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Axons of callosal neurons bifurcate transiently at the white matter before consolidating an interhemispheric projection

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Abstract

The main alternative output routes of adult cortical axons are the internal capsule and the corpus callosum. How do callosal axons choose their trajectories? We hypothesized that bifurcation followed by elimination of one branch is a developmental strategy for accomplishing this aim. Using embryonic and postnatal mice, we labelled cortical projecting neurons and quantified their axonal bifurcations in correlation with the mediolateral position of their somata. Bifurcating axons were numerous in the younger brains but declined during further development. Most bifurcating axons pertained to neurons located in the dorsolateral cortex. Moreover, callosal neurons bifurcate more often than subcortically projecting cells. We then quantified bifurcations formed by dissociated green fluorescent cells plated onto cortical slices. Cells grown over dorsolateral cortex bifurcated more often than those grown over medial cortex, irrespective of their positional origin in the donor. Removal of intermediate targets from the slices prevented bifurcation. We concluded that transient bifurcation and elimination of the lateral branch is a strategy employed by developing callosal axons in search of their targets. As cell body position and intermediate targets determine axon behaviour, we suggest that bifurcations are regulated by cues expressed in the environment.

Introduction

The cerebral cortex is the most complex structure of the mammalian brain, whose areas form precise connections with each other and with subcortical regions. Cortical circuitry develops perinatally, sculpted by progressive and regressive mechanisms until mature connections become fully functional.

Two basic neuronal types can be identified in all cortical layers: projection and local-circuit neurons. While the latter have short axons that usually do not extend beyond a given cortical area, projection neurons extend axons connecting different cortical and subcortical regions (Douglas & Martin, 2004). Among pyramidal neurons, those located in layers 2–3 project to ipsi- and contralateral cortical areas, whereas those in layers 5–6 project to cortical (ipsi- and contralateral) and subcortical regions. Moreover, pyramids within the same layer have different hodology and activity patterns. In rodent layer 5 there are at least two subpopulations. One has a thick apical dendrite reaching layer 1, produces spike bursts, is located in upper layer 5 and projects to subcortical targets. The other has a smaller soma and a shorter apical dendrite arborizing in upper layers without reaching layer 1, fires single spikes, occupies lower layer 5 and projects to the contralateral cortex and striatum (Klein *et al*., 1986; Hübener & Bolz, 1988; Chagnac-Amitai *et al*., 1990; Hallman *et al*., 1988; Hübener *et al*., 1990; Larkman & Mason, 1990; Silva *et al*., 1991; Kasper *et al*., 1994; Reiner *et al*., 2003; Tsiola *et al*., 2003; Molnar & Cheung, 2006).

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In addition, some molecules (Protein 36, SMI-32, FNP-7 and N-200, Nogo-A and calretinin) and genes (*Otx1*, *Kv3.1*, *Mu-Crystallin*, *S4B′*, *FezI*, *Encephalopsin*, *Crim1*, *Lmo4*, *Ctip2* and *Cux2*) are either specific to different subpopulations of subcortically projecting cells or to callosal neurons (Stanfield & Jacobowitz, 1990; Frantz *et al*., 1994; Akemann *et al*., 2004; Voelker *et al*., 2004; Zimmer *et al*., 2004; Arlotta *et al*., 2005; Molyneaux *et al*., 2005; Molnar & Cheung, 2006; Shin *et al*., 2006). Although most of them are reportedly expressed postnatally, it is believed that some of these genes are involved in early fate specification of projecting cells (Molyneaux *et al*., 2005; Arlotta *et al*., 2006).

Although layer 5 subpopulations can be discerned in adults, these neurons are born within the same embryonic time-window and remain morphologically similar during early development (Koester & O'Leary, 1992). It is only by the end of the first and second postnatal week, respectively, that callosal cells loose their apical dendrite (Koester & O'Leary, 1992) and achieve their full electrophysiological features (Kasper *et al*., 1994; Franceschetti *et al*., 1998). Subcortical projections, however, develop by embryonic day (E)14 in the mouse (De Carlos & O'Leary, 1992) and callosal projections cross the midline by E16 (Ozaki & Wahlsten, 1998).

Considering this background, the following question arises: among projection neurons, how do axons from callosal cells come to head medially towards the midline while those of subcortically projecting cells take an antipodal direction? We hypothesised that this might be achieved through a developmental strategy by which growing axons of callosal cells produce two opposite branches when arriving at the white matter. These would simultaneously seek their way towards lateral and medial intermediate targets, to later eliminate the lateral

branch. We here provide *in situ* and *in vitro* evidence for this hypothesis.

Materials and methods

Animals

All surgical procedures involving the use of living animals were approved by the Committee on Animal Care of the Institute of Biomedical Sciences, UFRJ.

For the *in situ* assays 17 Swiss mice at E18.5 (E1 denotes the day of conception) and 90 of different postnatal (P) ages (P1, P3, P6, P9 and P11; P1 denotes the day of birth) were used. For the *in vitro* assays, we used 76 E17.5 F2 mouse embryos obtained from timed-pregnant heterozygous transgenic females bred with heterozygous males expressing the enhanced green fluorescent protein (EGFP; Okabe *et al*., 1997). The resulting litters contained on average 50% wild-type and 50% $EGFP^+$ embryos, both used in the same experiments.

DiI insertions and histological preparations

Pregnant females were anaesthetized with chloropent–nembutal (50 mg/kg). Embryos were extracted by cesarean surgery and immediately decapitated. The heads were fixed by immersion in buffered 4% paraformaldehyde at 8 °C. Postnatal animals were anaesthetized with ether and perfused transcardially with 0.9% NaCl followed by 4% buffered paraformaldehyde.

Brains were removed from the skull and kept in 4% paraformaldehyde. To label callosal neurons in both embryos and postnatal animals, we used two different approaches: (i) insertion of a 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, USA) crystal into the cortex of whole brains, or (ii) insertion of a crystal directly into the white matter, visualized after a parasagittal section close to the midline in one cerebral hemisphere. This strategy was used to allow us to distinguish between cells projecting to contralateral targets (cortex and striatum) or only to the contralateral cortex (Reiner *et al*., 2003). As in early stages of callosal development fibres do not penetrate the cortex, insertions into the cortex were only used for P3–9 whereas insertions into the white matter were possible for all ages analysed.

To label subcortical projection neurons, DiI crystals were inserted into the internal capsule of embryos and postnatal animals. This fibre structure was localized by transillumination from a ventral angle of view, after sectioning the brain horizontally at the level of the anterior commissure. The position of the DiI crystals was checked under the microscope after cutting the brain, and only those within the limits of the cortical grey or white matter, or within the internal capsule, were considered for analysis.

Brains were stored in the dark at room temperature for 1–6 months, depending on the animal's age, until the relevant pathways were completely labelled. Brains were then encased in 2.5% agarose and sectioned coronally at 200 μm using a vibratome. Sections were counterstained with diamino-phenyl-indol (DAPI; Molecular Probes), mounted on gelatin-coated slides and analysed with a Zeiss Axioplan microscope equipped with fluorescence filters and a camera lucida, and with a Zeiss LSM.510 confocal microscope.

