NEURODEVELOPMENT

Synaptic transmission from subplate neurons controls radial migration of neocortical neurons

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The neocortex exhibits a six-layered structure that is formed by radial migration of excitatory neurons, for which the multipolar-to-bipolar transition of immature migrating multipolar neurons is required. Here, we report that subplate neurons, one of the first neuron types born in the neocortex, manage the multipolar-to-bipolar transition of migrating neurons. By histochemical, imaging, and microarray analyses on the mouse embryonic cortex, we found that subplate neurons extend neurites toward the ventricular side of the subplate and form transient glutamatergic synapses on the multipolar neurons just below the subplate. NMDAR (*N*-methyl-D-aspartate receptor)–mediated synaptic transmission from subplate neurons to multipolar neurons induces the multipolar-to-bipolar transition, leading to a change in migration mode from slow multipolar migration to faster radial glial-guided locomotion. Our data suggested that transient synapses formed on early immature neurons regulate radial migration.

The six-layered neocortex develops through radial migration of excitatory neurons in an inside-out manner (*1*, *2*). Defects in this construction process lead to brain disorders such as lissencephaly (*3*). In the developing neocortex, excitatory neurons are generated from progenitors known as radial glial cells in the ventricular zone (VZ) (Fig. 1A). Newly generated neurons exhibit a multipolar shape and meandering movement in the subventricular zone (SVZ) and intermediate zone (IZ) (multipolar migration) (*2*). These multipolar neurons (MpNs) then change to a bipolar shape (multipolar-to-bipolar transition) and migrate rapidly in the cortical plate (CP) toward the marginal zone (MZ) via radial glial-guided locomotion (locomotion). Subplate neurons (SpNs) and Cajal-Retzius cells are the first neurons born in the neocortex and reside in the subplate (SP) and MZ, respectively (*2*, *4*). Cajal-Retzius cells guide the inside-out layering of excitatory neurons by secreting reelin (*5*). SpNs build connections between the thalamus and the cortex (*4*). Here, we examined the possibility that SpNs also regulate radial migration.

To evaluate the roles of SpNs in radial migration, we observed the migration of green fluorescent protein (GFP)–labeled neurons by time-lapse recording of the slices from embryonic day 16 (E16) cortices (movie S1). We noticed that MpNs paused migration at the boundary between the IZ and the SP (Fig. 1A and fig. S1). After this stationary period, MpNs changed their morphology to a bipolar shape and began locomotion. Morphometric analysis revealed that the SP demarcated the boundary between the upper cortical region where bipolar neurons were distributed and the lower region where MpNs were distributed (fig. S2). In the SP, migrating neurons exhibited branched and twisted leading processes with features intermediate between bipolar and multipolar neurons (Fig. 1B). These observations raised the possibility that the SP plays roles in the multipolar-to-bipolar transition of migrating neurons.

The SP consists mainly of SpNs, developing axon tracts, and extracellular matrix (*6*). We hypothesized that MpNs receive signals from SpNs, which induce changes in their polarity. To investigate this possibility, we used two methods to visualize SpNs and migrating neurons separately in the same cortical slices: (i) in utero double electroporation and (ii) in utero single electroporation using Lpar1-EGFP (enhanced GFP) mice (*7*). In the VZ, SpNs are generated at E10 as one of the first-born neurons, and then excitatory neurons are generated in a sequential order. Therefore, SpNs and migrating neurons can be labeled separately by sequential in utero electroporations of GFP expression plasmid at E10 and red fluorescent protein (RFP) expression plasmid at E14, respectively, to the same embryos. In addition, various expression constructs can be introduced selectively into SpNs by in utero electroporation at E10 (fig. S3). In the embryonic cortex of the Lpar1-EGFP mouse, subsets of SpNs express EGFP (Fig. 1C). Thus, in utero electroporation of RFP expression plasmid into the cortex of this Tg mouse at E14 enables us to visualize GFP-positive SpNs and RFP-positive migrating neurons in the same slices.

