Original Article

Cortical Divergent Projections in Mice Originate from Two Sequentially Generated, Distinct Populations of Excitatory Cortical Neurons with Different Initial Axonal Outgrowth Characteristics

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Abstract

Excitatory cortical neurons project to various subcortical and intracortical regions, and exhibit diversity in their axonal connections. Although this diversity may develop from primary axons, how many types of axons initially occur remains unknown. Using a sparse-labeling in utero electroporation method, we investigated the axonal outgrowth of these neurons in mice and correlated the data with axonal projections in adults. Examination of lateral cortex neurons labeled during the main period of cortical neurogenesis (E11.5–E15.5) indicated that axonal outgrowth commonly occurs in the intermediate zone. Conversely, the axonal direction varied; neurons labeled before E12.5 and the earliest cortical plate neurons labeled at E12.5 projected laterally, whereas neurons labeled thereafter projected medially. The expression of Ctip2 and Satb2 and the layer destinations of these neurons support the view that lateral and medial projection neurons are groups of prospective subcortical and callosal projection neurons, respectively. Consistently, birthdating experiments demonstrated that presumptive lateral projection neurons were generated earlier than medial projection neurons, even within the same layer. These results suggest that the divergent axonal connections of excitatory cortical neurons begin from two types of primary axons, which originate from two sequentially generated distinct subpopulations: early-born lateral (subcortical) and later-born medial (callosal) projection neuron groups.

Key words: birthdate, callosal projection neuron, electroporation, laminar fate, subcortical projection neuron

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Introduction

The proper development of cortical projections is essential for the assembly and coordination of information processing within and across different cortical areas and other regions of the brain. This output system is primarily formed by excitatory cortical neurons, the major neuronal type in the cerebral cortex. These neurons include the pyramidal neurons of layers II/III, V, and VI and the spiny stellate neurons of layer IV (Feldman 1984; Jones 1984; DeFelipe and Farinas 1992; Lübke and Feldmeyer 2010). They are classified into subtypes on the basis of their highly diverse axonal projections; however, most can be broadly divided into two principal populations (Molyneaux et al. 2007): callosal projection neurons, which extend axons across the corpus callosum and reside in the supragranular (II/III) and infragranular layers (V and VI), and subcortical projection neurons, which extend axons away from the cortex and exclusively reside in the infragranular layers.

The diverse projections of these neurons develop in 3 phases: primary axon extension, delayed collateral branching formation, and selective axon elimination (O’Leary and Koester 1993). For example, in rats, layer V subcerebral projection neurons establish area-specific projections by initially forming a primary descending axon toward the spinal cord, developing a common set of collateral branches along the primary axon, and eliminating specific branches in an area-dependent manner (O’Leary and Terashima 1988). Similarly, a population of neurons in layers II/III and V of the rat somatosensory cortex establishes association projections by first extending a primary axon to the contralateral cortex, forming projections to the ipsilateral cortical area, and finally losing some of the initial callosal projections (Mitchell and Macklis 2005). Notably, elimination of supernumerary axons from a subset of initial callosal projections has also been observed in the developing rhesus monkey (Lamantia and Rakic 1990). Therefore, it is likely that these divergent projections originate from a restricted number of primary axons with identical structural properties. However, it is unknown how many types of axonal projections initially develop from excitatory neurons because the retrograde-labeling method used in previous studies did not allow examinations of initial axonal outgrowth from these neurons.

Recently, we successfully sparsely labeled mouse excitatory cortical neurons by in utero electroporation (Hatanaka and Yamauchi 2013). This labeling method permits the examination of morphological changes in developing excitatory cortical neurons at the single-cell level. Using this method, we showed that neurons labeled at embryonic day (E)12.5 initiated directed axonal outgrowth in the intermediate zone (IZ) before migrating into the cortical plate (CP; Hatanaka and Yamauchi 2013). Application of this method to different embryonic stages can visualize neurons in most layers, and allows us to examine initiation of primary axons that differentiate into divergent projections.

Here, we analyzed the axonal outgrowth of lateral cortex neurons labeled at different times during the main period of cortical neurogenesis. We demonstrated that these neurons commonly initiated axonal outgrowth when located in the IZ but that the outgrowth direction was lateral for early-born neurons and medial for later-born neurons. Therefore, cortical projection divergence appears to originate from two populations. We also investigated the molecular identity, laminar fate, and birthdates of these neurons.

Materials and Methods

Experimental Animals

Timed-pregnant Institute of Cancer Research mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Embryonic day (E)0.5 was defined as noon on the day of vaginal plug observation. The day of birth was designated as postnatal day (P)0. All the experiments adhered to the guidelines for the use of laboratory animals of the Nara Institute of Science and Technology and the National Institute for Physiological Sciences.

In Utero Electroporation

For dense labeling with green fluorescent protein (GFP; flat-mount preparations, population studies of axonal outgrowth and immunohistochemistry for postnatal brains), pCAGGS:EGFP (final concentration: 1 μg/μL) was used. For sparse neuronal labeling, the following mixtures of plasmids were used (Hatanaka and Yamauchi 2013): pCALNL5:mCherry (1 μg/μL), pCAGGS:Cre (0.05–0.1 ng/μL) and pCAGGS:EGFP (1 μg/μL; reference for injection site), or pCALNL5:memEGFP (1 μg/μL), pCAGGS:Cre (0.05–0.1 ng/μL), and pCAGGS:mCherry (1 μg/μL; reference for injection site). For direct examination of projection patterns and molecular identities, plasmids for the Supernova system (pCAGGS-loxP-STOP-loxP-EGFP-ires-TTA-WPRE, 1 μg/μL), pTRE-cre, 4–5 ng/μL; Mizuno et al. 2004), which label cells sparsely but more strongly, were used. This system enabled us to identify long axons easily and identify the site of electroporation without the reference, thereby allowing us to analyze samples immunostained with 2 markers. Plasmids were purified using the Endofree Plasmid Maxi Kit (Qiagen N.V., Venlo, Limburg, the Netherlands) and dissolved in phosphate-buffered saline (PBS; pH 7.4). In utero electroporation was performed as described previously (Hatanaka et al. 2004; Hatanaka and Yamauchi 2013) except that the number of electric pulses was reduced from 5 to 4. For young embryos (E11.5, E12.0, and E12.5), four 30-V electric pulses were applied with CUY650P3 forceps-shaped electrodes (Unique Medical Imada K.K., Natori, Japan). Labeling was performed approximately at noon (E11.5, 12.5, or 13.5), at midnight (E12.0), or in the daytime (E14.5 or 15.5).