Co-culture and slice overlay assay

Swiss and EGFP embryos were used at E17.5, when the main intermediate targets of cortical projection axons (telencephalic midline and internal capsule) are already formed. Pregnant females were anaesthetized, embryos were removed by cesarean section and immediately decapitated, and their heads were kept in chilled Gey's solution. $EGFP^+$ and $EGFP^-$ animals were sorted under a UV light source by the characteristic green fluorescence of the former.

Brains from wild-type mice were dissected, encased in sterile 2.5% agar and sliced in a vibratome at 250 μm with an angular tilt of 40° necessary to preserve both the callosal midline and the internal capsule in the same slice (modified from Agmon & Connors, 1991). After 30 min in Gey's solution these slices were plated onto poly-L-lysine-treated coverslips and remained in culture medium in the incubator before dissociated cells were placed over them. In some experiments, the midline region or the internal capsule (plus subcortical targets), or both, were removed before plating the slices.

Medial and dorsolateral strips of cortex were dissected from EGFP brains and subjected to mechanical and enzymatic dissociation. Because cell–cell contact modulates the formation of neurites (Davenport *et al*., 1999), a low number of dissociated cells (100 000–200 000) were left to decant over the wild-type slices.

The cocultures were then kept in DMEM-F12 (Invitrogen, USA) with 10% fetal bovine serum, under 5% CO_2, at 37 °C and appropriate humidity. After 3 days *in vitro*, slices were fixed in 4% paraformaldehyde, counterstained with DAPI and mounted on gelatin-coated slides.

Quantification of bifurcating cells

The following criteria were used to identify labelled cells (bifurcating or nonbifurcating) in *in situ* and *in vitro* assays. In the former, infragranular cortical cells retrogradely labelled with DiI were counted only when an axon could be discerned emerging from the cell body and penetrating the white matter, bifurcating therein. Bifurcations had typically the form of an inverted 'T' or 'Y', with the two branches heading in opposite directions. Branches within the grey matter were disregarded. In the culture assays, dissociated EGFP cortical cells could be easily identified by their strong green fluorescence over the cortical slices. Only those with a small cell body (diameter < 10 μm) within the cortical plate, close to the intermediate zone, and with an axon perpendicular to the ventricular surface were quantified. The term 'homotopic' is used hereafter to indicate EGFP cells from medial or dorsolateral cortex positioned over Swiss medial or dorsolateral sectors of the cortical slice, respectively. Similarly, 'heterotopic' is employed for medial EGFP neurons over dorsolateral wild-type cortex or vice-versa. In a second set of experiments, we repeated the assay after removal of the midline or of the internal capsule plus subcortical structures, or both.

Axons were discerned from other neurites *in vitro* as the longest neurite emerging from the soma, with at least twice the size of the others (Dent *et al*., 2004). We also immunoreacted the slices with SMI 312 (pan-axonal marker; Sternberger Monoclonals Inc.) to ensure quantification of axons and better visualization of the intermediate zone.

We classified cells as nonbifurcating if their axons deflected in only one direction (Fig. 1C), or as bifurcating if their axons ramified at the border of or within the white matter, giving off a lateral and a medial branch that could be followed for several micrometers (Figs 1A). In parallel, the medial–lateral localization of each cell in cerebral cortex was recorded in three adjacent sectors of the cortex: medial, dorsal and dorsolateral. The atlas by Paxinos & Franklin (2001) was used as a guide to define these cortical sectors under the camera lucida.

