen 1954; Klingler et al. 2015; Wahlsten 1981), we did not find any approaching or crossing the ML as previously reported (Schneider et al. 2011).

AC pioneer axons continued to elongate towards the ML at E14 and some had reached the ML region, but without crossing (Fig. 2F-I, arrowheads). Interestingly, we observed that commissural axons initially clustered in small fascicles prior to converging into a primary fascicle that is the nascent AC (Fig. 2H). Up to this point, the AC pioneer axons adopted a “progressive strategy” of growth based on a process of extension and elongation (Lent and Guimaraes 1990; Lent and Guimaraes 1991). This strategy was also observed at E15 by the arrival of more axons (“follower axons”) following the path established by pioneer axons towards the ML (Fig. 2J, K), producing a thickening of the AC. However, all pioneer axons arriving to the ML paused their growth prior to invading the contralateral parenchyma (Fig. 2L, M), and remained proximal to the ML in the first evidence of a “regressive strategy” of growing not previously reported for the AC.

By E16, commissural axons restarted a progressive strategy, fasciculated into a single tract and massively crossed the ML to the contralateral parenchyma (Fig. 2N, O). The pioneer axons were still visible and continued to lead the navigation process (Fig. 2N', arrowhead). One day later, at E17, AC axons reached the internal areas of the contralateral olfactory cortex, where they were positioned in the deeper mass of cells comprising the cortical plate along the cortico- striatal border (CSB) in the anterior regions, or the cortico-amygdaloid border (CAB) in the posterior regions (Fig. 2P, Q). At this location, axons began to defasciculate and spread out along the anterior-posterior axis to cover the extension of the contralateral PC (Fig. 2P, Q, arrowheads). At this age we recognized a second “regressive event” where AC axons stopped their growth and remained stationary in the CSB/CAB without invading the contralateral PC until P1 (Fig. 2R, arrowheads). This second regressive event resembled similar events that occur during the development of other systems containing distal targeting neurons such as the CC (Lent et al. 1990; Wise and Jones 1976), the commissure of the superior colliculus (Chebat et al. 2006) or the OB (Treloar et al. 1999). At P0, AC axons continued to spread out along the anterior-posterior axis without invading the layers of the contralateral PC (Fig. 2R). Our data contrast with the previously reported in hamsters, in which the AC followed exclusively progressive strategies (Lent and Guimaraes 1990; Lent and Guimaraes 1991).

Ipsi- and contralateral arborization of anterior commissure axons

We next investigated the AC axonal innervation and arborization of contralateral PC following the second waiting period established at E17-P1. Our analysis focused on the

following events: (1) the ipsilateral innervation of PC from the PC; (2) the contralateral innervation of PC from AC axons; (3) innervation of the contralateral olfactory bulb (OB) from the aPC.

To evaluate whether the ipsi- and contralateral cortico-cortical innervation of PC were established simultaneously, we first addressed innervation of the aPC from neurons labeled in the ipsilateral pPC. During the second regressive event at E17-P0, there was an absence of parallel axons along the layers of the aPC, indicating that axons from the pPC had not yet reached the anterior part (Fig. 3A, B) and, - confirming that cortico-cortical innervation in both hemispheres might occur simultaneously. Later at P1-P3, numerous centrifugal projections were seen arriving in the aPC and running parallel to the surface of layers I and III, and some crossing layer II of the aPC (Fig. 3C, D). Finally, at P7 we observed a more elaborated innervation of aPC predominantly in layers I and III (Fig. 3E).

Simultaneously, in the contralateral PC the AC growth cones that remained beneath the CSB/CAB during the second regressive event (Fig. 3F, G, arrowheads), initiated the contralateral innervation of PC from the deep layer III at P1 (Fig. 3H, arrowhead). At P3, more axons arrived in layer III and a few of them penetrated and crossed layer II to reach layer I (Fig. 3I). Finally, - commissural axons arborize densely the contralateral PC at P7 (Fig. 3J), arborizing predominantly layers I and III. Since layer Ib has been reported to receive associational fibers from PC (Haberly and Price 1978a), we presumed that fibers observed in layer II were in transit to layer I. Note that many growth cones were still visible at P7 predominantly in layers I and III of PC (Fig. 3J, arrowhead), indicating the arrival of axons and suggesting that the maturation of connections continued.