Proliferation Activity of Sparsely Labeled Cells

Pregnant mice were injected intraperitoneally with 5-ethynyl-2'-deoxyuridine (EdU, 25 mg/kg; Invitrogen, Carlsbad, CA, USA; Salic and Mitchison 2008) 3 times (4-h intervals) from the time 0, 24, or 48 h after electroporation at E12.5.

Preparation of Slices and Sections After Electroporation

Heads of E13.5–15.5 or brains of E16.5–18.5 mice were immersion-fixed in 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.4) or PBS. Postnatal brains (3 weeks old) were fixed by intracardial perfusion with the fixative under deep sodium pentobarbital anesthesia (>100 mg/kg). For prepro-cholecystokinin (ppCCK) and Purkinje cell protein 4 (PCP4) immunohistochemistry, intracardial perfusion was performed with a prefixative (8.5% w/v sucrose/5 mM MgCl2/0.02 M phosphate buffer, pH 7.4), followed by the fixative. After storage overnight at 4°C in the fixative (except for Ctip2 staining of postnatal brains), the heads or brains were washed with PBS. Brains of E13.5–15.5 mice were removed from the heads. To examine axonal outgrowth, brains were embedded in 4% agarose/PBS, cut into 150-μm-thick coronal slices using
a vibratome (VT1000S; Leica Microsystems GmbH, Wetzlar, Germany), directly collected onto Matsunami adhesive silane (MAS)-coated glass slides (Matsunami Glass Ind., Ltd, Osaka, Japan), and coverslipped in Mowiol mounting medium (0.1 M Tris–HCl, pH 8.5, 25% glycerol, 10% Mowiol 4-88 Reagent from Calbiochem and 2.5% 1,4-diazabicyclo[2.2.2]-octan from Sigma-Aldrich, St. Louis, MO, USA). To directly examine projection patterns and molecular identities, 100-μm-thick coronal slices were prepared and processed for immunohistochemistry. To prepare frozen coronal sections for immunohistochemistry (Ctip2, Satb2, or Tbr1 for embryonic, and ppCCK or PCP4 for postnatal staining), brains were immersed in 30% sucrose/PBS overnight at 4°C, embedded in optimal cutting temperature compound (Sakura Finetecnical Co., Ltd, Tokyo, Japan), cut to a 30-μm thickness with a cryostat (CM3050, Leica Microsystems), and collected in PBS. For Ctip2 immunohistochemistry for postnatal days, brains were cut to a 50-μm thickness with the vibratome and collected in PBS. To prepare flat-mount sections, lightly fixed (1–2 h) cortical hemispheres were placed between glass slides with spacers and fixed overnight. The hemispheres were processed as described above and frozen sections (30 μm thickness) were directly collected onto glass slides.

Immunohistochemistry and Counterstaining
Sections or slices were treated at least overnight (or >48 h for slices) with the appropriate primary antibodies diluted in 5% normal goat serum/0.3% Triton X-100/PBS and with the corresponding secondary antibodies overnight at 4°C. The following primary antibodies were used: mouse anti-5-bromo-2-deoxyuridine (BrdU; BU33, 1:1000; Sigma-Aldrich Corporation; non-cross-reacting secondary antibodies were used: mouse anti-5-bromo-2-deoxyuridine antibody [anti-guinea pig and anti-rabbit IgG (1:1000; Vector Labs, Burlingame, CA, USA), respectively], and subjected to tyramide-based signal amplification (Ueta, Hirai, et al. 2013) with Alexa 488 anti-mouse IgG (1:400); and Cy3 anti-mouse IgM (1:300). For ppCCK and PCP4 signal enhancement, sections were treated with biotinylated secondary antibodies (anti-guinea pig and anti-rabbit IgG (1:1000; Vector Labs, Burlingame, CA, USA), respectively), and subjected to tyramide-based signal amplification reaction (Ueta, Hirai, et al. 2013) with Alexa 594-streptavidin (1:1000). To analyze layer positions, coronal sections of postnatal brains were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (1 μg/mL) for 10 min. Floating sections and slices were subsequently mounted onto MAS-coated glass slides in Mowiol mounting medium.

Examination of Axonal Initiation
Labeled neurons in the lateral portion of the brain corresponding to the presumptive primary somatosensory cortex (SI) and parts of the secondary somatosensory cortex (SII) were examined. Well-isolated labeled neurons in CP and IZ were arbitrarily selected and a z-series of images were taken at different magnifications by confocal laser scanning microscopy (TCS SP5; Leica Microsystems/LSM 510 META; Carl Zeiss AG, Oberkochen, Germany/V1000; Olympus Corporation, Tokyo, Japan). These stacks confirmed that all processes of the neuron selected for further analysis were found within the slice. We excluded neurons with axons that had been cut during slice preparation. All CF neurons, and IZ neurons with a process >100 μm in length were subjected to subsequent quantitative analysis. The axonal direction was determined by examining the direction (lateral, medial, or other, including toward the pial and ventricular surfaces) in which the distal portion of the axon was positioned. To directly examine the axonal direction and molecular identity, the axonal directions of CF neurons were first documented with a ×20 objective lens, and an image of the immunoreactive profile of the labeled neurons was then constructed from a z-series (0.99-μm intervals) captured with a ×63 objective lens on an LSM 510 META confocal laser scanning microscope.

1,1′-Dioctadecyl-3,3′,3′-tetramethylindocarbocyanine Perchlorate Labeling
A small crystal of 1,1′-dioctadecyl-3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR, USA) was placed in the cerebral wall of each fixed brain from E15.5 or E17.5 mice. The brains were stored in the dark for 3–4 weeks in 4% PFA/PBS at room temperature. These brains were then cut parasagitally (400 μm) or horizontally (300 μm) and examined under a fluorescence dissection microscope (SZX16; Olympus Corporation) equipped with a CCD camera (DP70; Olympus Corporation).

