Excitatory Cortical Neurons with Multipolar Shape Establish Neuronal Polarity by Forming a Tangentially Oriented Axon in the Intermediate Zone

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The formation of axon-dendrite polarity is crucial for neuron to make the proper information flow within the brain. Although the processes of neuronal polarity formation have been extensively studied using neurons in dissociated culture, the corresponding developmental processes in vivo are still unclear. Here, we illuminate the initial steps of morphological polarization of excitatory cortical neurons in situ, by sparsely labeling their neuroepithelial progenitors using in utero electroporation and then examining their neuronal progeny in brain sections and in slice cultures. Morphological analysis showed that an axon-like long tangential process formed in progeny cells in the intermediate zone (IZ). Time-lapse imaging analysis using slice culture revealed that progeny cells with multipolar shape, after alternately extending and retracting their short processes for several hours, suddenly elongated a long process tangentially. These cells then transformed into a bipolar shape, extending a pia-directed leading process, and migrated radially leaving the tangential process behind, which gave rise to an "L-shaped" axon. Our findings suggest that neuronal polarity in these cells is established de novo from a nonpolarized stage in vivo and indicate that excitatory cortical neurons with multipolar shape in the IZ initiate axon outgrowth before radial migration into the cortical plate.

Keywords: cortical efferent, mouse, radial migration, slice culture, time-lapse imaging

Introduction

The establishment of neuronal polarity is fundamental to information processing in individual neurons. In vitro studies using dissociated hippocampal neurons have yielded a prevailing view on the establishment of neuronal polarity (Dotti et al. 1988; Arimura and Kaibuchi 2007; Barnes and Polleux 2009): neurons, initially nonpolarized, extend several neurites and become polarized when one neurite rapidly elongates to form an axon.

Because dissociated neurons are in an artificial environment, in which they have lost native external cues and 3D structure, the processes of neuronal polarization need to be examined in vivo, or at least in situ, to further explore the mechanisms underlying polarization. Recently, cortical γ-aminobutyric acid-ergic (GABAergic) interneurons have been shown to establish their polarity from a nonpolarized stage in explant culture (Yamasaki et al. 2010), which is consistent with the previous studies using dissociated hippocampal neurons. However, for retinal ganglion cells and bipolar cells, the apicobasal polarity of neuroepithelial progenitors has been shown to play a predominant role in determining neuronal polarity in vivo (Morgan et al. 2006; Zolessi et al. 2006). The discrepancy between these results may be due to differences in migration history. For retinal ganglion cells and bipolar cells, which migrate radially, parallel to their neuroepithelial progenitors, inheritance of their predecessors’ apicobasal polarity may influence subsequent definition of the site of axonal specification and dendrite extension (Barnes and Polleux 2009); however, this may not hold true for GABAergic interneurons, which develop their axons postnatally after they have lost the apicobasal polarity during tangential migration.

Excitatory cortical neurons migrate radially, giving rise to a highly polarized structure similar to that of retinal ganglion cells: their dendrites and axons extend toward the pial surface and the white matter, respectively. Their neuroepithelial progenitors, located in the ventricular zone (VZ), produce neurons as well as intermediate neuronal progenitors (IPs), both of which migrate to the subventricular/intermediate zone (SVZ/IZ) and assume a multipolar morphology (Tabata and Nakajima 2003; Miyata et al. 2004; Noctor et al. 2004). Then these neurons, including those from the IPs, migrate radially, extending leading and trailing processes (Rakic 1972; Hatanaka and Murakami 2002; Noctor et al. 2004). Since the leading and trailing processes appear to develop into an apical dendrite and an axon, respectively (Hatanaka and Murakami 2002), they have already established their neuronal polarity. Importantly, before this radial migration, the neurons exhibit "multipolar migration" behavior, dynamically extending and retracting multiple processes (Tabata and Nakajima 2003; Noctor et al. 2004), which is reminiscent of the dynamic behavior of dissociated hippocampal neurons before axon formation (Goslin et al. 1990). This raises the possibility that, without inheriting the apicobasal polarity of their neuroepithelial progenitors, these neurons establish their polarity from a nonpolarized stage.