Additionally, we also assessed the contralateral innervation of the OB of axons coming from the aPC traveling through the ALAC. The first growth cones arrived in the OB peduncle at E17-P0 (Fig. 3K, L, arrowheads) to reach the inner granule cell layer (GCL) of the OB at P1; a few axons were seen innervating the superficial GCL (Fig 3M, arrowhead). From P3 to P7, the contralateral innervation spread out in the OB and predominantly within the GCL, although some axons appeared to arborize in the mitral cell layer (MCL) (Fig. 3N, O, arrowheads).

In summary, we established a precise timeline of the mouse AC development that occurs as follows (Fig. 4): AC axon outgrowth began at E13, with pioneer axons reaching the ML using a progressive strategy at E14. At this time pioneer axons paused outgrowth and adopted a regressive strategy, while the AC follower axons, which began outgrowing at E14, continued growing towards the ML until E15; ultimately both pioneer and follower axons fasciculated and extended through the contralateral parenchyma at E16–17 in a progressive manner. From E17 to P1, AC axons reached the internal border of the contralateral PC and once again adopted a regressive strategy (or a second pause), subsequently invading the contralateral layers of PC. Finally, commissural axons innervated and arborized the contralateral PC during the first postnatal week of the development in the third progressive growth event.

Influence of chondroitin sulfate proteoglycans in sorting the anterior commissure axons

Although many different families of molecules have been reported as acting as chemoattractant or chemorepellent signals inside the extracellular matrix (ECM) during the axonal guidance process (Dickson 2002; Kaprielian et al. 2000; Lent et al. 2005), the full orchestra of molecules has not yet been fully delineated. We describe here for the first time a distribution of chondroitin sulfate proteoglycans (CSPG) relative to the AC and propose them as candidates to influence the development of the AC. The proteoglycans (PGs) are an abundant family of molecules in the embryonic ECM (Iozzo 1998; Maeda 2015; Maeda et al. 2011; Margolis and Margolis 1997), and may be involved in the formation of brain commissures (Conway et al. 2011; Feraud-Espinosa et al. 1996; Inatani et al. 2003; Klambt and Goodman 1991; Pires-Neto et al. 1998). Here, we used the monoclonal antibody CS56 to recognize both chondroitin-4-sulfate and chondroitin-6-sulfate, and versican to recognize a specific family of PGs part of the lectican family, in the brain at different embryonic and neonatal stages.

The expression of CS56 was initially detected as sparsely distributed within the brain parenchyma around the ML at E13 (Fig. 5A). When the initial pioneer axons approached the ML at E14, the staining for CS56 formed a CSPG tunnel around the nascent AC, possibly forming a molecular barrier to keep the axons enclosed in a bundle (Fig. 5B). The tunnel of CS56 was more obvious at E15 (Fig. 5C). Interestingly, at E16 expression of CS56 flipped from forming a tunnel to being within the lumen of the AC in the caudal half of the AC at the ML, which is the area through which the axons from the PLAC travel (Fig. 5D-F). To confirm that CS56 from E16 onwards might have a role in the pathfinding of AC axons within the ML, we performed IUE to specifically labeling the anterior cells that populated the aPC and extend axons through the ALAC. Indeed, the axons coming from the aPC and traveling through the ALAC avoided the area of CS56 overexpression at the ML (Fig. 5D-F). These data strongly suggested that CSPG could be involved in the sorting of AC axons coming from the anterior and posterior limbs and thus serve as both permissive and repulsive signals. This expression pattern was sustained during embryonic development until postnatal day 7 (Fig. 5G-I).

We next sought to identify at least one type of CSPG detected by the CS56 antibody. Immunohistochemistry against distinct CSPGs (Table 1) showed that only versican was expressed around the ML of the AC (Fig. 5J-R), while brevican and neurocan were absent (data not shown). Versican is a CSPG expressed during brain development and can function as either an attractive or inhibitory signal for axonal outgrowth (Schmalfeldt et al. 2000; Snyder et al. 2015; Wu et al. 2004). We initially detected versican in the neuroblasts migrating from the LGE at E12 (Fig. 5J) and around the ML at E13, predominantly in anterior areas (Fig. 5K). From E14 to E16, versican formed a tunnel around the AC with no expression detected within the AC (Fig. 5L-M), which is an expression pattern similar to that previously reported during the development of the internal capsule (Popp et al. 2003). The tunnel expression of versican was lost from E17–18, as it was restricted to the brain parenchyma surrounding the anterior side of the AC (Fig. 5O, Q). Conversely, in the area where the ALAC and PLAC converge at the ML, versican was overexpressed in the posterior edge of the ALAC and the anterior edge of the PLAC (Fig. 5P), supporting a role for

versican in helping axons maintain a correct trajectory. Interestingly, at P7 versican was also expressed inside the AC at the ML, but was restricted to the ALAC (Fig. 5R). As at P7 AC axons have already crossed the ML, the expression of versican in the lumen of the ALAC suggests a role in the stabilization of ALAC axons.