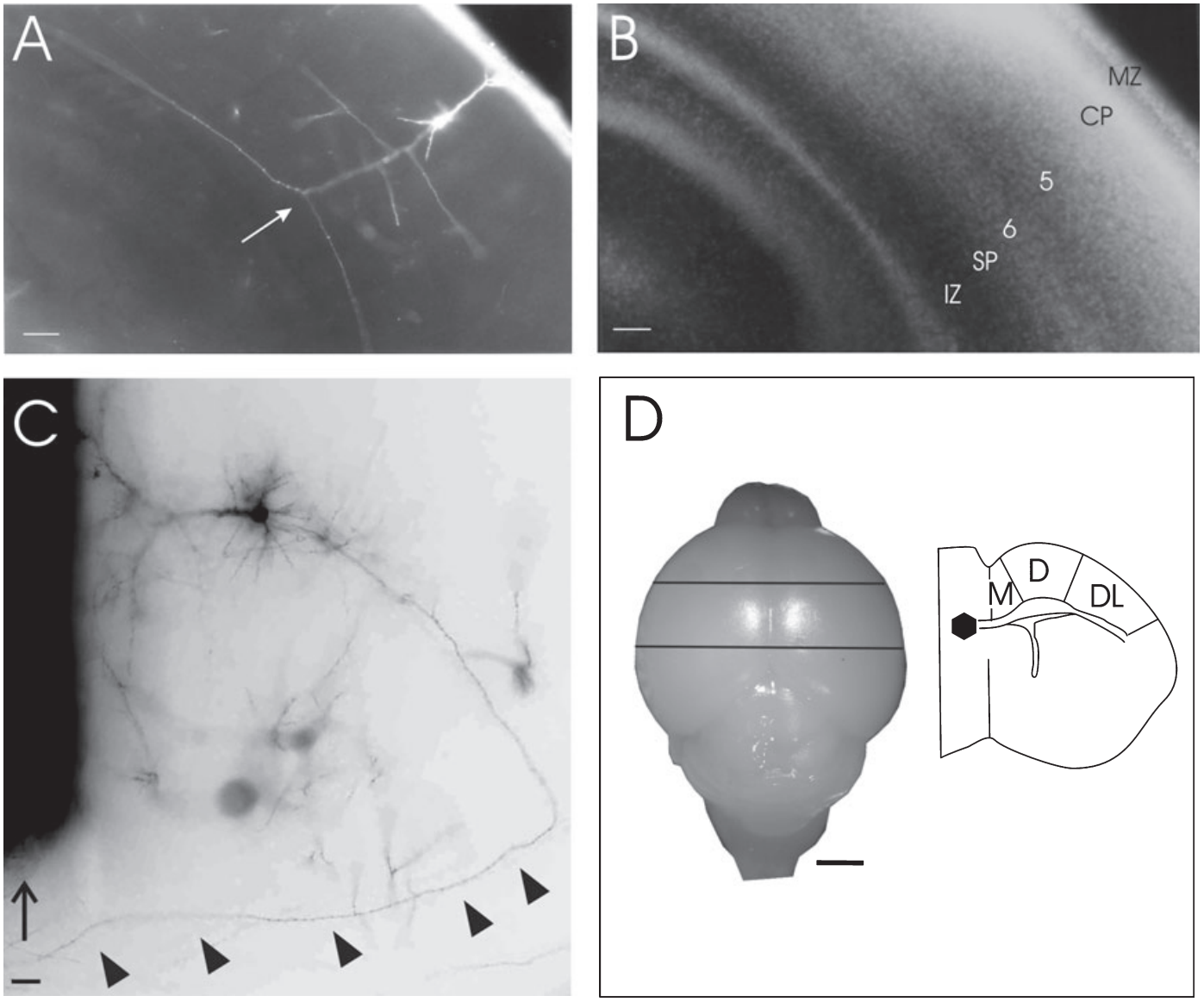

FIG. 1. Axonal behaviour of callosal cells in the white matter of different mediolateral cortical sectors. (A) Example of a retrogradely labelled callosal cell of a P1 animal, showing a typical bifurcation (arrow). (B) DAPI counterstaining of the same slice, used to localize the cell body within the corresponding (in this case, dorsolateral) sector of the cerebral cortex, and the bifurcation within the white matter and intermediate zone. (C) Example of nonbifurcating callosal neuron located in the medial sector of the cortex of a P6 mouse. The arrow indicates the midline and arrowheads indicate the callosal axon. (D) Dorsal view of a P6 mouse brain (left), showing the territory (between straight lines) where labelled cells were found. At the right is a schematic representation of a coronal hemisection through a P6 brain within this territory, showing the locations of an implanted crystal (black polygon) and the three cortical sectors where labelled cells were quantified. CP, cortical plate; D, dorsal; DL, dorsolateral; IZ, intermediate zone; M, medial; MZ, marginal zone; SP, subplate; 5 and 6, cortical layers 5 and 6. Scale bars, 100 μm (A and B), 250 μm (C), 1 mm (D).

To circumvent the high variability of labelling with carbocyanines, which depends on crystal size and position in each brain, we used a relative index to express the bifurcation rate: B/(B + NB), where B represents the number of labelled cells with bifurcating axons and NB is the number of nonbifurcating cells. (This bifurcation index is shown as B/T in Figs 2–4; T is the total number of cells, i.e. B + NB). To compare different ages and different cortical regions we used a nonparametric Student's *t*-test, with levels of significance $P < 0.05$ and $P < 0.01$ reported.

For *in vitro* and *in situ* assays, camera lucida drawings were made to record the positions of the DiI crystals and of the labelled cell bodies. Representative examples of bifurcating and nonbifurcating cells were photographed.

Results

Bifurcation of callosal axons is spatially and temporally regulated

Bifurcating callosal neurons could be identified after backlabelling with a DiI crystal inserted into the contralateral white matter (black symbol in Fig. 1D). Both the cell bodies and bifurcating branches showed up very clearly against the background (Fig. 1A). The number of callosal cells that bifurcate was quantified using a bifurcation index relative to the total number of labelled cells (see Materials and Methods). To test for any differential distribution of neurons that originate bifurcating axons, we divided the cortex into three adjacent sectors: medial, dorsal and dorsolateral (Fig. 1D). In addition, to test

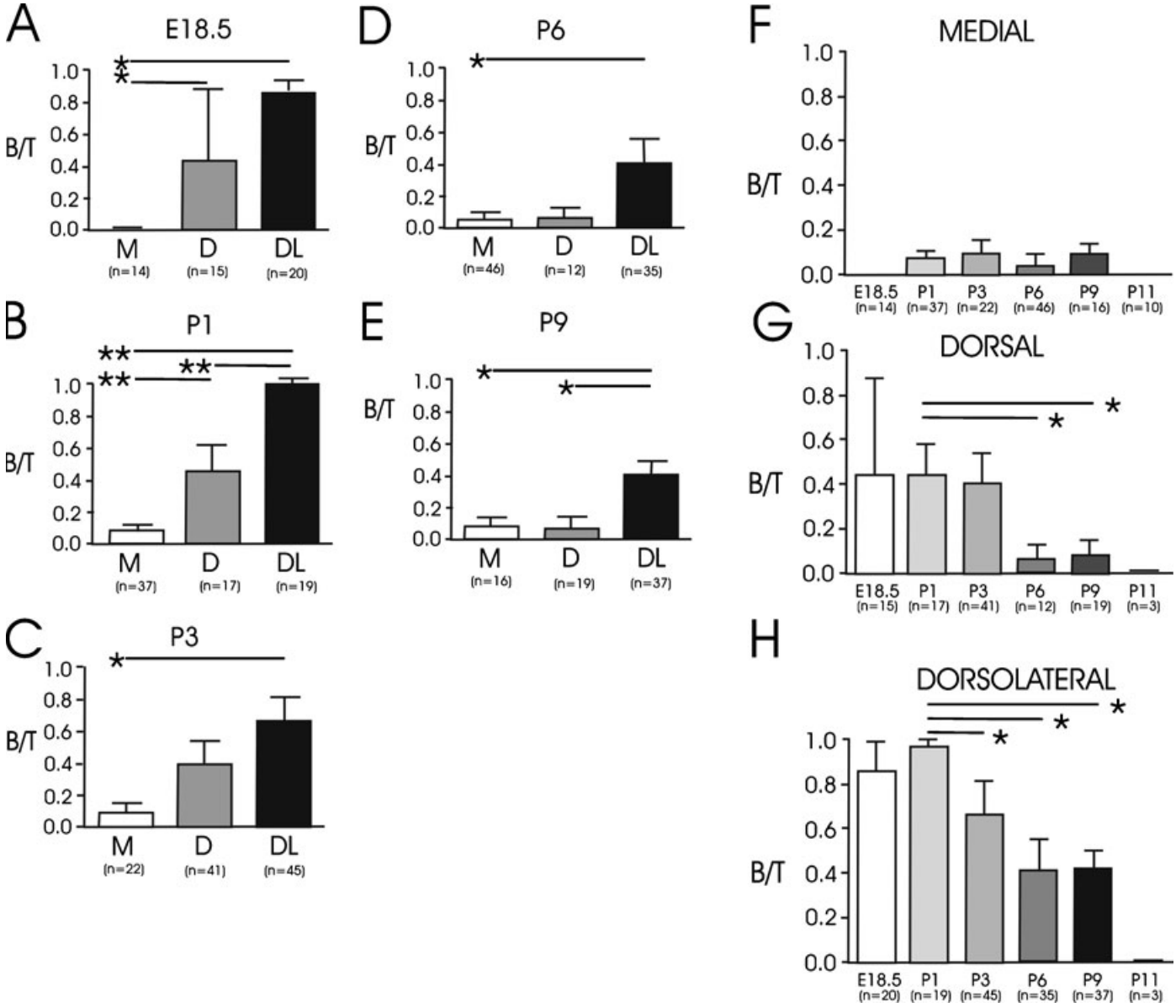

FIG. 2. Frequencies of axonal bifurcations of callosal cells in different mediolateral cortical sectors and through development. (A–E) Histograms represent the bifurcation index (B/T, where B is the number of bifurcating cells and T is the total, i.e. bifurcating + nonbifurcating) of callosal cells in the different ages and sectors, as indicated. (F–H) Developmental variation of the bifurcation index of callosal cells at medial, dorsal and dorsolateral cortex, respectively, at all the ages analysed. Notice that for all ages the bifurcation index (B/T) is low medially, whereas it increases in the dorsal and dorsolateral cortex. *$P < 0.05$, **$P < 0.01$; *n*, number of quantified cells. D, dorsal; DL, dorsolateral; M, medial.

for any temporal regulation of bifurcations we performed the same analysis at different ages (E18.5, P1, P3, P6 and P9). We also analysed separately cell populations labelled by crystals positioned within the contralateral cortex or in the contralateral white matter, assuming that the former labels only projections to the opposite cortex whereas the latter labels projections to any contralateral target (cortex and striatum). As both populations showed a similar proportion of bifurcating cells, we pooled these results, as presented in Fig. 2. At all ages analysed, the bifurcation index was significantly higher in the dorsolateral cortex than in the other sectors (Fig. 2A–E).