In this study, we examined axonal development of excitatory cortical neurons in situ. We show that multipolar cells, whose immature processes underwent repeated growth and retraction, suddenly elongated a tangential process that became an axon. These neurons thus appear to establish their neuronal polarity de novo from a nonpolarized stage.

Materials and Methods

Animals

Timed-pregnant ICR mice were purchased from SLC Japan. Noon of the day of vaginal plug observation was defined as embryonic day (E) 0.5. All experiments were conducted in compliance with the guidelines for use of laboratory animals of Nara Institute of Science and Technology and National Institute for Physiological Sciences.
Plasmids

pTol:1-EYFP, pCAGGS:EGFP (Hatanaka and Murakami 2002), pCAGGS:Cre; and pCAGGS:Cre were used. mCherry; memEGFP (Gap13; EGFP; Moriyoshi et al. 1996), and Kif5Cg60-EGFP (EGFP-tagged truncated Kinesin-1, whose stable accumulation in a single neurite is one of the earliest markers of axonal specification; Jacobson et al. 2006; Toriyama et al. 2010) were cloned into the pCALN5 vector (Riken Biosource Center, RDB1826; Kanegae et al. 1996) to produce pCAGGS-loxP-neo-loxP-mcherry (pCALN5:mcherry), pCAGGS-loxP-neo-loxP-memEGFP (pCALN5:memEGFP), and pCAGGS-loxP-neo-loxP-Kif5Cg60-EGFP (pCALN5:Kif5Cg60-EGFP), respectively. Plasmids were purified using a Plasmid Maxi Kit (QIAGEN) and dissolved in phosphate buffered saline (PBS, pH 7.4).

In Utero Electroporation

To sparsely label cells (Morin et al. 2007), mixtures of plasmids were used as follows: 1) for morphological examination, pCALN5:m-Cherry (final concentration 1 μg/μl) and pCAGGS:Cre (0.05–0.1 ng/μl); 2) for time-lapse imaging, pCALN5:memEGFP (1 μg/μl) and pCAGGS:Cre (0.05–0.1 ng/μl). To identify the labeled region, pCAGGS:EGFP or pCAGGS:mCherry (1 μg/μl) was also added. For Kif5Cg60-EGFP expression, a mixture of pCALN5:mCherry (1 μg/μl), pCALN5:Kif5Cg60-EGFP (0.01–0.02 μg/μl), and pCAGGS:Cre (0.05–0.1 ng/μl) was used. Fast Green (0.05%) was used to monitor the injection. In utero electroporation was performed as previously described (Hatanaka et al. 2004).

Preparation and Morphological Examination of Fixed Brain Slices

The lateral VZ cells of E12.5 mice were labeled by in utero electroporation. After 24, 30, or 40 h, labeled heads were collected and immersion fixed in 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.4 at 4 °C overnight. Brains were removed, embedded in 4% low-melting temperature agarose (Agarose LM; Nacalai Tesque) in PBS, and cut into 150- or 200-μm thick coronal slices with a vibrating blade microtome (VT1000S, Leica Microsystems). Coronal slices at the middle level along the anteroposterior axis were collected on a glass slide. Whole cell structure was constructed with Z-series images by confocal microscopy (TCS-SP5, Leica Microsystems); we confirmed that a single cell’s processes were entirely within the slice being observed.

Dissociated Culture

E12.5 mice were collected in PBS. About 1 μl of pTol:1-EYFP (5 μg/μl) was injected into the lateral ventricle, after which the head was placed between forceps-shaped electrodes (CUY60PS; Unique Medical Imada). Electroporation comprised five 50-ms pulses (at 75-ms intervals; 15 V). Labeled cortices, together with the number of unlabeled cortices, were cut out and dissociated as previously described (Hatanaka and Murakami 2002). Cells were plated at 3.5 × 104 per dish (μ-Dish, ib80136; ibidi) coated with polyethyleneimine (Sigma-Aldrich). They were cultured for up to 66 h in Dulbecco’s modified Eagle’s medium and F12 medium containing 10% fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100 μg/mL), and images were captured at each indicated time point by an inverted microscope equipped with a CCD camera (DS-2M, Nikon).