Postnatal maturation of the AC studied by axonal myelination

We next evaluated the maturation of the AC while it forms a functional axon tract. Here, we focused in analyzing the myelination within the AC from P0 to P21 by detecting the expression of CNPase, an enzyme involved in the maturation of oligodendrocytes during myelination (Raasakka and Kursula 2014). Our data demonstrate that the first oligodendrocytes expressing CNPase were found around the AC at P0 (Fig. 6A) and continue invading the AC at P7 (Fig. 6B). The first evidence of myelination was observed at P14 and continues until P21 (Fig. 6C, D). The fraction of AC covered by the CNPase staining increased dramatically from P7 to P14 (Fig. 6E), indicating that most of the myelination process occurred near P14; these support the recently reported *rapid myelination period* occurring in the AC between P13–15 (Collins et al. 2018). The AC thickness also increased predominantly during the peak of myelination at P14, coinciding with an increase in the number of cells per unit area (Fig. 6F).

Finally, we studied the ultrastructure of myelin in the ALAC at P22 as representative of a mature AC. First, we established the density of AC axons to be 517 axons/100 μm^2 . We then determined that the percentage of myelinated axons was approximately 20% (Fig. 7B, C). This number coincided with the myelination rate measured with EM in older animals at P32–35 (Sturrock 1975; Sturrock 1976), although the diameter of axons, 0.52 μm for myelinated 0.25 μm for unmyelinated (Fig. 7D) was similar to animals of the same age (Sturrock 1975; Sturrock 1976). The external architecture of the AC revealed a boundary of parenchyma composed predominantly of unmyelinated neuronal fibers (Fig. 7E), although there was no clear evidence of a specialized anatomical structure that might restrict the AC axons. We observed the somata of oligodendrocytes with processes engulfing axons distributed throughout the AC (Fig. 7F, arrows). These cells were identified due to their characteristic dark cytoplasm with long cisternae of rough endoplasmic reticulum, and irregular nuclei with clumped chromatin both beneath and within the nucleoplasm (Sturrock 1974a).

Characterization of molecular profiles in the anterior commissure

We further characterized the anatomy of the mature AC by detecting different subpopulations of neurons and glial cells by immunohistochemistry (Table 1). Among the neurons, we found at least five subpopulations of cells, including: (1) some scattered NeuN⁺ neurons (Fig. 8A); (2) a few Reelin (Reln)-expressing neurons exclusively located in the PLAC (Fig. 8B). In contrast to reports in rats, we did not find Reln cells in the ML (Misaki et al. 2004); (3) neurons expressing the postmitotic neuronal marker MAP2, which represented approximately the 35% of the total and displayed small and fusiform cell bodies (Fig. 8C). These cells distributed homogeneously through the lumen of the AC and were generally aligned to the longest cross-sectional axis of the commissure. Densely packed neuronal processes expressing calretinin (CR) in the nucleus accumbens surrounded the

ALAC (Fig. 8C); (4) a group of neurons found beneath the bed nucleus of the stria terminalis, lateral division, posterior part (BSTLP), which expressed a CR⁺/MAP2⁺ phenotype. This phenotype was shared by the BSTLP neurons and, because they displayed a single process pointing ventrally from the BSTLP, it is suggestive of a migratory behavior of cells infiltrating from the BSTLP (Fig. 8D-F). To date, the presence and function of these last two type of cells in the AC is unknown; (5) a sparse group of neurons expressing glutamic acid decarboxylase 65/67 (GAD65/67) and displaying the typical morphology of inhibitory interneurons (Fig. 8G, arrowheads). Interestingly, we found in the core of the ALAC the expression of the immature neural markers doublecortin (DCX) and polysialylated neural cell adhesion molecule (PSA-NCAM), typical of neuroblasts and suggesting the presence of an immature profile within this region (Fig. 8H, I, dotted lines). The marker DCX was first detected at E14 (data not shown) and increased in expression throughout embryonic development until eventually declining after birth (Supplementary Fig. 1A-C).

The analysis of glial cells showed abundant astroglial linages with immature cells expressing S100 and mature cells expressing GFAP (Raponi et al. 2007) in the AC (Fig. 8J, K), as well as subpopulations of microglial cells detected by IBA1 expression (Fig. 8L). Whereas the microglial marker IBA1 was only detected postnatally, the astroglial markers GFAP and S100 showed progressively increased expression in the AC from E18 to postnatal stages (Supplementary Fig. 1D-F).