Birthdating Analysis of Excitatory Cortical Neurons in Layers V and VI
EdU/BrdU injection and detection
Intrapерitoneal injection of EdU into pregnant mice (50 mg/kg) was performed once at noon (E11.5, E12.5, E13.5, and E14.5) or midnight (E13.0 and E14.0). Mice with E13.0 embryos that received one injection of EdU at E13.0 received an intraperitoneal injection of BrdU (Sigma-Aldrich Corporation; 50 mg/kg) after 12 h. Brains of newborn pups (P0) were harvested and immersion-fixed in fresh fixative (0.2% picric acid/2% PFA/PBS) for 1 h on ice with gentle agitation. These brains were then washed with PBS, immersed in 20% sucrose/PBS, cryosectioned (coronal; 8 μm), and directly collected onto MAS-coated glass slides. Two (from E11.5, E12.5, E13.5, E14.0, and E14.5 mice) or 3 (from E13.0 mice: EdU and BrdU injected) 1-in-5 series of sections were used. The first series of sections was incubated sequentially as follows: a mixture of anti-Cux1, -Satb2, and -Ctip2 antibodies, followed by a mixture of Alexa 405 anti-rabbit, Alexa 488 anti-mouse, and Cy3 anti-rat IgG. EdU was detected via Alexa 647 azide labeling using Click-IT EdU HCS Assays with rinse buffer (Invitrogen). The second series of sections was treated as follows: a mixture of anti-Cux1, -Satb2, and -Ctip2 antibodies, followed by a mixture of Alexa 405 anti-rabbit, Alexa 488 anti-mouse, and Cy3 anti-rat IgG. EdU was detected via Alexa 647 azide labeling using Click-IT EdU HCS Assays with rinse buffer (Invitrogen). The second series of sections was treated as follows: a mixture of anti-Satb2 and -Nurr1 antibodies, followed by a mixture of Alexa 488 anti-mouse and Alexa 594 anti-rabbit IgG. The third series of sections (E13.0 mice only) was incubated sequentially as follows: first, a mixture of anti-Satb2 and -Nurr1 antibodies, followed by a mixture of Alexa 488 anti-mouse and Alexa 594 anti-rabbit IgG. The third series of sections (E13.0 mice only) was incubated sequentially as follows: first, a mixture of anti-Satb2 and -Nurr1 antibodies, followed by a mixture of Alexa 488 anti-mouse and Alexa 594 anti-rabbit IgG.
the rostrocaudal axis (40 μm apart, totaling 9 sections from 3 mice, 12 sections from 4 mice or 21 sections from 7 mice) at the most anterior part of the hippocampus using a ×4 objective lens under a fluorescence microscope (BX60; Olympus Corpor-

Data analysis

Cell counts were performed in 400-μm-wide strips throughout the thickness of layer V (and layer VI for Satb2+ cells) of the SI. Layer V was defined as the region lacking Cux1+ neurons and containing large-diameter Ctip2+ cells. For BrdU sections, the patterns and distributions of Ctip2+ and Satb2+ cells as well as Cux1+ cells in neighboring sections were used to define layer V. We used intensity and size thresholds for EdU+ (and BrdU+), Ctip2+, and Satb2+ cells. First-generated neurons could be identified as heavily labeled EdU+ (or BrdU+) cells (>50% of maximum intensity; Poileux et al. 1997). These included cells with homogeneous nuclear EdU (or BrdU) staining or those in which the staining was intense but punctate, with multiple spots that covered most nuclei. Cells with only a few spots were excluded from further analysis. Ctip2+ cells can be subdivided into 2 subpopulations: one with large and heavily immunoreactive nuclei, which apparently correspond to prospective subcerebral projection neurons, and the other with small and weakly immunoreactive nuclei, which likely correspond to GABAergic neurons (Arliotta et al. 2005; Ueta, Otsuka, et al. 2013). Furthermore, we noticed that Satb2 was expressed in nearly all excitatory neurons at P0, although it is known as a determinant of callosal neurons (Alcamo et al. 2008; Britanova et al. 2008). However, Satb2 expression was usually very faint in large-diameter and Ctip2+ cells. Satb2 has been suggested to repress the expression of Ctip2 in callosal neu-

Results

Most Cortical Axons Run Along the Mediolateral Axis During the Mid and Late Embryonic Stages

In adults, cortical efferents are highly diverse. For example, intracortical projections comprise callosal projections that connect the same or different areas on opposite hemispheres, as well as associational projections between areas on ipsilateral hemisphere. These long-range connections are believed to be established by the passage of the main axon through the white matter. Before investigating axonal orientation, we examined the axonal orientations of these neurons in the IZ (future white matter) at embryonic stages. Cortical ventricular zone (VZ) cells were labeled with pCAGGS:EGFP by in utero electroporation at either E12.5 or E14.5, when they primarily produce infragranular and supragranular neurons, respectively (Hatanaka et al. 2004). The labeled hemispheres were collected after 3 days, flattened, and cut parallel to the pial surface, and the orientations of axonal processes at the IZ level were examined (Fig. 1; E15.5, n = 5; E17.5, n = 5). In both cases, most labeled processes were oriented along the mediolateral axis (Fig. 1A,D). This orientation was supported by immunostaining for TAG1 (Fig. 1B,E), which is expressed in cortical efferents (Wolfer et al. 1994). In addition, neurofilament immunostaining, which is observed in a broader population of axons that includes cortical afferents and efferents, further confirmed this uniform orientation along the mediolat-

Excitatory Cortical Neurons Initiate Directed Axonal Outgrowth Before Entering CP