Time Lapse Imaging

Thirty-two or 37–38 h after the in utero electroporation at E12.5, labeled brains were removed, embedded in 4% low-melting temperature agarose in PBS, and cut into 300-μm thick coronal slices with a vibrating blade microtome (VT1000S). Care was taken to preserve their intact meninges. They were placed onto a glass bottom dish (5% CO2, 95% O2 at 37 °C; 0.45). After addition of 0.3 mL of the culture medium described above, the dish was transferred to a temperature- and gas-controlled incubation chamber (5% CO2, 95% O2 at 37 °C; ONICS; Tokai Hit) fitted onto a confocal microscope (Radiance 2000, BioRad). The lid of the dish was replaced by FoilCover (Zeiss).

Labeled cells were viewed through a ×20 objective (N.A. = 0.45). Images were captured with 488-nm excitation and 500- to 560-nm emission filters at 30-min intervals. Since the shape of the slice gradually deformed, data were obtained within 40 h. At each time point, a stack of images was collected from a series of 8–10 consecutive images taken along the z-axis at 10-μm intervals or 10–12 images at 5-μm intervals. Images containing labeled cells were extracted at each time point and merged by ImageJ (1.40 g, Java 1.5.0_16, http://rsbweb.nih.gov/ij/). After brightness and contrast were adjusted, assembled images were created by Fiji (http://pacific.mpi-cbg.de/wiki/index.php/Fiji). We chose the slice in which radial migration had occurred, to avoid analysis of damaged preparations. Moreover, we excluded cells that apparently differed from mCherry-labeled cells in vivo in position and morphology, to minimize the likelihood of any artificial effect of labeling and culture. Kif5Cg60-EGFP-labeled slices were prepared in a similar manner, cultured for 2–3 h (5% CO2, 95% O2 at 37 °C), and fixed briefly (~20 min at room temperature) without time-lapse imaging. Images of cells extending a tangential process and expressing both mCherry and Kif5Cg60-EGFP were captured by a confocal microscope (FV1000, Olympus).

Immunohistochemistry

Labeled heads were fixed overnight (for Tbr1 and Tbr2) or for 1–2 h (for NeuroD). Brains were removed, immersed in 30% sucrose in PBS, embedded in OCT compound (Sakura Finetechical), and cut coronally at a thickness of 10 μm. The following primary and secondary antibodies were used: rabbi anti-Tbr1 (1:500, abcam); rabbit anti- Tbr2 (1:600, abcam); goat anti-NeuroD1 (1:400, N-19, Santa Cruz); Alexa 647 anti-rabbit IgG (1:500, Molecular Probes); and biotinylated anti-goat IgG (1:100, Chemicon). Cy5-conjugated streptavidin (1:1000, Jackson) was also used. Sections were examined under a confocal microscope (FV1000). Images were acquired in a vertical plane spanning the cerebral wall after adjusting the laser power in which the brightest cell (Tbr2 and NeuroD in SVZ/IZ and Tbr1 in CP) showed a maximal pixel value. More than one hundred labeled cells in at least 3 brains were examined for each antibody staining.

Results

VZ Cell-Derived Neuronal Progeny Initiate Long Process Extension Within ~40 h in Dissociated Culture

To determine the possible time window for axon formation of excitatory cortical neurons, we first examined the time course of process formation in dissociated VZ cell progeny, which give rise to excitatory cortical neurons. We labeled VZ cells at E12.5, when deep-layer neurons are produced, with pTol:1-EYFP to drive EYFP expression in neuronal precursors and young neurons (Hatanaka and Murakami 2002). Mixing of labeled cells with nonlabeled cells allowed us to observe the morphology of each labeled cell clearly in dissociated cultures of high plating density. By 30 h in culture, cells extended several immature processes (Fig. 1.A,B). They were less than 40 μm in length at this stage (Fig. 1F, 42 cells at 24 h and 41 cells at 30 h). However, by 40 h in culture, cells extending one outstandingly long process had appeared (Fig. 1C). The proportion of cells with a process extending >100 μm was 20% (9/45 cells) at 40 h. Then they showed a monotonic increase: 62% (39/62) by 48 h. These results indicate that most VZ cell progeny labeled at E12.5 developed an outstandingly long process within 3 days, with the first cells doing so between 30 and 40 h in culture.
**Excitatory Cortical Neurons in the IZ Initiate a Long Tangential Process Extension In Vivo**