Secondary to the discovery of two new neuronal subpopulations not previously reported (CR⁻/MAP2⁺ and CR⁺/MAP2⁺), we investigated their appearance during development. Interestingly, the first evidence of both cell types occurred postnatally at P7, at which time the expression of both cell types increased abruptly to reach maturity one week later at P14. No evidence of later maturation stages was found when we analyzed the tissue at P21 (Supplementary Fig. 2).

DISCUSSION

The AC represents the most important tract to establish interhemispheric connections in the paleocortex and predominantly connects olfactory areas of the brain (Brodal 1948; Haberly and Price 1978a; Haberly and Price 1978b; Ramon y Cajal 1901). Because of the variety of areas connected via the AC, this tract is formed by a complex mixture of myelinated and unmyelinated axons of diverse origin (Brunjes 2013; Collins et al. 2018; Sturrock 1975), that follow a strictly regulated program during the development. To augment understanding of AC development, in this work we describe a detailed program of progressive and regressive strategies utilized by pioneer and follower axons during the elongation process from the PC. We also found that some families of CSPG may be involved in regulating the sorting of axons coming from the anterior and posterior limbs of AC in the ML. Postnatally, we show that AC myelination occurs predominately after the second week of life in a rapid myelination period, consistent with recently reported data (Collins et al. 2018), to finally reach a 20% level of myelination at P22. Additionally, we report two new subpopulations of neurons detected by CR and MAP2, one homogeneously distributed throughout the lumen of

the commissure and displaying small- enlarged cytoplasm (CR⁻/MAP2⁺), and the other infiltrating from the BSTLP (CR⁺/MAP2⁺) with unknown functions.

During the last two decades there has been increasing evidence that failures in the development of the AC may mediate serious neurologic disorders (Adler et al. 2004; Doderio et al. 2013; Hulshoff Pol et al. 2004; Kikinis et al. 2015; Saxena et al. 2012). Despite this evidence, the knowledge we have regarding the mechanisms of AC development are still very limited. Accordingly, we performed a thorough and detailed analysis of AC axonal wiring during the embryonic and postnatal development by applying both classical (DiI injections) and more innovative (piggyBac transposon system) techniques. The quality of DiI labeling to track developmental axons has been previously reported to poorly discriminate individual fibers (Lent and Guimaraes 1991). In our work, we employed DiI injections to roughly approach the AC development but could not distinguish the precise sequence of events for axonal extension and elongation we sought using only DiI. In order to effectively illustrate axon outgrowth in the AC we thus used a piggyBac technique to label progenitor cells, which we and others have previously employed to study cell lineages to good effect (Figueres-Onate et al. 2016; Garcia- Marques and Lopez-Mascaraque 2013; Garcia-Moreno et al. 2014; Martin-Lopez et al. 2013; Martin-Lopez et al. 2017). We were thereby able to precisely track axon growth through the AC and could describe the “progressive” (growth) and “regressive” (pauses, waiting periods and fiber pruning) strategies observed in other brain tracts, including the CC, the commissure of the superior colliculus or the afferent innervation of the OB (Chebat et al. 2006; Lent et al. 1990; Treloar et al. 1999; Wise and Jones 1976). However, our current results contradict what was previously reported in hamsters, whose AC develop using only progressive events (Lent and Guimaraes 1990; Lent and Guimaraes 1991). Our interpretation of these discrepancies is based on the method used to label the AC axons using DiI, as they may not be effective in revealing the regressive events during development of axonal tracts. Therefore, we demonstrate that the piggyBac-transposon multicolor method is a powerful technique for tracking axons during the development of the AC pathfinding.

Consequently, we could establish a detailed timeline of developmental events in the AC of mouse (Fig. 4). These events include the arrival of the first axons in the ML around E14, one day later than the previously reported at E13.5 (Kallen 1954; Silver et al. 1982), although we agree that ML is initially crossed at E14.5 (Wahlsten 1981). However, a more recent work reported AC axons reaching and even crossing the ML at E13.5 (Schneider et al. 2011). Discrepancies could be due to differences in the mouse strain or targeting of distinct neurons subpopulations, such as subplate neurons projecting through the internal capsule showing that behavior (De Carlos and O’Leary 1992). In our opinion, and considering that the first subpopulations of PC cells start migrating through the LCS at E11 (Pax6 cells from pallial origin) and E13 (Dlx2 cells from subpallial origin) (Carney et al. 2006), the suggestion that commissural axons reached (and even crossed) the midline at E13 seems unlikely. Our data, on the other hand, supported the results obtained from magnetic resonance (Zhang et al. 2005) and AC labeling using L1CAM (Klingler et al. 2015).