In a previous study, we demonstrated that VZ-derived cortical neurons, labeled at E12.5 by in utero electroporation, initiate directed axonal outgrowth in the IZ (Hatanaka and Yamauchi 2013). This was also indicated by recent studies of neurons labeled at E12.5–13.5 (Namba et al. 2014; Sakakibara et al. 2014). However, it remains uncertain whether excitatory cortical neurons across all layers (II–VI) exhibit directed axonal outgrowth in the IZ before entering the CP. To investigate axonal initiation, we labeled VZ cells sparsely in the lateral cortex using a mixture of Cre and floxed plasmids (Morin et al. 2007; Hatanaka and Yamauchi 2013) at days E11.5, E12.0, E12.5, E13.5, E14.5, or E15.5, thus spanning the main period of cortical neurogenesis (Smart and Smart 1982). Because most labeled cells did not incorporate a thymidine analog, EdU, after 2 days (Fig. 2A,B), it is likely that “labeled neurons” under the present condition were the progeny of VZ cells that underwent only a few cell divisions after electroporation. Therefore, we could expect to label excitatory cortical neurons in a stage-specific manner. Neurons were examined 40, 48, 72, or 96 h after labeling, when axonal outgrowth became evident (except for 96 h). A gradual increase in the time required for the appearance of axonal outgrowth was likely due to the lengthening of the cell cycles (Takahashi et al. 1995). Because most axons were oriented along the mediolateral axis at these
stages (Fig. 1), we prepared coronal slices to maximize the possibility that the entire cellular profile and axonal projections were contained in a single slice. Axonal processes of CP neurons (nearly all already extending >100 μm; Hatanaka and Yamauchi 2013) and those of IZ neurons extending 100 μm or more were documented and further analyzed.

First, we examined the axonal outgrowth from neurons labeled at E11.5 after 40 h (Fig. 2C–E). Although only the preplate (PP)—rather than the CP—is formed and the IZ is found only in the most lateral cortical regions at this stage (ca. E13.5; Del Rio et al. 2000), virtually all labeled PP (98%, 83/85 cells; see Fig. 2L for details) and some IZ neurons had extended long tangential processes, suggesting that these cells initiate axonal outgrowth before entering and thus form the PP. Consistent with our previous study (Hatanaka and Yamauchi 2013), all CP and some IZ neurons had extended long tangential processes in the IZ (Fig. 2D–F, Dl–F). Similarly, the vast majority of CP and a substantial number of IZ neurons labeled at E13.5, E14.5 (Fig. 2G–I, Gj–Ji), or E15.5 (data not shown) extended a long tangential process, consistent with the view that directed axon formation occurs when cells still reside in the IZ. Continuous elongation of these processes toward the midline and formation of an “L-shaped” pathway, a projection pattern typical of excitatory cortical neurons, confirmed their axonal properties (Fig. 2K, Ki). Taken together, these findings suggest that directed axonal outgrowth in the IZ is a general feature of the majority of, if not all, excitatory cortical neurons.

The Axonal Outgrowth Direction Depends on the Labeling Stage

Interestingly, the axonal direction appeared to be related to the time of labeling. Neurons labeled earlier often displayed a more lateral axonal outgrowth (Fig. 2C–E), whereas neurons labeled later tended to grow in a medially oriented fashion (Fig. 2F–K). To quantify the relationships between the axonal direction and the labeling stage, we divided axonal processes from both CP and IZ neurons into 3 groups on the basis of their orientation:
lateral, medial, or otherwise oriented (e.g., toward the pial or ventral surfaces). We selected >100-μm processes because axonal formation is a highly dynamic process during which shorter processes (<50 μm) are alternately extended and retracted; however, once the process abruptly exceeds 100 μm, it usually continues to elongate and becomes an axon (for the criteria for assessing process length, refer to Hatanaka and Yamauchi 2013).

Almost all neurons labeled at E11.5 or E12.0 directed their processes laterally 40 h after electroporation (Fig. 2L; E11.5, 4 brains, 101 cells, lateral 98%, medial 0%, other 2%; E12.0, 5 brains, 108 cells, lateral 97%, medial 3%, other 0%). In contrast, neurons labeled at E12.5 directed their processes either laterally or medially (Fig. 2E, F). The earliest (first-generated) neurons labeled at E12.5, which were located in the CP, projected almost all laterally (dark gray bar, 94%, 17/18 cells), whereas those in the IZ (probably including later-generated neurons) projected either laterally (light gray bar, 65%, 20/31 cells) or medially (35%, 11/31 cells). Therefore, a total of 74% of neurons labeled at E12.5 extended their
processes laterally at this stage (8 brains, 50 cells, medial 24%, other 2%). Conversely, most neurons labeled at E13.5 extended their processes medially at 48 h after electroporation (9 brains, 52 cells, lateral 0%, medial 98%, other 2%).

This switch in direction was further confirmed by dense labeling with the pCAGGS:EGFP plasmid (Supplementary Fig. 1); the earliest neurons labeled at E12.5 predominantly extended their long axons laterally after 40 h. A day later, medially extending axons appeared, likely because the subsequent generation of labeled neurons extended axons to a visible length by this time (data not shown). In contrast, all neurons labeled at E13.5 extended their axons medially at 48 h.

The preference for medially directed axonal outgrowth was also evident among neurons sparsely labeled at E14.5 and E15.5. Most axons projected medially at 48 h after electroporation, the stage at which labeled neurons were only found in the IZ but had not yet migrated into the CP (Fig. 2L; E14.5, 7 brains, 62 cells, lateral 12%, medial 83%, other 5%; E15.5, 3 brains, 32 cells, lateral 18%, medial 72%, other 10%). This preference for the medial direction was maintained at 72 h after electroporation, when some labeled neurons had arrived in the CP (E14.5, 3 brains, 60 cells, lateral 2%, medial 98%, other 0%; E15.5, 6 brains, 64 cells, lateral 10%, medial 76%, other 14%). The increase in medial projection from 48 to 72 h was probably due to a reduction in the number of IZ neurons with transient long processes that would be later retracted. (This usually occurs, albeit at a low frequency, with processes <100 μm in length arising from neurons labeled at E12.5 [Hatanaka and Yamauchi 2013]. However, it may occur in longer processes arising from neurons labeled at later stages; therefore, the scored lateral projections from IZ neurons may include partly immature processes that have not yet been determined definitively as axons. Even so, axonal specification should mostly occur in the IZ because almost all labeled CP neurons extend an apparently long axon). Therefore, the direction of axon outgrowth is directly related to the embryonic stage at which labeling occurs; most neurons labeled at E11.5 and E12.0 and many labeled at E12.5 projected laterally, whereas neurons labeled later projected medially.