The great majority (> 80%) of neurons located in the medial cortex were found to be nonbifurcating (Figs 1C and 2A–E), leading to a low bifurcation index that remained unchanged throughout all ages analysed (Fig. 2F). In the dorsal neocortex, on the other hand, the bifurcation index was higher on E18.5, P1 and P3 than on P6 and P9 (Fig. 2G). A much stronger decrease in the bifurcation index with age took place in the dorsolateral cortex, which showed a very high index in late embryonic and early postnatal development (E18.5 and P1), followed by a pronounced decrease at subsequent ages (Fig. 2H). Conversely, the numbers of nonbifurcating cells increased significantly from P3 to P11 in the dorsolateral cortex, suggesting that bifurcations are transient along development.

Bifurcation is not as robust for subcortical projection neurons as for callosal cells

In order to test whether transient bifurcation would be a general strategy of developing cortical efferents, we inserted DiI crystals into

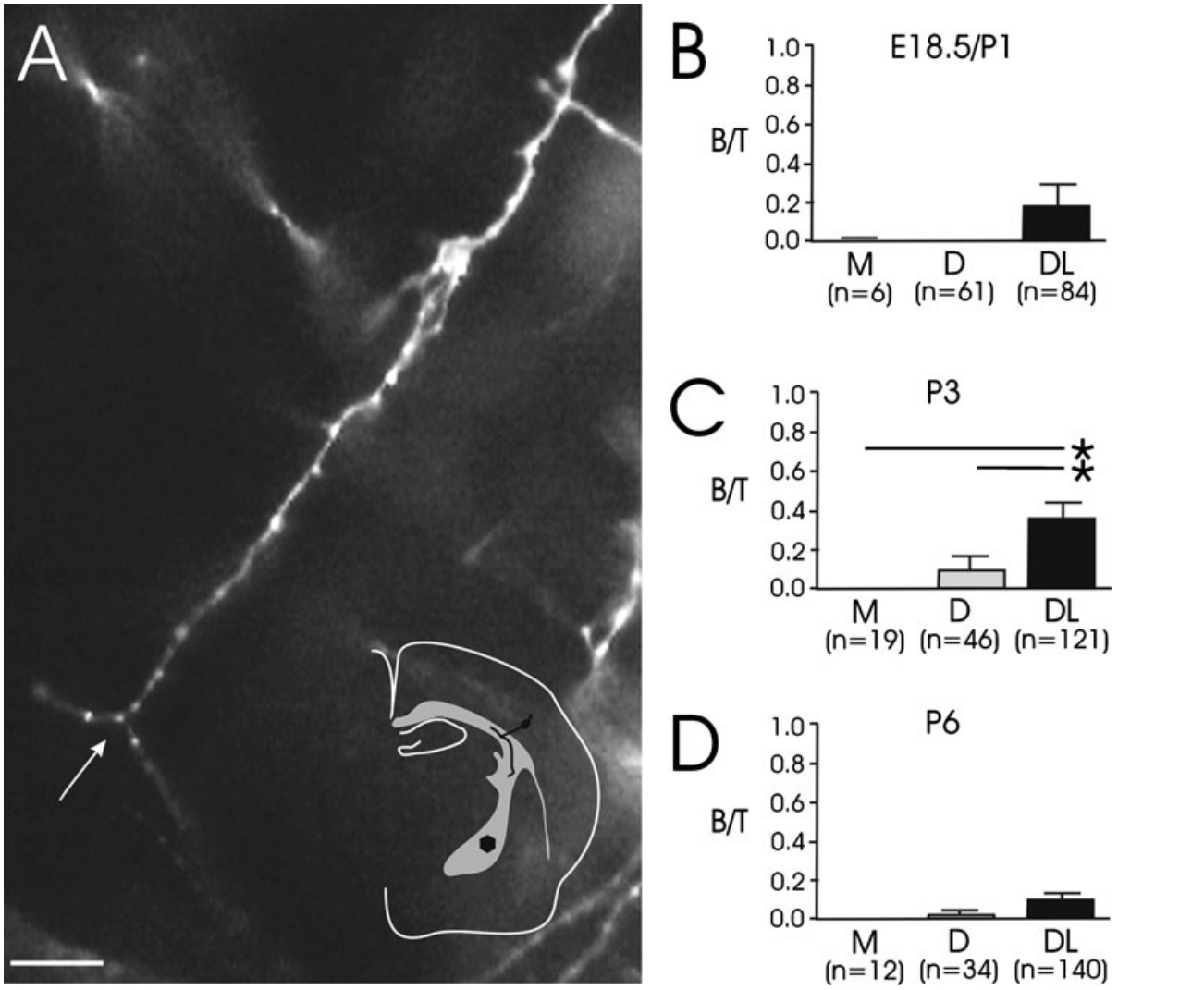

FIG. 3. Frequencies of axonal bifurcations of subcortically projecting cells in different mediolateral cortical sectors. (A) A bifurcating neuron in the dorsolateral cortex at P1, retrogradely labelled after insertion of a DiI crystal in the internal capsule (inset). (B–D) Quantification of subcortically projecting bifurcating cells through development, after insertion of DiI into the internal capsule. The majority of the cells were nonbifurcating in all sectors and at all ages. $*P < 0.05$; *n*, number of cells analysed. B/T, bifurcation index; D, dorsal; DL, dorsolateral; M, medial. Scale bar, 100 μm.

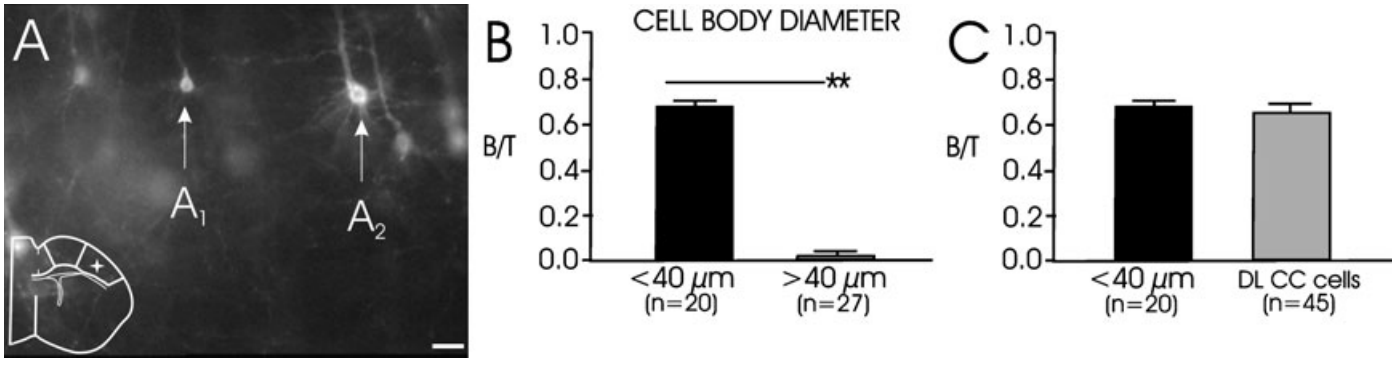

FIG. 4. Relation between cell body size and bifurcation index. (A) Two cells (arrows) at layer 5 of dorsolateral neocortex of a P3 mouse with different morphology, backlabelled after insertion of a DiI crystal in the internal capsule. (B) Bifurcation index (B/T) on P3, showing that cells with large somata (> 40 μm) have a low index while smaller cells (< 40 μm) have the highest index. (C) B/T on P3, showing that callosal cells and small subcortical projecting cells (< 40 μm) have similar bifurcation behaviour. $**P < 0.01$; *n*, number of cells analysed. Scale bar, 100 μm.