Besides our interest in establishing the timeline of AC development, we wanted to further investigate the influence CSPG may have on guiding axons through the AC to incorporate

this family of molecules to the other types influencing the development of brain commissures (Chedotal 2014; Dickson 2002; Kaprielian et al. 2000; Nawabi and Castellani 2011; Suarez et al. 2014). The CSPG are a family of glycoproteins that have been reported to form boundary tunnels around the AC and act as guidance cues (Fernaund-Espinosa et al. 1996; Inatani et al. 2003; Pires- Neto et al. 1998), although the roles of specific CSPG remain enigmatic. Despite the difficulties in understanding the function of PGs in vivo, we know they might act by generating instructive “sugar codes” to instruct axonal guidance (Holt and Dickson 2005). Here, we speculated that some CSPG such as versican, may be playing a similar role in sorting axons traveling between the ALAC and PLAC. We first mapped the expression of all CSPGs by analyzing staining with anti-CS56, an antibody that recognizes all types of CSPGs. Our data showed that CS56 was overexpressed in the PLAC at the level of the ML, an expression pattern that strongly implied that CSPG may have both permissive and repulsive behaviors depending on the type of axon. This dual behavior of CSPG has been reported before and is related to the intrinsic properties of neurons (Kantor et al. 2004; Shen 2014). It is therefore reasonable to speculate with the existence of differing intrinsic mechanisms expressed by ALAC and PLAC axons. Among the CSPG investigated, only versican was observed in and around the AC during development. The expression pattern was found to be similar compared with that previously described around the internal capsule (Popp et al. 2003), which makes it reasonable to speculate that this CSPG could have similar activities in the axonal guidance in the AC.

Our next step was analyzing the postnatal maturation of the AC by describing the innervation and arborization of the contralateral PC and the myelination of AC axons. The arborization process in the contralateral PC was found to be similar to the arborization of callosum fibers, which exhibit a waiting period in the deeper layers before arborizing the contralateral cortex while cortical neurons mature (Lent et al. 1990; Wise and Jones 1976). This process contradicts what was observed in hamsters (Lent and Guimaraes 1990). On the other hand, the myelination process studied by the CNPase staining agreed with the recently described studies made with other myelin markers and EM (Collins et al. 2018). In contrast, the myelination rate we found of 20% (103 ± 5 axons/100 μm^2) was higher than the reported recently (Collins et al. 2018) and more similar to the previously described for more mature animals at P35 (Sturrock 1975; Sturrock 1976) and P240 (Sturrock 1974b). Indeed, our recent data in PC describe a mature architecture and organization of PC by the weaning day at P21 (Martin-Lopez et al. 2017; Sarma et al. 2011) suggesting that the AC axons are similarly mature by this age. The consistency in our data may be accounted for in part by the large number of axons counted per animal, $> 2,000$ (Sturrock 1975), and differences in the maturation rates might be related to the use of different mouse strains (Wahlsten 1981). However, we did not rule out the possibility of a later maturation of the tract as immature markers like DCX and PSA-NCAM were still detected in the core of the AC at this age.

Finally, characterization of cell populations in the AC revealed two previously unrecognized subpopulations of neurons: one expressing MAP2 ($\text{CR}^-/\text{MAP2}^+$), and the other expressing both MAP2 and CR ($\text{CR}^+/\text{MAP2}^+$) located in the ML region ventral to the BSTLP (Fig. 8). The $\text{CR}^-/\text{MAP2}^+$ were homogeneously distributed within the AC and may represent a subpopulation of neurons that could be involved in olfactory circuits and derived from the adjacent septal nuclei (Sturrock 1977). However, the absence of staining of the lateral

dendrites with MAP2 also suggested they could belong to the population of AC interfascicular neurons (Larriva-Sahd et al. 2002). The CR⁺/MAP2⁺ phenotype exhibited a larger cytoplasm and extended a single cell process pointing ventrally in a behavior suggesting cell migration infiltrating from the adjacent BSTLP. This behavior has been previously described by a population of neurons infiltrating the AC from the amygdaloid complex in the caudal parts of the AC (Gurdjian 1925). Regardless of their origin, the function of both populations of neurons remains a mystery.