We next examined process extension in vivo, with reference to the time course observed in dissociated culture. Lateral cortical VZ cells at E12.5 were labeled with mCherry by in utero electroporation, and the position and morphology of their progeny were analyzed in brain preparations fixed 40 h later. Since the boundary between the SVZ and IZ was difficult to identify in the thick slice, we collectively treated them as the IZ hereafter.

At this stage, the majority of the labeled cells had left the VZ, and some had reached the cortical plate (CP). We scored 16 and 85 well isolated and randomly selected CP and IZ cells, respectively, in coronal slices (5 brains). All the CP cells extended a long (>100 μm) tangential process that ran through the IZ (Fig. 2A,B, and B'). The IZ cells were morphologically diverse but frequently displayed several thin processes and sometimes a thick process. The length of these processes was mostly less than 50 μm (Fig. 2C,F, type 1 cells, see below), but sometimes one of the thin processes was outstandingly long.

**Figure 1.** Time course of appearance of excitatory cortical neurons with a long process in dissociated culture. Cortical VZ cells at E12.5 were labeled with Tau1.EYFP, dissociated, and cultured in vitro. (A–E) Images of cells at 24, 30, 40, 48, and 66 h in culture. Pair-like closely associated cells were often observed, suggesting the occurrence of a final mitosis shortly after plating. A long process extension was observed from 40 h in culture (arrowheads). (F) Box-and-whisker plot showing the length distribution of the longest process in cells at 24 and 30 h in culture (n = 83). The line within the box marks the median, and the extended whiskers mark 1.5 times the interquartile range. The asterisk and circles indicate the average and outliers, respectively. (G) Percentage of cells extending a long (>100 μm) process. Scale bars: 20 μm (A–C); 50 μm (D and E).

**Figure 2.** All CP and some IZ cells show a long process oriented tangentially in vivo. VZ cells were sparsely labeled at E12.5 with mCherry and examined 40 h later. Serial z-stacked images were captured by confocal microscopy and assembled as a single image (A–E, except for B'). The pial surface is above. (A, B) CP cells extending a long tangential process in the IZ. Arrowheads indicate a long tangential process. (B') Three-dimensional representation of the image shown in B, confirming that a single cell’s processes were within a coronal slice. (C) Type 1: IZ cells extending only several short processes. (D) Type 2: IZ cell extending a long tangential process and several short processes. (E) Type 3: IZ cell extending a pia-directed thick process and a long tangential process. The presence of pair-like closely associated cells (asterisks in C) suggests that labeled IZ cells had differentiated from intermediate neuronal progenitors. (F) Box-and-whisker plot (elements as in Fig. 1F) showing the length distribution of the longest process in cells of type 1 (n = 59). (G) Labeled CP and IZ cells at 24, 30, and 40 h after electroporation were categorized into 4 types according to their position and morphology, as described in A–E. Earliest long process extension occurred between 24 and 30 h in vivo. CP, cortical plate; IZ, intermediate zone. Scale bar: 20 μm (A–E).
(>100 μm in type 2 and 3 cells; the length of the second-longest process of these cells was <50 μm, data not shown). We defined >100-μm processes as long, and the rest as short. For descriptive purposes, IZ cells were categorized into 3 types: 1) cells extending only short processes (59 cells, 69%, Fig. 2C, corresponding to multipolar (Tabata and Nakajima 2003) or stellate cells (Shoukimas and Hinds 1978; Hatanaka et al. 2004); 2) cells extending one long tangential and several short thin processes (18 cells, 21%, Fig. 2D); and 3) cells extending one long tangential process and a pia-directed thick process (8 cells, 9%, Fig. 2E). Thus, 40 h after electroporation, long process extension was observed in all CP cells and in 30% of IZ cells (types 2 and 3).