Live imaging of the SpNs of Lpar1-EGFP mice revealed that they extended axon-like processes toward the ventricular side (Fig. 1D and movie S2). RFP labeling of migrating neurons indicated that these processes are closely associated with MpNs just below the SP (Fig. 1E and movies S3 and S4). The SpN processes had many varicosities, which were reminiscent of en passant synaptic boutons and often attached to the cell bodies of MpNs (Fig. 1, F and G). Similar contacts between SpNs and MpNs were observed in the in utero double-electroporated cortices (fig. S4). Immunohistochemical analysis showed that a part of the SpN neurites contacting with MpNs were vesicular glutamate transporter (VGLUT2)–positive, suggesting that they formed glutamatergic synapses (Fig. 2A). On average, 19 VGLUT2-positive puncta were detected per MpN (fig. S5). By using electron microscopy, we found synapse-like contacts on the cell bodies of MpNs in the upper IZ, which were characterized by a few vesicles of 43 ± 1 nm diameter, as well as electron dense structures reminiscent of active zones and postsynaptic densities (Fig. 2, B to E, and fig. S6). About 70 to 80% of the junctional structures around MpNs showed no vesicles, which are presumably immature or degenerating synaptic contacts (Fig. 2, F and G). The pre- and postsynaptic plasma membranes often showed a wavy shape (Fig. 2, F and G), which may reflect the transient nature of these synapses. We confirmed that this type of junctions was formed between MpNs and SpNs by double-label immunoelectron microscopy (fig. S7).

To investigate the roles of synapses between MpNs and SpNs, we examined the neuronal activity of SpNs by Ca^{2+} imaging using GCaMP3. SpNs exhibited Ca^{2+} transients (Fig. 3A and movie S5), suggesting that they were neuronally active as early as E15. We also tried to visualize the presynaptic activity of SpNs using cortical slices electroporated with synaptophysin-pHluorin constructs (*8*) at E10. Synaptophysin-pHluorin signals were observed at the upper IZ (Fig. 3B and movie S6), which were not observed when tetanus toxin (TeNT) was coexpressed (Fig. 3B). TeNT cleaves synaptobrevin and thus blocks exocytosis of synaptic vesicles, indicating that these signals were specific. Moreover, upon K^+ stimulation, synaptophysin-pHluorin signals were augmented around several cells, presumably MpNs, just below the SP (Fig. 3B and movie S7). These observations suggested that SpNs release neurotransmitters around MpNs.

We examined whether neuronal activities of SpNs were necessary for radial migration by overexpressing the inwardly rectifying potassium channel Kir2.1 (*9*), which causes a suppression of neuronal excitability. The embryos were doubly electroporated with Kir2.1 and GFP constructs at E10 and subsequently with RFP construct at E14. In contrast to the control cortices, where many RFP-labeled neurons entered the CP, they were accumulated just below SP when Kir2.1 was expressed in the SpNs (Fig. 3C, arrows). We further tried to suppress neurotransmitter release from