CONCLUSIONS

In summary, our findings demonstrate: (1) the development of AC in mouse follows dynamics of *progressive* (extension and elongation) and *regressive* (waiting periods and pauses) strategies during development, in contrast to that described for other rodents; (2) the ipsilateral and contralateral innervation of the PC occurs later in the development than the projections to the OB; (3) the sorting of anterior and posterior axons of the AC at the level of the ML may be regulated by the expression of selective chondroitin sulfate proteoglycans; (4) the myelination of the AC occurs postnatally in a *rapid period* involving an increase in the number of cells after P14; (5) the myelination rate of CD1 mouse at P22 show a maturation degree homologous to older animals from other strains; (6) we report two new subpopulations of neurons in the AC: one with small and enlarged cell bodies expressing CR⁻/MAP2⁺ phenotype and distributed uniformly throughout the AC; and one other expressing the CR⁺/MAP2⁺ phenotype as neurons in the BSTLP that could be infiltrating from that nucleus; (7) these both subpopulation of neurons are generated after postnatal day 7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Development of the anterior commissure. DiI was injected into fixed brains along the anterior-posterior axis of PC to label both anterior (ALAC) and posterior (PLAC) limbs. Images are shown in the horizontal plane as described in the “atlas of the prenatal mouse brain” (Schambra et al. 1992). (A) Section at E13 showing the first growth cones. (B) Section at E14 showing pioneer axons approaching the ML (arrowhead in B'). (C) First evidence of AC axons crossing the ML at E15. (D) AC axons crossing the ML at E16 and general thickening of the tract. (E) Thickness of AC is seen equally in both sides of the ML at P0. (F) Aspect of a mature AC at P21 showing both the ALAC and PLAC. Nuclei counterstained with DAPI (blue). Scale bars = 1 mm in F; 500 m in A-E; 100 m in insets (A'-D').

Fig. 2.

Timeline of anterior commissure development as demonstrated by labeling piriform cortex precursor cells with the piggyBac transposon system (Martin-Lopez et al. 2017). (A, B) Coronal section and diagram at E13 illustrating the first commissural axons (“pioneer axons”) extending from “PC commissure pioneer neurons”. AC axons elongate while PC precursor cells migrate ventrally through the LCS. (C-E) Horizontal sections and diagram showing pioneer axons navigating towards the ML at E13. (F, G) Coronal section and diagram at E14 illustrating the AC pioneer axons approaching the ML and PC neuroblasts migrating through the LCS. (H, I) Horizontal section and diagram showing the first AC pioneer axons reaching the ML at E14 (arrowhead in H’). (J, K) Horizontal section and diagram at E15 showing the thickening of the AC in the ipsilateral parenchyma. Follower

axons coalesce upon the pioneer to form a thicker AC bundle. (L, M) Horizontal section and diagram illustrating pioneer axons crossing but staying near the ML at E15 (arrowhead). Pioneer axons around the ML represent the first “regressive event” during the AC development. (N, O) Horizontal section and diagram of the AC at E16 showing the axonal fasciculation to form a tract elongating through the contralateral parenchyma. No evidence of pioneer axons is observed arriving to the contralateral PC (arrowhead). (P, Q) Horizontal section and diagram at E17 showing the AC axons reaching the contralateral inner border of the PC formed by the CAB/C SB. Axons defasciculate and spread out along the anterior-posterior axis (arrowheads) at E17. (R) Horizontal section at P0 showing a similar morphology observed at E17, with no axons innervating the contralateral PC. E17-P0 represent the second “regressive event” of the AC development. Abbreviations: CAB: cortico- amygdaloid border; CSB: cortico-striatal border; LCS: lateral cortical stream; ML: midline. Scale bars = 500 μ m in A, C, F, H, J, L, N, P and R; 50 μ m in A', D, F' H' L' N'; 5 μ m in J'.

Fig. 3.

Ipsi- and contralateral cortico-cortical innervation of piriform cortex during embryonic and neonatal development. Animals received unilateral IUE with tdTomato-expressing piggyBac transposon at E11. (A-E) Ipsilateral cortico-cortical projections of aPC with axons from the pPC. (A, B) No axons are seen approaching the aPC coming from the pPC. (C-E) At P1-P7 we observe the first evidence of ipsilateral innervation in aPC, represented by the arrival of parallel fibers crossing layer II and invading layers I and III. (F-J) Contralateral innervation of PC. AC axons approach to the CSB/CAB at E17, where they stall (regressive event) without innervating the PC (F and G, arrowheads). At P1, some axons begin to innervate the contralateral layer III of PC (H, arrowhead) and slowly arborize until P3 (I). At P7, the