Molecular Profiles of Lateral and Medial Projection Neurons

In the adult cortex, excitatory cortical neurons form 2 major distinct projection groups: subcortical and callosal (Molnar and Cheung 2006; Molyneaux et al. 2007). The sequential appearance of lateral and medial projections and their specific directions raised the possibility that lateral and medial projection neurons constitute prospective subcortical and callosal projection groups, respectively. To examine this possibility, we analyzed the expression of neuronal subtype-specific markers. Ctip2 is highly expressed in subcortical projection neurons, including corticospinal motor neurons in layer V, during development (Arlotta et al. 2005). Satb2 is expressed in callosal projection neurons (Arlotta et al. 2008; Britanova et al. 2008). We prepared neurons labeled sparsely at either E12.5 or E13.5, the period of transition from lateral to medial projection, and examined immunoreactivity to these markers at E14.5 or E15.5 using thin sections. As immunoreactivity to these markers was very faint in the IZ, we focused on labeled neurons in the CP.

Most (90.3 ± 3.2%) CP neurons labeled at E12.5 and examined at E14.5, which likely projected laterally (dark gray bar in Fig. 2I), were Ctip2+ (Fig. 3A,F first column; 3 brains; 48/50, 84/99, 54/60, number of positive cells/total cells). This proportion decreased to 43.2 ± 3.5% at E15.5 (Fig. 3B,F second column; 4 brains; 31/66, 9/24, 56/150, 24/47), probably because of an increase in the contributions of subsequent generations of labeled neurons, many of which projected medially. Notably, only 9.2 ± 2.9% of CP neurons labeled at E13.5, which probably projected medially (Fig. 2I), were Ctip2+ at E15.5 (Fig. 3C, negative case, F third column; 3 brains; 3/60, 4/51, 9/61).

Satb2 expression in the CP at E14.5 was weak or undetectable, consistent with our observation that CP neurons at this stage predominantly project laterally (Fig. 2I). For this reason, we examined the Satb2 immunoreactivity of labeled neurons at E15.5. In contrast to Ctip2 immunoreactivity, 72.9 ± 2.7% of CP neurons labeled at E12.5 were Satb2+ at E15.5 (Fig. 3D,G second column; 4 brains; 58/85, 16/20, 93/135, 35/47) and nearly all CP neurons labeled at E13.5 and examined at E15.5 were Satb2+ (Fig. 3E,G third column; 96.2 ± 3.8%; 3 brains; 36/38, 62/64, 62/64). Notably, CP neurons labeled at E14.5 or E15.5 and examined at P0 were virtually all Satb2+ (data not shown). The total percentages of Ctip2+ and Satb2+ cells exceeded 100, likely because of concurrent Ctip2 and Satb2 expression in some neurons (Alcamo et al. 2008; Britanova et al. 2008). Nevertheless, these results support the idea that sequentially appearing lateral and medial projection neurons form distinct subcortical and callosal projection neuron groups, respectively. The observation of Tbr1 expression, which is strong in corticostriatal neurons (McKenna et al. 2011), in some neurons labeled at E12.5 but not E13.5 also supports this idea (Supplementary Fig. 2).

Subsequently, we attempted to directly relate the neuronal projection type with the molecular profile in thick slices. We labeled E12.5 VZ cells with brighter fluorescence using the Super Nova system (Mizuno et al. 2014) and examined the process directions and immunoreactivities of labeled progeny at E15.5 when we can expect to see both lateral and medial projections (15 brains; see Fig. 3B,D,F,G). At this stage, CP neurons had already extended axons >300 μm in length. In total, 94% of the lateral projection neurons were Ctip2+ (Fig. 3H–Iii,J; 31 cells), 3% were Ctip2+ /Satb2− (1 cell), and the remaining 3% were Satb2+ (1 cell). Strikingly, no medial projection neurons were Ctip2+, whereas 18% were Ctip2+/Satb2+ (6 cells), 79% were Satb2+ (Fig. 3J–Iii,J; 27 cells), and 3% were negative for both markers (1 cell). These data clearly showed that most lateral and medial projection neurons constitute prospective subcortical and callosal projection neuron groups, respectively.

Layer Destinations of Lateral and Medial Projection Neurons

It has been established that newly generated excitatory neurons migrate through earlier-born neurons, thus forming the characteristic six-layered structure in an inside-first and outside-last fashion (Angevine and Sidman 1961). This principle predicts that lateral projection neurons reside in deeper layers than medial projection neurons because the former appear earlier than the latter. If this is true, it may contradict the view that lateral and medial projection neurons give rise to the subcortical and callosal projection neuron groups, respectively, because in infragranular layers V and VI, both subcortical and callosal projection neurons are intermingled. To address this issue, we examined the neurons’ layer destinations. Neurons were sparsely labeled at E12.5, E13.5, E14.5, or E15.5 to target neurons in most layers and were examined 3 weeks postnatally, by which time the layer structure had been well formed.