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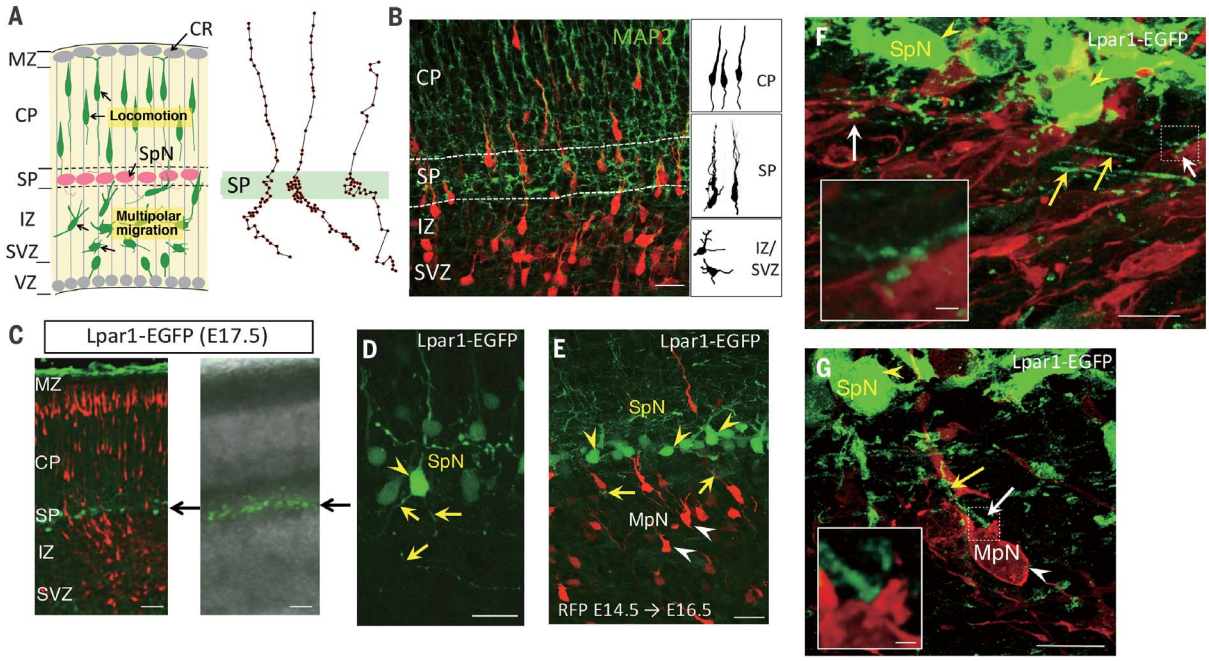

Fig. 1. SP as a strategic position for radial migration. (**A**) Newborn neurons first show multipolar migration. After multipolar-to-bipolar transition, they initiate locomotion (left). Three migrating neurons shown in movie S1 were tracked (right). (**B**) In the SP, migrating neurons showed a transitional form between multipolar and bipolar morphologies. (**C**) SpNs and migrating neurons were doubly labeled with GFP (green) and RFP (red), respectively, in the Lpar1-EGFP mouse cortex. The SP layer (arrow) can be visualized as the dark zone under bright-field microscopy. (**D**) SpNs (yellow arrowheads) extend axon-like processes (arrows). (**E** to **G**) SpNs (yellow arrowheads) and MpNs (white arrowheads) were visualized in cortical slices of Lpar1-EGFP mice. Yellow arrows indicate axon-like processes of SpNs. Enlarged images showed that neurites of SpNs contacted with migrating MpNs just under the SP. The contact sites often showed a varicosity-like structure (white arrows). Scale bars: 50 μm for (C); 20 μm for (B), (D), and (E); and 1 and 10 μm for (F) and (G).