the internal capsule to backlabel those cortical cells projecting to subcortical targets and thus be able to estimate a similar bifurcation index as for callosal neurons. Figure 3A shows a subcortically projecting cell with a bifurcating axon in the cortical white matter. The bifurcation index was found to be rather low at all ages and in all cortical sectors, indicating that nonbifurcating cells were dominant in this case (Fig. 3B–D). Nevertheless, the dorsolateral cortex still showed a higher index of bifurcation than dorsal and medial cortex at one of the ages (P3).

In addition, we noticed that subcortically projecting bifurcating neurons always had smaller cell bodies than nonbifurcating cells (Fig. 4). It has been reported that cortical layers 5 and 6 are heterogeneous, containing cells with different morphologies and, especially, cell body sizes (Wise & Jones, 1976, 1977). Callosal cells have smaller somata than subcortically projecting neurons (Hübener & Bolz, 1988; Hübener *et al*., 1990). To clarify this issue further, we classified labelled cells based on the size of their somata (cell diameters $<$ or >40 μm; Fig. 4A) and compared them with the bifurcation indices (Fig. 4B). It was found that larger cells bifurcate less than smaller ones, and that the mean index found for the latter is similar to those found for the dorsolateral callosal cells (Fig. 4C), thus strengthening the hypothesis that callosal cells are responsible for most of the bifurcations identified. This would justify a lower number of bifurcations when one compares E18 and P1 with P3 dorsolateral cortex; the lateral branch of most bifurcated callosal cells would only penetrate the internal capsule by this age.

The lateral branch of bifurcating callosal axons reaches the internal capsule

By placing DiI crystals in the white matter near the midline in one hemisphere of P3 brains, we could often follow labelled callosal fibres far laterally, close to or even within the internal capsule of the opposite hemisphere (Fig. 5A). It was possible to reconstruct some of these callosal neurons to show they had in fact bifurcating axons at the internal capsule region, as shown in Fig. 5B. Similarly, crystals placed in the internal capsule produced labelled fibres in the corpus callosum (Fig. 5C). Labeled axons in the vicinity of the internal capsule were observed irrespective of the position of the crystal (contralateral white matter or contralateral cortex), suggesting that, at least transiently, cortical cells may project to both intermediate targets at the midline and the internal capsule.

The local environment influences axon bifurcating strategy

As a first step to investigating possible cellular mechanisms underlying axonal bifurcation of cortical neurons, we used a slice overlay assay. For this, we dissociated E17.5 EGFP cells and placed them over organotypic cortical wild-type E17.5 slices. The dissociated EGFP cells were isolated from either medial or dorsolateral cortices, and the slices contained midline tissue as well as the internal capsule. We first quantified the homotopic experiments, in which medial cells were placed over the medial cortex and dorsolateral cells over the dorsolateral cortex. Figure 6A shows a bifurcating cell located dorsolaterally over the slice. Dorsolateral cells bifurcated more frequently over dorsolateral cortex than medial cells over medial cortex (Fig. 6D). Thus, these slice ovelay experiments recapitulate the *in situ* situation. Bifurcation could be cell-autonomous or could depend on the cortical microenvironment, or both. In order to distinguish between these possibilities we analysed the heterotopic experiments, in which medial cells were plated over dorsolateral

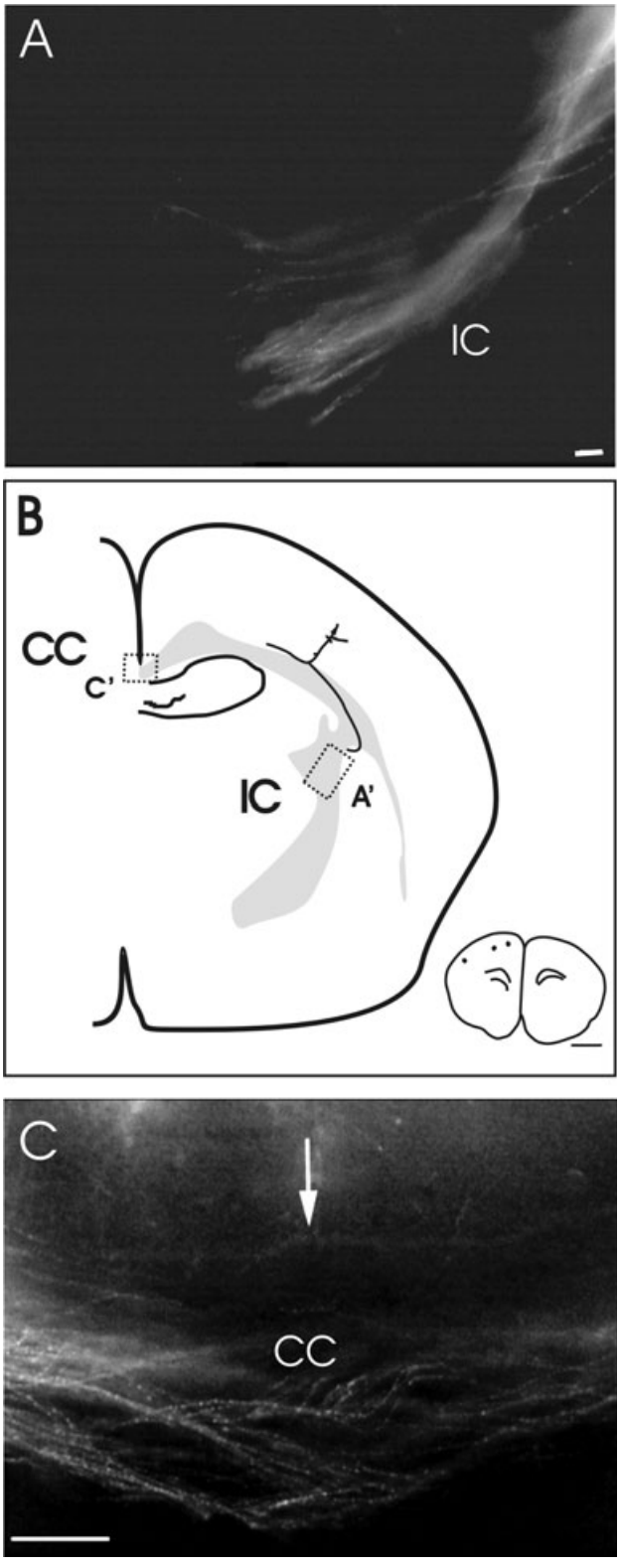

FIG. 5. Lateral and medial extension of bifurcating neurons. (A) Axons labelled with a DiI crystal positioned in the opposite hemisphere reach and penetrate the internal capsule (IC). (B) Camera lucida reconstruction showing a P9 callosal cell whose bifurcating axon reaches the internal capsule. Inset shows the position of the crystals in the contralateral hemisphere. Squares show the location of micrographs in A and C. (C) Fibres labelled with a DiI crystal placed into the internal capsule coursing through the corpus callosum (CC) at the midline (arrow). Scale bars, 100 μm (A), 500 μm (B and C).