Neurons labeled at E12.5 comprised lateral and some medial projection neurons and were primarily distributed in layers V and VI (Fig. 4A,E; 4 brains, total 333 cells; 52% in layer VI, 37% in...
Figure 3. Lateral and medial projection neurons have distinct molecular identities as prospective subcortical and callosal projection neurons. (A–E) The molecular identities of CP neurons labeled with mCherry at E12.5 (A, B, and D) or E13.5 (C and E). Expression of Ctip2, a marker of subcortical projection neurons, and Satb2, a marker of callosal projection neurons, was examined in labeled CP neurons at either E14.5 (A) or E15 (B–E). Insets: higher magnification images of labeled neurons indicated by yellow arrowheads. Marker positivity and negativity are indicated by (+) and (−), respectively. (F and G) Bar histogram showing the percentages of (F) Ctip2+ and (G) Satb2+ neurons among the total population of labeled CP neurons. N.D.: not determined because of weak Satb2 immunoreactivity in the CP at E14.5. The earliest migrants from E12.5 VZ cells, which reach the CP within 2 days of electroporation, were presumptive lateral projection neurons and were nearly all Ctip2+ (90.3 ± 3.2%). One day later, the percentage of Ctip2+ neurons decreased (43.2 ± 3.5%), probably because of the contributions of subsequently generated, labeled medial projection neurons. CP neurons labeled at E13.5, which corresponds to medial projections, were mostly Satb2+ (96.2 ± 0.7%). Values represent the means and standard errors of the mean. The expression patterns and temporal changes of markers in labeled neurons correlated with the appearances of lateral and medial projection neurons at the population level. (H–J) The direct relationship between projection patterns and marker expressions. (H) Lateral or (I) medial projection neurons were labeled with GFP at E12.5 using the Supernova system and examined at E15.5. Original images were inverted. Arrowheads indicate axons. Some axons are out of focus or were cut off during slice preparation. (Hi–Hiii and Ii–Iiii) Asterisks mark GFP fluorescence and immunoreactivity in the cell of origin in (H and I). (Hi and Ii) merged view, (Hiii and Iii) Ctip2 and (Iii and Iiii) Satb2 immunoreactivity. The lateral projection neuron shown in H was Ctip2+/Satb2−, whereas the medial projection neuron shown in I was Ctip2−/Satb2+. (J) Bar histogram showing the percentages of lateral (red) or medial (green) projection neurons that were Ctip2+/Satb2−, Ctip2+/Satb2+, Ctip2−/Satb2+, or Ctip2−/Satb2−. The numbers in parentheses indicate the numbers of cells in each group. Scale bars: 20 μm in (A–E) and (Hi–Hiii); 100 μm in (H and I).
layer II/III). Importantly, neurons labeled at E13.5, corresponding to medial projection neurons, were also found to a significant extent in layers V/VI, although most were located in upper layers II-IV (Fig. 4B,E; 5 brains, total 621 cells: 8%, 25%, 56%, and 11%, respectively). Labeling of VZ cells at E14.5 or E15.5, producing mostly lateral projection neurons, resulted in an exclusive distribution of their progeny in upper layers (Fig. 4C,E; E14.5, 3 brains, total 380 cells; 0%, 0%, 59%, and 40%; and Fig. 4D,E; E15.5, 3 brains, total 202 cells; 0%, 0%, 2%, and 98%). Therefore, the inside-first and outside-last sequence was generally preserved. However, the precise neuronal distribution was consistent with the view that lateral and medial projection neurons differentiate into subcortical and callosal projection neuron groups, respectively; of neurons labeled at E12.5, many exhibited lateral projections and were located in the infragranular layers, whereas of those labeled at E13.5 or later, most exhibited medial projections and were distributed in all layers, including the infragranular layers (e.g., 33% of neurons labeled at E13.5).

This view was further supported by the expression of Ctip2 in differentiated neurons. In adults, Ctip2 is expressed in corticopontine projection neurons, but not callosal projection neurons, in layer V (Iuta, Hirai, et al. 2013). When we examined layer V neurons that had been labeled densely at E12.5, a considerable number of cells were Ctip2+ (Fig. 4F). In contrast, nearly all layer V neurons labeled at E13.5 were Ctip2- (Fig. 4G). Collectively, these results reinforced the notion that sequentially appearing lateral and medial projection neurons comprise prospective subcortical and callosal neuron groups, respectively.

Lateral and Medial Projection Neurons in Layer V Are Sequentially Generated in Overlapping But Slightly Different Time Windows

Although a previous study of rats did not find any differences in birthdates between subcortical and callosal neurons in layer V (Koester and O’Leary 1993), our observations raised the possibility that lateral and medial projection neurons might be generated sequentially, even when located in the same layer. To address this possibility, we injected EdU at defined times during embryonic development (Fig. 5A). As the cell cycle length of VZ cells in an E12–13 mouse is approximately 11 h (Takahashi et al. 1995), pregnant mice received a single injection of EdU at E11.5 or at 12-h intervals between E12.5 and E14.5, when the deep layer neurons undergo their final mitosis (Smart and Smart 1982). We utilized Ctip2 and Satb2 immunohistochemistry to correlate EdU staining with the projection types of these cells. Layer V neurons in the presumptive SI were examined at P0 (Fig. 5B) when the borders of the deep layers (IV/V/VI) become apparent. As the birthdates were not the same across the cortical areas (Smart and Smart 1982), we focused on a single area for a precise evaluation. We first examined layer V neurons because high levels of Ctip2 expression are maintained at this stage (Arlotta et al. 2005). In our analysis, we only included cells that were
strongly labeled with EdU (>50% maximum labeling) and expected these to correspond to first-generated neurons that undergo a round of DNA replication and cell division after EdU injection (Polleux et al. 1997). We divided the cells into 3 populations: 1) Ctip2+ neurons, which presumably represent lateral projection neurons; 2) Satb2+ neurons, which presumably become medial projection neurons; and 3) Ctip2+/Satb2+ double-positive neurons, which are unidentified projection neurons.

Consistent with previous reports, our EdU labeling analysis indicated that the overall population of layer V neurons was