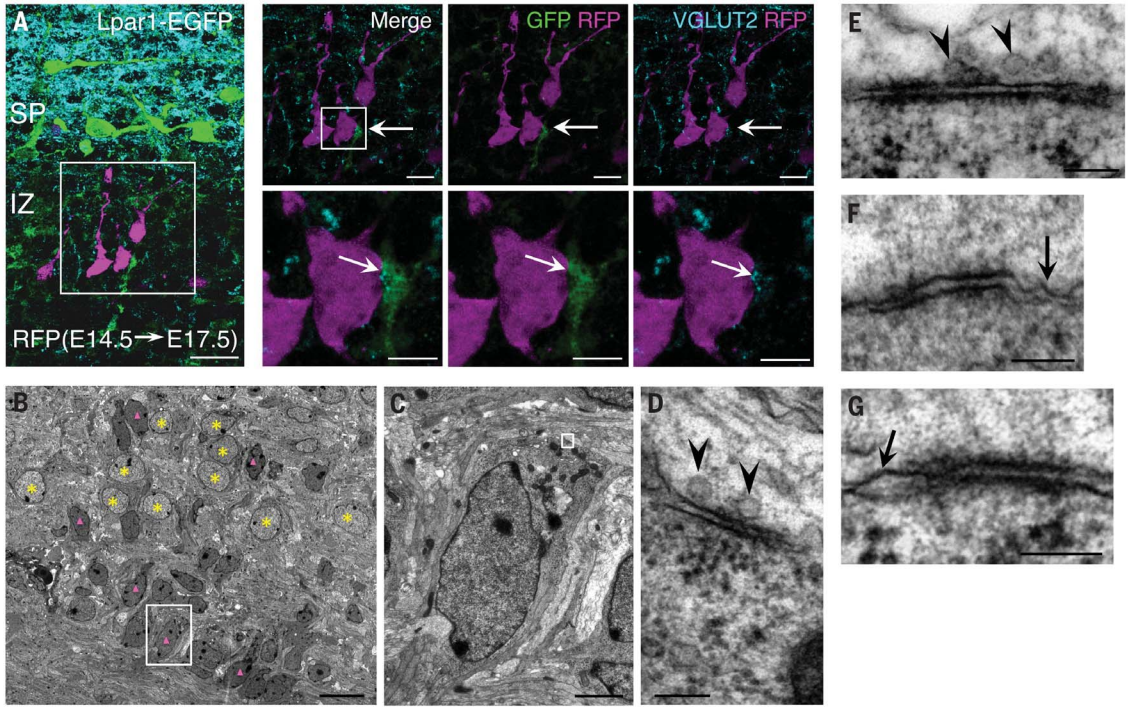

Fig. 2. Glutamatergic synaptic contacts between SpNs and MpNs. (**A**) SpNs and migrating neurons were doubly labeled with GFP (green) and RFP (magenta), respectively, in the cortex of Lpar1-EGFP mice. The SpN neurites showed VGLUT2 immunoreactivity (cyan) at the contact sites with MpNs (arrows). The insets show the enlarged areas indicated by rectangles. (**B**) E16 cortical sections were observed by electron microscopy. SpNs and migrating neurons are marked with asterisks and triangles, respectively. (**C** to **E**) The areas indicated by rectangles in (B) and (C) are enlarged in (C) and (D), respectively. Arrowheads in (D) and (E) show synaptic vesicles. (**F** and **G**) The junctional structures without synaptic vesicles. Arrows indicate wavy plasma membranes. Scale bars: 20 μm for (A), left; 10 μm for (A), right upper layer, and (B); 5 μm for (A), right lower layer; 2 μm for (C); and 100 nm for (D) to (G).

SpNs using TeNT knock-in mice, in which TeNT is expressed in a Cre-dependent manner (*10*). Using this mouse, in utero double electroporation of Cre expression plasmid at E10 and RFP constructs at E14 was performed. As in the case of Kir2.1 experiments (Fig. 3C), radial migration was inhibited just below the EGFP/TeNT-expressing SpNs (Fig. 3D, arrows). These results suggested that the synaptic transmission from SpNs to MpNs is critical for MpNs to initiate locomotion, although some deep CP neurons might also contribute to the synaptic transmission (fig. S3).