Since a considerable percentage of the cells at 40 h have already extended the long process, we examined CP and IZ cells at 24 and 30 h after electroporation, using the above categorization, to investigate the process extension in earlier stages (Fig. 2G). At 24 h, there were no labeled cells in the CP, and all IZ cells were type 1 (n = 110 cells, 3 brains; 5 cells extended a >50-μm but <100-μm process). At 30 h, a few labeled cells appeared in the CP (1%, 2 cells among 184 cells, 3 brains). The majority of IZ cells were type 1 (91%, 166 cells), and the rest were types 2 (7%, 13 cells) and 3 (2%, 3 cells). Together, these results suggest that VZ cell-derived excitatory cortical neurons initiate a long process extension in the IZ, with the first cells doing so between 24 and 30 h after electroporation in vivo.

Excitatory Cortical Neurons with Multipolar Shape Initiate Tangential Axon Formation

From the varied morphologies of VZ cell progeny in vivo, together with the morphological changes of these cells in dissociated culture, we hypothesized a developmental sequence in which they 1) transform into multipolar cells upon arrival at the SVZ/IHZ; 2) initiate axon outgrowth oriented tangentially; 3) extend a pia-directed leading process; and finally 4) migrate radially, leaving a trailing process which becomes the radial part of the axonal projection.

To directly examine the dynamic process of axon formation, we carried out in situ time-lapse imaging using slice culture. We used memEGFP (Moriyoshi et al. 1996) instead of mCherry to label E12.5 VZ cells because its uniform membrane distribution allowed us to examine even a thin process clearly. Thirty-two hours after electroporation, most labeled cells had already left the VZ, and IZ cells extended only short processes (59 cells, 69%, Fig. 2C). We further examined the nature of the tangential process using one of the earliest markers of axon specification, Ki67EGFP, whose stable accumulation occurs in the tip of an axonally committed process in dissociated hippocampal neurons (Jacobson et al. 2006; Toriyama et al. 2010). We labeled E12.5 VZ cells with both Ki67EGFP and mCherry by electroporation. After 36–38 h, cortical slices were prepared and cultured for 2–3 h. We examined IZ cells that had extended a tangential process longer than >50 μm and were expressing a detectable level of EGFP in any region of the cell. Among 8 cells examined (their process lengths were 60, 80, 80, 90, 95, 95, 115, and 160 μm), all showed a prominent accumulation of EGFP at the tip of the process (Fig. 4D), indicating that the tangential process already displays axon-specific properties before its subsequent long extension.

These time-lapse imaging analyses, together with our finding that Ki67EGFP accumulates at the tip of the tangential process, thus indicate that excitatory cortical neurons with multipolar shape initiate axon outgrowth oriented tangentially. Since these cells showed no apparent polarity, axon formation in excitatory cortical neurons appears to occur directly from a nonpolarized stage in situ.

Nature of the Labeled IZ Cell

Because excitatory cortical neurons originate either directly from the VZ cells or through the intermediate neuronal progenitors (IPs; Miyata et al. 2004; Noctor et al. 2004, 2008), it is possible that the IZ cells observed here include IPs. To examine the origin of the labeled IZ cells, we characterized these cell at 24 h after electroporation with the following transcription factors: Tbr2, which is a marker of IPs (Englund et al. 2005; Arnold et al. 2008; Sessa et al. 2008), NeuroD, expressed in both IPs and immature neurons, and Tbr1, expressed in neurons in the upper IZ and the CP (Hevner et al. 2006). We found that most IZ cells were weakly positive for Tbr2 (88/110 cells = 80%, Fig. 5A; the remaining negative cells tended to distribute in the upper part of the IZ), strongly positive for NeuroD (92/102 cells = 90%, Fig. 5B) and weakly positive for Tbr1 (99/101 cells = 98%, Fig. 5C). Thus, it is likely that the most, if not all, of the IZ cells are IPs or immature neurons that have differentiated from IPs.
Discussion