arborization is more elaborated and complex in layers I and III (J), with some axons crossing layer II. Some, growth cones are still visible at P7 suggesting that maturation continue (J, arrowhead). (K-O) Contralateral innervation of the OB from ALAC. Growth cones reach the OB peduncle at E17-P0 (K, L, arrowheads). From P1, axons penetrate in the GCL (M, arrowhead), to arborize in the GCL and MCL at P3-P7 (N, O, arrowheads). In all images, layer II of the PC is defined as the region of highly packed cell bodies shown by darker spherical nuclei. Layer I and III are the areas above and below layer II respectively. Abbreviations: ALAC: anterior limb of the anterior commissure; aPC: anterior piriform cortex; CAB/CSB: cortico- amygdaloid border/cortico-striatal border; GCL: granule cell layer; MCL: mitral cell layer; OB: olfactory bulb; pPC: posterior piriform cortex. Scale bars = 100 μ m F-O; 50 μ m A-E.

Fig. 4.

Diagram illustrating the progressive (growth and elongation) and regressive (pauses and waiting) periods during the timeline of AC embryonic and postnatal development.

Fig. 5.

Expression of chondroitin sulfate proteoglycans during the development of anterior commissure. Images show horizontal planes (Schambra et al. 1992) of the ML region, illustrating the anterior pole (rostral) in the upper part of the images, and the posterior pole (caudal) at the bottom. (A-I) CSPG distribution in the ML during the AC development detected with antibody against CS56. (A) Intense staining is observed in the brain parenchyma around the ML region at E13. (B, C) From E14 to E15, CS56 staining forms a tunnel surrounding the area through which the AC axons navigate (arrowheads). (D-F) At E16, CS56 expression reaches the region from which axons extend from the PLAC. AC axons coming from the aPC that form the ALAC were specifically labeled by using regionalized IUE in the anterior LGE (red); these axons are seen navigating exclusively through the area absent of CSPG (green). This CS56 expression pattern is maintained until the end of embryonic (E17-E18) (G, H) and postnatal (P7) (I) development. (J-R) Expression of the versican CSPG around the AC region during embryonic-postnatal development. (J) Expression of versican by neuroblasts migrating from the LGE at E12. (K) Expression of versican in the brain parenchyma around the ML at E13; (L-N) Formation of a versican tunnel around the AC at E14-E16 and absence of expression inside the AC. (O) At E17, versican is only expressed in the rostral part of the AC (arrowheads) and the caudal edge of the ALAC in the area where coalesce with axons from the PLAC (P, arrowheads). (Q) At E18, versican expression forms longitudinal stripes within the lumen of AC in the ALAC area (Q). (R) Overexpression of versican in the ALAC region at P7 (arrowheads). Nuclei counterstained with Draq5 (blue). Abbreviations: ALAC: anterior limb of anterior commissure; CSPG: chondroitin sulfate proteoglycans; LGE: lateral ganglionic eminence; ML: midline; PLAC: posterior limb of anterior commissure. Scale bars = 100 μm.

Fig. 6. Temporal dynamics of anterior commissure myelination. (A-D) Expression of myelin-associated enzyme CNPase (green) in the ML of AC at the postnatal stages P0-P21. Nuclei counterstained with Draq5 (blue). The first cells expressing CNPase were observed at P0 (A) around the ML. At P7 (B) there is an invasion of CNPase cells in the lumen of the AC, but no evidence of myelination was observed. At P14 (C) there is an abrupt increase in CNPase staining showing myelinated fibers throughout the lumen of the AC (*rapid myelination process*) that stabilize until P21 (D). These processes are shown graphically after performing Quantification of the CNPase stained area (E). Maturation of AC showed by an increase of the number of cells (Draq5 nuclei) in the AC during postnatal development. Statistical significance (* = $p < 0.01$). Abbreviations: AC: anterior commissure; CNPase: 2',3'-Cyclic-nucleotide 3'-phosphodiesterase; ML: midline. Scale bar = 100 μ m.

Fig. 7.

Characterization of the anterior commissure ultrastructural profiles and myelin at P22. (A) Brightfield image of the ALAC in the coronal plane showing the subdivisions made for myelin quantifications (areas A to F); dark staining is the product of pre-treatment with osmium before processing for electron microscopy (EM). (B) Representative cross-sectional EM image of AC axons showing myelinated axons surrounded by myelin sheaths. (C) Comparison of myelinated unmyelinated axons at P21 in the ALAC represented as # axons/100 m^2 ($t = -7.62$; $* = p < 0.01$). (D) Comparison in the diameter between myelinated and unmyelinated axons ($t = 5.43$; $* = p < 0.01$). (E) External boundary between the lumen of the AC occupied by myelinated/unmyelinated axons and the surrounding tissue (absence of myelinated axons). (F) Representative image of two oligodendrocytes found inside the ALAC, characterized by a dark cytoplasm and irregular nuclei with clumped chromatin. Scale bars = 100 μm in A; 2 μm in F; 1 μm in B and E.