Figure 5. Sequential generation of presumptive lateral and medial projection neurons in layer V. (A) Time schedule of 5-ethynyl-2'-deoxyuridine (EdU) and 5-bromo-2'-deoxyuridine (BrdU) administration. EdU was administered alone to each pregnant mouse at E11.5, E12.5, E13.0, E13.5, E14.0, or E14.5 (navy blue arrows) or was followed by BrdU after 12 h at E13.5 (blue arrow). Pups were analyzed postnatal day 0 (P0). (B) The presumptive SI region (S1, framed area, 400 μm wide) was subjected to analysis. (C) EdU signals and Ctip2 and Satb2 immunoreactivity above the threshold value (50% over baseline and ≥44 μm² in size) in layer V. Ctip2+ and Satb2+ neurons presumably represent lateral and medial projection neurons, respectively. Left panels: EdU injection at E13.0; right panels: EdU injection at E13.5. From top to bottom: EdU signal, Ctip2/EdU signal, Satb2/EdU signal, and Ctip2/Satb2/EdU signal. Double- and triple-positive cells are indicated by circles and triangles, respectively. Many Ctip2+ cells were EdU+ (magenta) after the injection of EdU at E13.0. In contrast, many Satb2+ neurons were EdU+ (pale blue) after the injection of EdU at E13.5. (D) Line histogram showing the percentage of EdU+ cells among each neuronal type in layer V. Neurons were divided into Ctip2+ (red, ca. 30% of layer V neurons), Ctip2+/Satb2+ (orange, ca. 5%), or Satb2+ (green, ca. 65%) groups (300–600 total cells per brain were counted; E11.5: 3 brains; E12.5: 3 brains; E13.0: 7 brains; E13.5: 4 brains; E14.0: 4 brains; E14.5: 3 brains). Ctip2+ neurons exhibited an earlier neurogenesis peak than Satb2+ neurons. Values represent the means and standard errors of the mean. Significant differences between Ctip2+ and Satb2+ neurons were compared at E13.0 and E13.5 in the same set of sections. Paired two-tailed Student’s t test, *P ≤ 0.05. (E) BrdU signal and Ctip2 and Satb2 immunoreactivity above the threshold value. BrdU was injected at E13.5 after injecting EdU at E13.0. Many Satb2+ neurons were BrdU+ (pale blue). Compare with panels in (C). (F) Line histogram showing the percentages of EdU+ or BrdU+ cells among each neuronal type in layer V in the same brain (broken gray lines). Colored lines represent the means (300–400 cells total cells per condition were scored; 4 brains) and standard errors of the mean. Paired two-tailed Student’s t test, *P ≤ 0.05. Scale bar: 100 μm in (C and E).
generated between E12.5 and E14.5 (Fig. 5D; Smart and Smart 1982). Notably, the generation of Ctip2+ and Ctip2+/Satb2+ neurons occurred within an earlier and somewhat narrower time window, with a peak at E13.0, whereas Satb2+ neurons developed within a slightly later and broader time window, with a peak at E13.5 (Fig. SC,D). As the generation times of layer V Ctip2+ and Satb2+ neurons differed only slightly, we further investigated this difference in the same animal. We administered both EdU at E13.0 and BrdU (another thymidine analog) at E13.5 to each pregnant mouse and analyzed the incorporation of EdU and BrdU in Ctip2+ and/or Satb2+ neurons in adjacent or near-adjacent sections (Fig. SE,F; also compare with SC). In all 4 mice, Ctip2+ and Ctip2+/Satb2+ neurons exhibited a higher labeling frequency at E13.0, whereas Satb2+ neurons exhibited a higher labeling frequency at E13.5.

If lateral projection neurons are generally born earlier than medial projection neurons, lateral projection neurons in layer V also should exhibit earlier birthdating than medial projection neurons in layer VI. Indeed, the peak birthdate for Ctip2+ neurons in layer V appeared marginally earlier than that for Satb2+ neurons in both layers V and VI (Supplementary Fig. 3). Among layer VI neurons, the difference in the time windows for lateral and medial projection neuron generation appeared to be consistent. In adults, PCP4 and ppCK expression have been well correlated to corticofugal and corticocortical projection neurons, respectively, in layer VI (Watakabe et al. 2012). At 3 weeks postnatally, PCP4+/EdU+ neurons, which presumably developed from lateral projection neurons, were found in brains labeled with EdU from E12.5 onward, whereas ppCK+/EdU+ neurons, presumably generated from medial projection neurons, were found in brains labeled with EdU from E13.0 onward (in the SI; Supplementary Fig. 4). This is remarkably consistent with the findings of Tbr1 expression in progeny from VZ cells labeled at E12.5 but not at E13.5 (Supplementary Fig. 2).

Therefore, we concluded that lateral and medial projection neurons in layer V (or probably across layers V and VI), each defined by a distinct molecular identity, are sequentially generated in overlapping but slightly different time windows.

Discussion

In the lateral region of the mouse cortex, we found that 1) most excitatory cortical neurons initiated axonal outgrowth upon migration through the IZ (Fig. 6); 2) the axonal outgrowth direction was either lateral or medial; 3) lateral projection neurons appeared earlier than medial projection neurons; 4) most lateral and medial projection neurons were Ctip2+ and Satb2+, respectively; 5) lateral projection neurons differentiated into neurons located primarily in the infragranular layers, whereas medial projection neurons were distributed across layers II–VI; and 6) even in the same layer (V), lateral and medial projection neurons were sequentially born within slightly different time windows. Therefore, based on the direction of initial axonal outgrowth, excitatory cortical neurons can be divided into two distinct, sequentially developed subpopulations that probably correspond to the prospective subcortical and callosal projection neuron groups.

Common Axonal Initiation from Excitatory Cortical Neurons in IZ

In the monkey, early axonal extension toward the target area has been suggested by the study of migrating neurons destined to layer III (Schwartz et al. 1991). Recent time-lapse imaging studies have extended this idea and have clearly shown that early-born excitatory cortical neurons establish a directional axon from cells, assuming a multipolar shape in the sub-VZ/IZ (neurons from E12.5 VZ; Hatanaka and Yamauchi 2013; those from E12.5
to 13.5 VZ, Namba et al. 2014; Sakakibara et al. 2014) before migrating into the CP. However, it remains unclear whether excitatory cortical neurons born in other time windows (or constituting other layers) initiate axonal outgrowth in the same manner. Our observations that numerous labeled neurons initiated axonal outgrowth in the IZ in the main period of cortical neurogenesis and that almost all labeled neurons extended a tangential axon by the time of CP entry suggests that axon formation in the IZ is a common property. In addition, the same axonal direction from the CP and IZ neurons, except for neurons labeled at E12.5 (containing both first-generated lateral and next-generated medial projection neurons), supports the idea of early directiononal axon specification. Although the behavior of IZ neurons was not examined directly, the presence of such cells with a multipolar shape suggests that these neurons establish their axons from nonpolarized cells de novo (Hatanaka and Yamauchi 2013), similar to the dynamic process observed in early-born neurons.