In this study, we analyzed the formation of axons and their subsequent extension in excitatory cortical neurons in situ. Axonal development in these neurons can be divided into 4 stages, as shown schematically in Fig. 6. In stage I, an excitatory cortical neuron with multipolar shape repeatedly extends and retracts its short processes in the SVZ/IZ for several hours. In stage II, a new process suddenly begins to elongate tangentially. In stage III, a pia-directed leading process is formed. Finally, in stage IV, the cell becomes bipolar and migrates radially to form an L-shaped axon. Our results indicate that polarity in excitatory cortical neurons is established in the IZ prior to radial migration into the CP. Since apicobasal polarity appeared to be lost in the multipolar stage, neuronal polarity seems to be established de novo from a nonpolarized stage.

Axon Formation from a Nonpolarized Stage In Vivo

Excitatory cortical neurons with multipolar shape initiated axon formation in the IZ. Although these cells might inherit the apicobasal polarity of their radial glial progenitors (Barnes and Polleux 2009), the observation that these progeny detach their endfeet on both the ventricular and pial surface and transform into multipolar cells in the SVZ/IZ (present study; Tabata and Nakajima 2003; Noctor et al. 2004) suggests that they depolarize when they leave the VZ. Moreover, the finding that most, if not all, intermediate neuronal progenitor cell division in the SVZ/IZ is accompanied by retraction of all visible processes (Miyata et al. 2004; Noctor et al. 2004, 2008) also supports the view that they have lost apicobasal polarity.

Other aspects of the behavior of multipolar cells also suggest a prolonged nonpolarized state, in which the cells do not...
exhibit fixed polarity but may display occasional fluctuating polarity, before axon formation. The cells alternately extended and retracted their thin short processes for several hours; and some of them retracted a thin process that had grown >40 μm and then extended a new one (see cell 1 in Fig. 3A). Thus, our present observations suggest that axon formation occurs directly from a nonpolarized stage.

Recently, cortical GABAergic interneurons in explant culture have been shown to develop their axon from a nonpolarized stage (Yamasaki et al. 2010). This and the present studies, together, indicate that the process of polarization is common to cortical neurons regardless of their migration mode (radial or tangential) or cell type (excitatory or inhibitory). Moreover, morphological examinations have found multipolar-like cells in the developing mouse and monkey hippocampus (Nowakowski and Rakic 1979; Nakahira and Yuasa 2005). The establishment of neuronal polarity from a nonpolarized stage may therefore not be atypical; indeed, it may be common in certain neuronal populations.

Axogenesis in cortical neurons, observed here, appears to be very similar to that in dissociated hippocampal neurons, which first emit several short processes that undergo repeated, random growth and retraction, followed by rapid extension of one of them to form an axon (Dotti et al. 1988; Goslin et al. 2000).
future studies will thus be to define, for example, where the centrosome localizes, and how microtubules are oriented, throughout the process of polarity formation.

The Nature of Multipolar IZ Cells

Twenty-four hours after electroporation in vivo, when all labeled IZ cells extended only short processes, the cells were expressing a low level of Tbr2. Since Tbr2 is a marker for IPs (Englund et al. 2005; Arnold et al. 2008; Sessa et al. 2008), its expression suggests that most, if not all, of these IZ cells are IPs and/or young neurons that have differentiated from IPs. Consistently, the IZ cells expressed a high level of NeuroD, as has been reported to occur in IPs and immature neurons (Hevner et al. 2006). Very weak expression of Tbr1 in these cells is also consistent with the view that they are committed to neuronal lineage or immature neurons. Furthermore, pairwise appearance of labeled cells in dissociated culture and in brain sections at early stages of observation can be explained if we assume that they have just divided from IPs. Thus, it is likely that IZ multipolar cells from which axon outgrowth is initiated may be neurons derived from IPs.