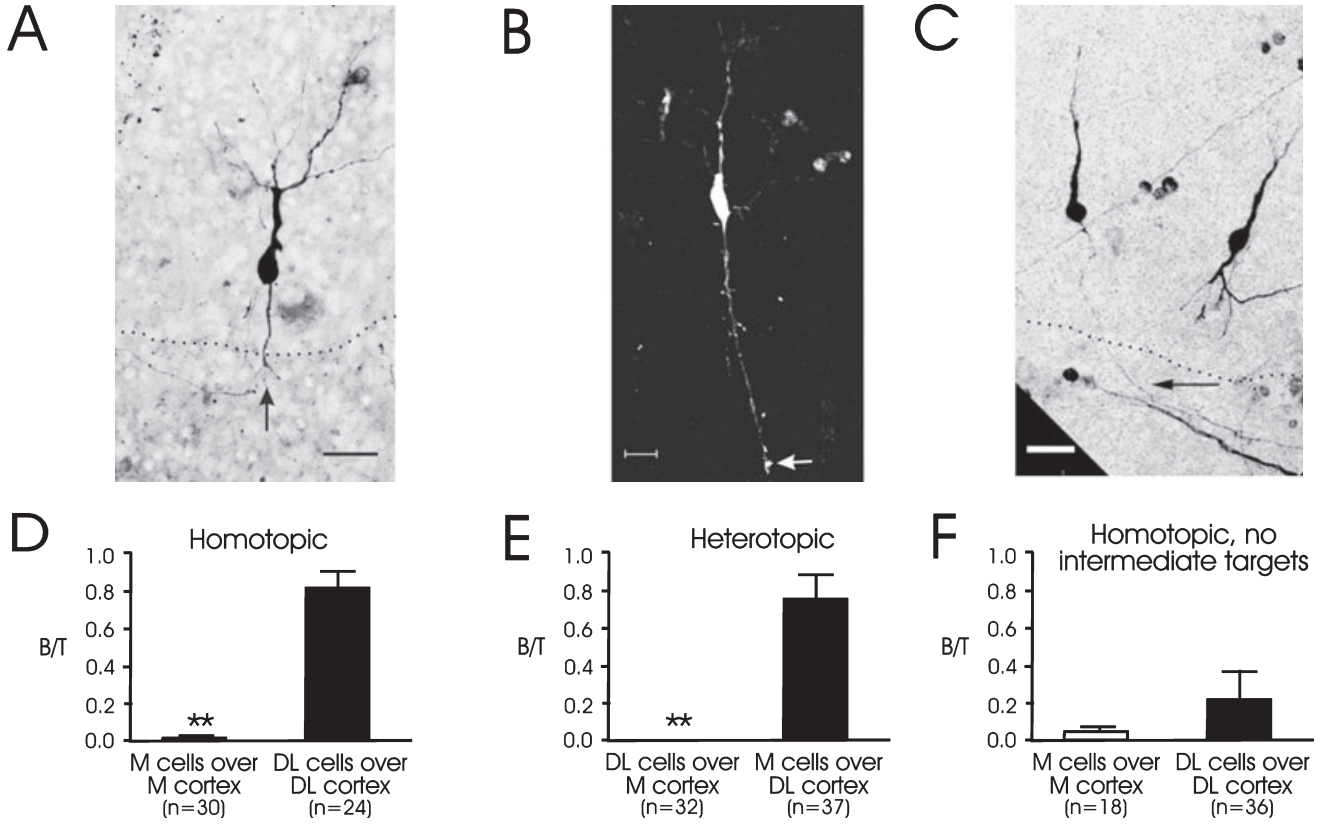

FIG. 6. Influence of the extracellular environment on bifurcation strategy of cortical cells. (A) Example of an EGFP cell, cultured for 3 days over dorsolateral cortex. A bifurcation (arrow) can be seen beyond the border of the intermediate zone (dotted line). The arrow points to the bifurcation and the dotted line represents the border of the intermediate zone. (B) Example of a cell (3 days *in vitro*) plated over a dorsolateral strip of cortex from which the midline and the lateral sectors were removed, showing no bifurcation when the intermediate targets are absent. The arrow points to the growth cone at the border of the intermediate zone. (C) Another cell (3 days *in vitro*) plated on the dorsolateral cortex from which only the midline was removed. Notice that the axon deflects but does not bifurcate at the intermediate zone. The arrow points to the axon analysed and the dotted line represents the border of the intermediate zone. (D) Quantification of homotopic assays, showing that dorsolateral cells over dorsolateral cortex bifurcate more than medial cells over medial cortex. (E) Data for the heterotopic assays, where medial cells are shown to bifurcate more often when cultured over dorsolateral cortex than in the reverse case. $^{**}P < 0.01$. (F) Similar quantification, except that the host slice lacks the medial and lateral intermediate targets; the bifurcation rate is shown to decrease pronouncedly. No significant difference was found between the two means in this graph; *n*, number of cells analysed. Scale bars, 20 μm (A and B), 20 μm (C).

cortex and dorsolateral cells over medial cortex. As illustrated in Fig. 6E, medial cells had a larger bifurcation index when cultured over dorsolateral cortex. In contrast to homotopic experiments, however, dorsolateral cells did not bifurcate. To address the question whether intermediate targets are necessary to induce axonal bifurcation, cells were plated on slices from which the midline and/or the internal capsule and subcortical structures had been removed. In strips of cortex lacking both intermediate targets, medial as well as dorsolateral neurons were shown to extend axons towards the intermediate zone without bifurcating (Fig. 6B and F). When cells were cultured on cortical slices lacking only one of the intermediate targets, either the midline or the subcortical structures, similar results were obtained. Again, there was no axonal bifurcation in the intermediate zone (Figs 6C and 7A).Based on these *in vitro* experiments, we propose that the bifurcation of cortical axons is at least in part controlled by extrinsic factors derived from the local environment.

Discussion

By using *in situ* labelling of long-projection cortical cells with DiI, we have shown that a proportion of cortical axons bifurcate in the white matter during postnatal development, one branch running laterally, the other medially. Bifurcations seem to be a feature typical of contralateral (callosal) axons, but less so of ipsilateral, subcortically projecting fibres. Callosal cells localized dorsolaterally in the cortex are more likely to bifurcate than dorsal and medial ones, but the bifurcation rate decreases with age, being time-regulated during the late embryonic and early postnatal period. In addition, our *in vitro* experiments demonstrated that dissociated cortical cells also bifurcate when cultured over the dorsolateral region of cortical slices, but not over medial regions, or when intermediate targets (i.e. midline and/or internal capsule) have been removed. We therefore conclude that axonal bifurcation may be a strategy of some developing callosal neurons seeking their targets, relying on environmental cues rather than on intrinsic cell-autonomous determinants.