**Determination of Initial Axonal Direction**

Most axons from excitatory cortical neurons were oriented along the mediolateral axis in the IZ during the prenatal period. We never observed the growth of two axons in opposite directions from a single individually labeled neuron in vivo at the initial stage. Therefore, excitatory cortical neurons direct their axonal outgrowth either laterally or medially. Because lateral and medial extension were observed in overlapping time windows within the same brain area (e.g., axons from neurons labeled at E12.5), it is likely that intrinsic factors play an important role in directing axonal outgrowth.

Reportedly, Fezf2, a transcription factor prominently expressed by subcortical projection neurons during embryonic stages, was shown to regulate the decision between subcortical and callosal neuronal fate (Chen, Rasin, et al. 2005; Chen, Schaevitz, et al. 2005; Molyneaux et al. 2005) via a Ctip2-dependent and/or independent pathway (Chen et al. 2008). Forced expression of Fezf2 in VZ cells at mid or late embryonic stages (Chen et al. 2008; Lodato et al. 2011; Rouaux and Arlotta 2013), or in neurons at early postnatal stages (into layer IV [De la Rossa et al. 2013] or layer II/III [Rouaux and Arlotta 2013]), reprogrammed these progenitors/neurons into subcortical projection neurons. In contrast, Satb2, a chromatin-remodeling protein, regulates callosal neuronal fate by suppressing Ctip2 via binding to its regulatory regions (Alcamo et al. 2008; Britanova et al. 2008). Both factors are candidates for the regulation of lateral and medial axonal outgrowth in the IZ, respectively. Indeed, forced expression of Fezf2 in VZ cells at E13.5 produced some lateral projection IZ neurons (data not shown). Accordingly, Fezf2 can truly direct initial axon in the IZ; however, the appearance of some corticofugal axons in Fezf2 mutant mice suggests that Fezf2 does not act alone (Chen, Schaevitz, et al. 2005; Molyneaux et al. 2005; Komuta et al. 2007). Satb2 is required for callosal axon projection (Alcamo et al. 2008); however, forced expression of Satb2 alone in VZ cells at E12.0 rarely produced medial projection IZ neurons (data not shown). Therefore, although it is possible that these factors determine the initial direction of axonal outgrowth, it is plausible that other currently unknown factors also contribute to this process.

**Cortical Divergent Projections from Lateral and Medial Projection Neurons**

Excitatory neurons can be subdivided into two distinct groups: lateral and medial projection neurons. The sequential generation, laminar location, and marker expression of these neurons are consistent with the view that lateral and medial projection neurons correspond to the prospective subcortical and callosal projection neuron groups.

How do these two groups correspond to the divergent projection types of adult neurons? It is logical that lateral projection neurons would correlate with subcortical projection neurons, which initially extend their axons laterally toward the internal capsule followed by elongation to reach their targets. If this is true, do medial projection neurons then relate to the projection types of all neurons other than subcortical projection neurons?

We observed that most, if not all, medial projection neurons continued to extend their axons toward the corpus callosum and projected to the contralateral side within several days after electroporation (data not shown). Therefore, these neurons actually comprise callosal projection neurons. They also likely include neurons with dual projection to both the contralateral and ipsilateral cortex (e.g., corticocortical associative neurons in the motor cortex [Veinante and Deschênes 2003] and in the SI cortex [Yamashita et al. 2013], and crossed corticostriatal neurons in the motor cortex [Wilson 1987]). A large population of corticocortical associative neurons from the SI to the motor cortex (Mitchell and Macklis 2005) appears to initially extend a callosal projection, thus supporting the idea that these types of neurons begin as medial projection neurons, although some lose their callosal projections at later stages. The layer destinations of medial projection neurons revealed that this group also included layer IV neurons (from labeling at E13.5), which lack long-range connections, at least in the adult SI. Indeed, immature layer IV neurons initially appear to possess callosal projection fibers, as shown by retrograde-labeling studies (Wise and Jones 1976; Ivy and Killackey 1981; O’Leary et al. 1981). In agreement with these findings, the elimination of supernumerary axons in the corpus callosum (ca. 70%) was reported in the developing rhesus monkey (Lamantia and Rakic 1990). The fact that some adult layer IV neurons retain contralateral projections (stellate cells in the barrel field [Ivy et al. 1984] and spiny multipolar neurons in the striate cortex [Martinez-Garcia et al. 1994]) is consistent with the view that they exhibit extended medial axons during early developmental stages.

**Birthdates and Neurons with Particular Projection Patterns**

The birthdates of cortical neurons are known to correspond to their particular cortical layer positions (Smart and Smart 1982). However, it remains unclear whether all types of neurons in a given layer are generated during the same temporal window during development. In rats, no differences in birthdate were reported between subcortical and callosal projection neurons in layer V (Koester and O’Leary 1993). In contrast, latexin-positive, corticocortical associative projection neurons, which are located in the deep layers of the rat lateral cortex, have a unique birthdate; their generation occurs within a temporal window distinct from other neuronal types within the same layer, including...
putative corticothalamic and callosal neurons (Arimatsu et al. 1994, 1999).

Our present birthdating analyses revealed that in layers where both subcortical and callosal projection neurons reside (layer V and VI), presumptive subcortical projection neurons were born in a slightly overlapping but earlier time window than presumptive callosal projection neurons in each layer. Furthermore, subcortical projection neurons in layer V exhibited an earlier birth peak than that of callosal projection neurons in layer VI, at least in the SI, although this difference was minimal. Therefore, it is very likely that excitatory cortical neurons are born in the following sequence: subcortical projection neurons in layers VI and V, followed by callosal projection neurons in layers VI, V, IV, and II/III. Although the inside-first and outside-last birthdate sequences exist, our results suggest that groups of lateral projection neurons are generally born earlier than those of medial projection neurons and that each layer comprises subsets of projection neurons for which the birthdate of each occurs within a unique time window; however, further studies are required to test this idea. It would be beneficial to examine this process in the monkey, which has a longer neurogenesis period (ca. 60 days in the visual cortex; Rakic 1974), in order to validate this possibility.

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

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