Fig. 8.

Characterization of neuron and glial cells in the anterior commissure by immunohistochemistry at P21. All images show cross-sectional planes of the AC with nuclei counterstained with Draq5 (blue). (A) NeuN neurons (red) located within the lumen of the AC (arrowheads). (B) Scattered Reln neurons (red) located exclusively in the PLAC (arrowhead). (C) Immunoreactivity to CR (green) and MAP2 (red) showing a population of MAP2⁺ neurons with small and enlarged cell bodies within the lumen of the ALAC and a strong staining for CR surrounding the AC. (D-F) AC section at the level of axons crossing the ML. A subpopulation of neurons expressing the CR⁺/MAP2⁺ phenotype depicted in the area underneath the BSTLP. A ventral cell process emerges from these cells, suggesting

migration from the BSTLP. (G) Expression of GAD65/67 (red) positive inhibitory neurons inside (arrowheads) and around the AC. (H) DCX staining (red) reveals the presence of immature neurons in the core of the AC (dotted line). The external edge of the AC is shown by arrowheads. (I) Labeling for PSA-NCAM (red) in the core of the AC (dotted line vs arrowheads). (J) Subpopulations of astroglial cells expressing S100 (red) inside the AC. (K) GFAP staining (red) showing astrocytes inside the AC. (L) Iba1 positive microglial cells (red) intermingled with other cells in the AC. Abbreviations: AC: anterior commissure; BSTLP: bed nucleus of the stria terminalis, lateral division, posterior part; CR: calretinin; MAP2: microtubule associated protein-2; ML: midline. Scale bars = 500 μ m in inset in D; 100 μ m in all other images.

Table 1.

Primary and secondary antibodies

Antigen	Primary AB	Source (Catalog#)	Dilution	Secondary Ab	Source	Dilution
Brevican	Mouse IgG1	BD Biosciences (610894)	1:200	Goat anti-Ms IgG1 Alexa 488	Invitrogen	1:1000
Calretinin	Mouse IgG1	EMD Millipore (MAB1568)	1:400	Goat anti-Ms IgG1 Alexa 488	Invitrogen	1:1000
CNPase	Mouse IgG1	Biolegend (836404)	1:1000	Goat anti-Ms IgG1 Alexa 488	Invitrogen	1:1000
CS56	Mouse IgM	Sigma-Aldrich (C8035)	1:200	Goat anti-Rb IgG Alexa 488	Invitrogen	1:1000
Doublecortin	Rabbit Poly.	Cell Signaling (4604)	1:500	Goat anti-Rb IgG Alexa 488	Invitrogen	1:1000
GAD65/67	Mouse IgG2a	EMD Millipore (MAB5406)	1:500	Goat anti-Ms IgG2a Alexa546	Invitrogen	1:1000
GFAP	Rabbit Poly.	Dako (Z0334)	1:500	Goat anti-Rb IgG Alexa 546	Invitrogen	1:1000
Ibal	Rabbit IgG	Wako (016–2000001)	1:200	Goat anti-Rb IgG Alexa 546	Invitrogen	1:1000
Map2	Chicken IgG	EMD Millipore (AB5543)	1:500	Goat anti-Chk IgG Alexa 568	Invitrogen	1:1000
NeuN	Mouse IgG1	EMD Millipore (MAB377)	1:500	Goat anti-Ms IgG1 Alexa 546	Invitrogen	1:1000
Neurocan	Mouse IgG	Chemicon (MAB5212)	1:200	Goat anti-Ms IgG Alexa 546	Invitrogen	1:1000
PSA-NCAM	Mouse IgM	EMD Millipore (MAB5324)	1:250	Goat anti-Ms IgM Alexa 555	Invitrogen	1:1000
Reelin	Mouse IgG	EMD Millipore (AB78540)	1:500	Goat anti-Ms IgG Alexa 546	Invitrogen	1:1000
S100	Mouse IgG1	Sigma-Aldrich (S2532)	1:100	Goat anti-Ms IgG1 Alexa 546	Invitrogen	1:1000
Versican	Rabbit Poly.	EMD-Millipore (AB1033)	1:250	Goat anti-Rb IgG Alexa 488	Invitrogen	1:1000