Tangentially Oriented Axon Outgrowth and Extension

Multipolar cells initiated axon outgrowth that was oriented tangentially. Although the physical manipulations may have caused a delay of process extension in dissociated cells, the finding that axon outgrowth in vivo seemed to occur at a comparable time to that in dissociated culture suggests that an intrinsic mechanism may regulate the timing of axon development. On the other hand, external cues may play an instructive role in defining the site of axon outgrowth (Esch et al. 1999; Randlett et al. 2011). It has been proposed that the axon is specified using a positive feedback signal that stabilizes process extension (Arimura and Kaibuchi 2007; Inagaki et al. 2011). We therefore speculate that, since pre-existing axons in the IZ are also oriented tangentially (along the dorsoventral axis), they may be the source of such stabilizing cues: they could help not only to define the site of axon outgrowth but also to guide its tangential elongation in the IZ. However, there may be other possible mechanisms that would account for the formation of tangentially oriented axons, especially pioneer axons.

Our current analyses were limited to neurons originating from the lateral region of the E12.5 mouse cerebral cortex. We therefore do not yet rule out the possibility that other IZ cells,
derived from different regions and stages, initiate their axon outgrowth in a different manner (e.g., de Anda et al. 2008, 2010). On the other hand, the fact that IZ cells in the lateral region, labeled at E13.5, E14.5, or E15.5, show a sequential population change from type 1 to type 3 in fixed preparations (Y Hatanaka, T Namikawa, K Yamauchi, unpublished observations) supports the view that the broader population of IZ cells, which become not only deep- but also upper-layer neurons, initiate axon outgrowth in a similar manner. Further investigation will thus be necessary to determine whether the observed tangentially oriented axon outgrowth from a non-polarized stage is common to all excitatory cortical neurons.

Axon Formation Before Radial Migration
In an electron microscopic study of the mouse cingulate cortex, Shoukimas and Hinds (1978) found that some IZ cells extend an axon-like tangential process. Golgi studies have also revealed IZ cells having a tangential process (see, e.g., Bayer and Altman 1991). However, because their images were static and lacked precise information about cellular identity, it has not been possible to give a clear description of how these IZ cells develop. The present study, by examining the dynamic behavior of VZ cell progeny using time-lapse imaging, shows clearly that excitatory cortical neurons in the IZ initiate axonal extension before radial migration into the CP.

The timing of axogenesis in retinal bipolar cells and ganglion cells differs from that in excitatory cortical neurons; the former two seem to initiate axogenesis while they are still undergoing interkinetic movement in the neuroepithelium or immediately thereafter (Morgan et al. 2006; Zelessi et al. 2006; Randlett et al. 2011). This difference in time, but not in mode, of migration may account for the distinct processes of axon formation between these neurons.

Implication for Appearance of Nonpolarized Cells before Axon Formation In Vivo and Its Regulation Mechanism
Why do excitatory cortical neurons assume a nonpolarized shape before axon formation in vivo? Axon formation is thought to require a coordinated rearrangement of the actin cytoskeleton and microtubules (Tahirovic and Bradke 2009), and critical role of local microtubule stabilization is directly demonstrated (Witte et al. 2008). Since the axonal extension of excitatory cortical neurons occurs in a direction perpendicular to the orientation of the radial glial progenitors, in which microtubules are critical for their highly polarized morphology (Li et al. 2003), rearrangement of their cytoskeleton may be necessary before axon formation. The fact that many excitatory cortical neurons are differentiated from IPs through 1--2 rounds of symmetric cell division (Miyata et al. 2004; Noctor et al. 2004, 2008) also suggests occurrence of cytoskeletal rearrangement (e.g., a bundle of microtubules that form spindle fibers in mitosis). We speculate that this rearrangement is achieved through decreasing stability of cytoskeleton, especially of microtubules, which leads to nonpolarized shape during this period.

The period of nonpolarized shape continues for several hours (present study; Tabata and Nakajima 2003). Then what mechanisms control the period of nonpolarized stage? First of all, it may take some time for newly differentiated neurons to produce sufficient number of molecules that are necessary for axon formation. Then, activation of signaling cascades such as the energy-sensing adenosine 5'-monophosphate-activated protein kinase pathway, whose activity is shown to suppress axon formation in dissociated hippocampal neuron (Amato et al. 2011), may contribute to maintaining the period.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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References