Deciding to grow medially or laterally

As a general rule of cortical development, there is an overproduction of axons, axonal branches and synapses (developmental exuberance), which are later selected and refined (recently reviewed by Innocenti & Price, 2005). The corpus callosum has been a major model for such studies and has brought much insight onto the mechanisms for establishing mature cortical connections (Innocenti, 1981; O'Leary

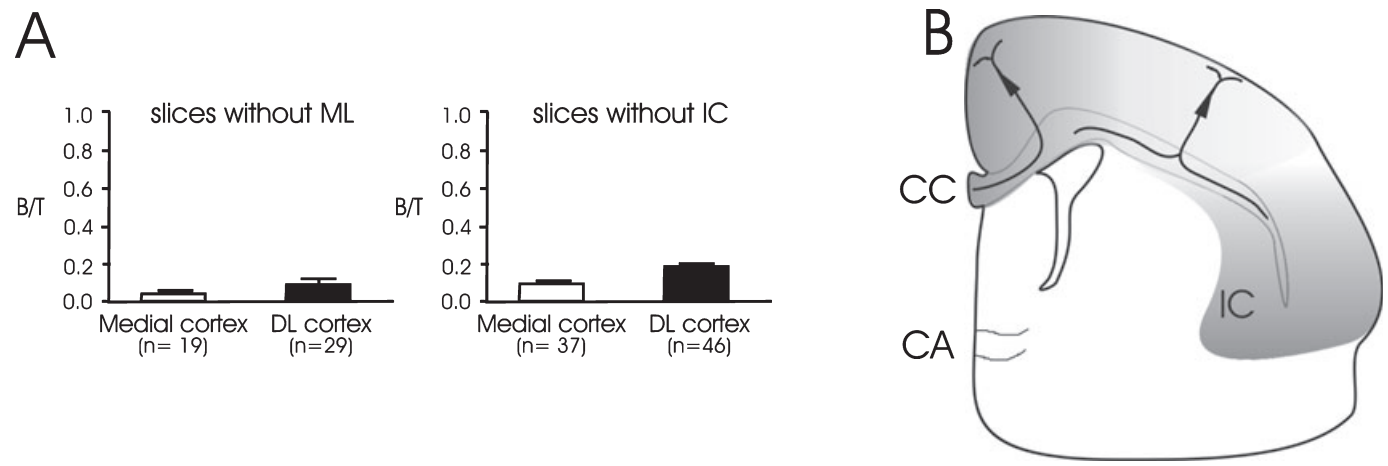

FIG. 7. Influence of the intermediate targets on bifurcation strategy of cortical cells. (A) Quantification of *in vitro* bifurcations where the midline (ML) or internal capsule (plus subcortical structures) (IC) were independently removed. Removal of one intermediate target reduced the bifurcation index in cells located in both medial and dorsolateral cortices. (B) Proposed existence of two gradients of diffusible guidance molecules from the main intermediate targets: ML and IC. The encounter of these gradients at regions equidistant from the molecular sources would promote callosal bifurcations in the dorsolateral white matter. Callosal neurons positioned medially would simply deflect towards the midline. This effect would be time-regulated as the number of bifurcations declines with age. AC, anterior commissure; CC, corpus callosum.

et al., 1981; Schwartz & Goldman-Rakic, 1982; Bullier *et al*., 1990; Dreher *et al*., 1990; Kadhim *et al*., 1993). Efferent cortical axons (callosal as well as ipsilateral subcortically projecting fibres), grow towards the intermediate zone as soon as they arise from the somata, attracted by a gradient of semaphorin 3C. However, they never penetrate the proliferative zones, from where they are repelled by semaphorin 3A (Bagnard *et al*., 1998). At this decision point, the two axonal populations must separate. Callosal axons must decide on a medial pathway towards the midline while subcortically projecting fibres must grow laterally towards the internal capsule. Information on this decision point is scarce, and most evidence concentrates on what happens after it.

Several guidance cues along their way, such as secreted Slit-2 and extracellular matrix molecules produced by midline glial cells, form borders and tunnels that contribute to callosal fibres crossing the midplane at the right place (Richards *et al*., 2004; Lent *et al*., 2005). In addition, the diffusible molecule Netrin-1 is expressed at the telencephalic midline (Serafini *et al*., 1996), and cortical-projecting cells express the Netrin-1 receptor Deleted in colorectal cancer (DCC; Shu *et al*., 2000), suggesting a role for Netrin-1 in the establishment of this pathway. Once crossed, callosal axons mostly innervate homotopic areas of the contralateral hemisphere, although many cells in the somatomotor cortex innervate the contralateral striatum (Hedin-Pereira *et al*., 1999; Reiner *et al*., 2003). It has been reported that callosal fibres employ a dual strategy to arborize in the contralateral cortex. The main axon overshoots its target region, but its collaterals penetrate the cortex (or the striatum) and arborize only at the appropriate region and layer, while the main shaft is eliminated (Innocenti, 1995; Hedin-Pereira *et al*., 1999). The cues used by callosal axons to find their target regions in the contralateral hemisphere are likely to be the same that thalamic axons use to find their cortical target areas (Bolz *et al*., 2004; Uziel *et al*., 2002; Dufour *et al*., 2003).

In this study we have focused on the decision point at the border of the developing white matter, where callosal and subcortically projecting fibres seem to separate. More specifically, we addressed the issue of how callosal axons choose to project medially towards the midline, leaving the lateral direction to the subcortically projecting axons. Based on our results, we propose that, contrary to common belief, most axons from callosal cells located in the dorsolateral neocortex do not simply deflect medially towards the midplane but rather bifurcate transiently when entering the embryonic white matter. The two antipodal branches grow in opposite directions to some extent and, in most cases, only one branch becomes consolidated, while the other is removed. We thus suggest that, at least for these dorsolateral callosal neurons, the decision to establish a medial projection is taken not when fibres reach the developing white matter but later in development. In fact, as discussed below, callosal axons have the machinery to respond to lateral cues that can drive them to the internal capsule or even to the anterior commissure.

Projecting transiently to both contralateral and ipsilateral intermediate targets

It has long been demonstrated that mature callosal connections are formed as a result of elimination of inappropriate projecting fibres. Neurons in the rat and cat cortex that project through the callosum at earlier but not at later postnatal ages do not die, but merely loose the contralateral process (Innocenti, 1981; O'Leary *et al*., 1981; Ivy & Killackey, 1982), maintaining their ipsilateral corticocortical axon. We have shown here that callosal cells bifurcate their axons in the white matter, one branch coursing laterally, the other medially. Implantation of DiI crystals into one hemisphere, near the midline, labels fibres in the internal capsule of the opposite hemisphere and even in the anterior commissure (Hedin-Pereira *et al*., 1992). Similarly, DiI crystals inserted into the internal capsule label fibres crossing to the opposite hemisphere. Hedin-Pereira *et al.* (1999) made anterograde tracer injections into one hemisphere of developing hamsters and reported that in some cases callosal axons penetrate subcortical structures, and terminal arbors were clearly seen within the striatum. Taken together, these data indicate that callosal fibres are able to follow similar cues as subcortically projecting neurons or those cells projecting through the anterior commissure. This, however, does not imply that these fibres reach thalamic, brain stem or spinal cord levels, as Koester & O'Leary (1993) reported that double-labelled cells were never found in the cortex after injections of different dyes into the ipsilateral pons and contralateral cerebral cortex of developing and adult animals.

Bifurcating axons have been seen in developing and adult cortex since the work of Cajal (1911). Although the number of bifurcating

axons was not previously quantified during development, it has been reported that a few bifurcating connections remain until adulthood. Based on our results, we suggest that the population of bifurcating cells is mainly composed of callosal cells, and that bifurcation would constitute a developmental pathfinding strategy employed by these cells to achieve the appropriate pattern of connections. There is now increasing evidence that the callosal population is not homogeneous but consists of multiple subpopulations that express different sets of markers early in development (Arlotta *et al*., 2006). In this work we have shown that, in at least one callosal subpopulation (located in the deep layers of dorsolateral cortex), cells adopt bifurcation as a strategy for pathfinding.

It is believed that bifurcating projections arise during development and are maintained through adulthood only if they become important in co-ordinating motor and multimodal associative information (Mitchell & Macklis, 2005). Some of this processing would be exclusively intracortical (Hedin-Pereira *et al*., 1992; Koester & O'Leary, 1993; Mitchell & Macklis, 2005), whereas some would deal with cortical and subcortical information (Ferino *et al*., 1987; McGeorge & Faull, 1987; Reiner *et al*., 2003). Using electrophysiological techniques, Ferino *et al.* (1987) showed that a subpopulation of projection neurons in the prefrontal cortex respond to stimulation of cells in the ipsilateral and contralateral striatum and contralateral prefrontal cortex. Although layer 5 neurons of the primary motor cortex most often project to the ipsilateral striatum, contralateral (callosal) projections are also seen after bilateral injections. These cells present small-to-medium perikarya and are preferentially located in upper layer 5 (Reiner *et al*., 2003), being therefore morphologically related to corticocortical callosal neurons. These projection loops are considered important in integrating motor processing of both sides (Ferino *et al*., 1987; McGeorge & Faull, 1987).

We propose here that when dorsolateral axons, equally distant from presumptive attractive cues at the midline and at the internal and external capsule, are confronted with the need to decide in what direction to grow, they are unable to simply deflect and instead they bifurcate and extend branches in both antipodal directions, to later eliminate the inappropriate connection (Fig. 7B). A subpopulation of callosal neurons eventually stabilize both branches and project simultaneously ipsi- and contralaterally (Reiner *et al*., 2003).

A proposed mechanism for callosal axon bifurcation in the cortical white matter

Until now, it has been assumed that, once developing corticofugal axons grow ventrally to penetrate the intermediate zone, they then either turn laterally to exit the dorsal telencephalon through the internal capsule (subcortical projections) or turn medially and cross the midline towards the contralateral hemisphere (callosal projection). It is now clear that cortical projection neurons consist of different subsets of neurons that are able to express specific markers since early development (see Molnar & Cheung, 2006 for a recent review). It is possible, however, that different types of projection neurons may respond in the same way to guidance cues, irrespective of their specific fate. In our *in vitro* experiments we observed that many of the overlaid cells bifurcate at the dorsolateral cortex. Formally, we cannot exclude the possibility that some of the bifurcating neurons we observed *in vitro* are noncallosal cells. However, the bifurcation pattern observed *in vitro* is very similar to the *in situ* pattern, where we selectively label callosal cells with DiI. As available cell markers label different subsets of noncallosal projection neurons, further work is needed to identify how subpopulations behave when confronted with a decision point at the border of the white matter.

Although the existence of bifurcating axonal profiles in the cortex has been known since Cajal, we here present evidence suggesting the novel concept that bifurcations can be considered a developmental strategy employed by growing callosal axons in dorsolateral regions to find final targets. The main issue, then, becomes the nature of the mechanisms that stimulate the bifurcating behaviour of growing callosal axons. Our hypothesis is that growing callosal axons are able to recognize diffusible molecular cues expressed by distant intermediate targets, such as the midline, the internal and external capsule or even the anterior commissure (Fig. 7B). Growth cones arriving at the white matter decision points would be confronted with a confluence of molecular gradients originated from these intermediate targets, being able to sense their relative levels and be forced to deflect (if one side's level dominates over the other) or bifurcate (if equivalent concentrations of signals are present). This hypothesis may explain why dorsolateral callosal neurons, equidistant from the midline and the internal capsule, are more likely to bifurcate than those at medial positions in the cortex, as shown by our experiments. In addition, it explains our *in vitro* experiments using dissociated cortical neurons grown over cortical slices devoid of the lateral and medial intermediate targets, whose axons are unable to bifurcate. Finally, the fact that the white matter border seems to be a decision point is substantiated by our observation that growth cones assume a complex morphology when they arrive therein.

Based on their temporal and spatial expression in the telencephalon, several candidate molecules could be involved in this phenomenon. The pattern of distribution of Netrin-1 and evidence that Netrin-1- and DCC-knockout animals are acallosal (Serafini *et al*., 1996; Fazeli *et al*., 1997; Metin *et al*., 1997; Richards *et al*., 1997; Braisted *et al*., 2000) make this molecule a strong candidate for this role. A considerable amount of evidence (Richards *et al*., 1997; Metin *et al*., 1997; Braisted *et al*., 2000) demonstrates that cortical axons are attracted by Netrin, which is expressed in the developing internal capsule. In addition, projecting axons from cortical populations express DCC during development, irrespective of their final target (Shu *et al*., 2000), so subcortical and anterior commissure fibres, as well as callosally projecting axons, are potentially able to respond to the presence of Netrin-1 at the internal capsule. Although there is no direct evidence that Netrin-1 specifically attracts callosal cells, Netrin mRNA is detected at the midline as early as E12.5 (Serafini *et al*., 1996). Therefore, it is conceivable that callosal cells can potentially respond to sources of Netrin at the midline as well as at the internal capsule.

Recently, Kalil and her collaborators (Dent *et al*., 2004; Tang & Kalil, 2005) tested the potential of different guidance factors as branching promoters *in vitro*. It became clear that Netrin-1, as well as FGF-2 but not semaphorin 3A, stimulates branching in dissociated cortical neurons by enhancing actin polymerization. Adding Netrin-1 to the culture bath they found a clear dose-dependent response, although not all neurons were sensitive to the presence of Netrin, suggesting the existence of subpopulations.

Taken together, the reported evidence indicates that axonal bifurcation may be viewed as a developmental strategy employed by callosal fibres in their way towards their targets. As the number of bifurcations declines with age, we suggest that one of the branches is eliminated, as it never reaches a viable target, whereas the other is stabilized. The branching factor acting on callosal axons remains to be identified, but Netrin-1 is considered the most probable candidate.

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Abbreviations

DAPI, diamino-phenyl-indol; DCC, Deleted in colorectal cancer (Netrin-1 receptor); DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; E, embryonic day; EGFP, enhanced green fluorescent protein; P, postnatal day.

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