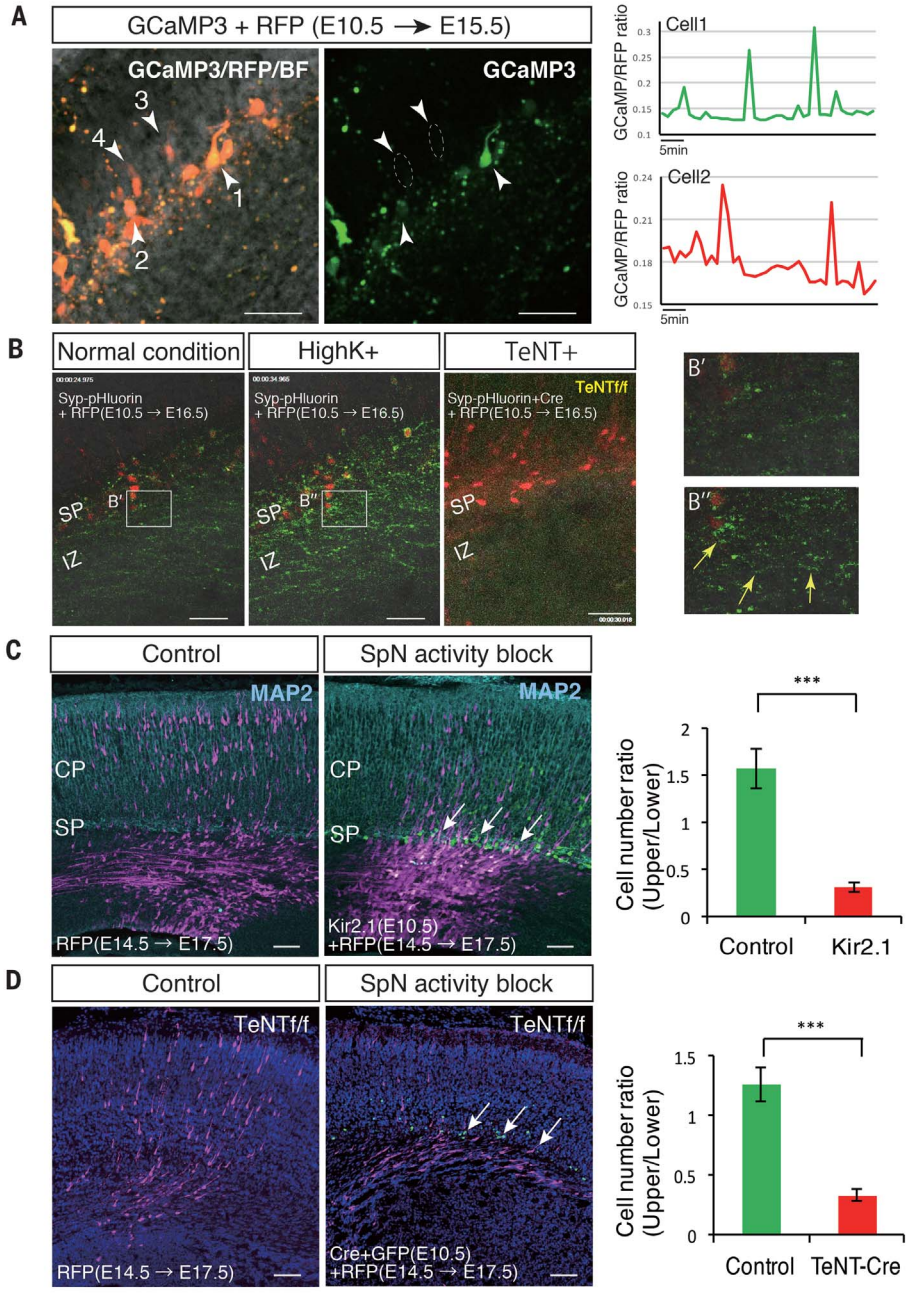

Fig. 3. Critical roles of neuronal activity of SpNs in radial neuronal migration. (**A**) Calcium imaging of SpNs (cells 1 and 2) electroporated with the GCaMP3 construct. Deep CP neurons scarcely showed Ca^{2+} rises (cells 3 and 4). (**B**) SpNs were electroporated with the synaptophysin-pHluorin construct, and the cortical slices were applied for imaging analyses. Synaptophysin-pHluorin signals were augmented after K^+ stimulation around many cells (**B′** and **B″**, arrows). The signals were suppressed when TeNT was coexpressed. (**C**) Neuronal migration was blocked when neuronal activity of SpNs was suppressed by Kir2.1. (**D**) Neuronal migration was blocked when neurotransmitter release from SpNs was impaired by EGFP-TeNT. Three brains of independent experiments were used, and two sections from each brain were analyzed for Kir2.1 experiments ($N = 6$). Two brains of independent experiments were used, and three sections from each brain were analyzed for EGFP-TeNT experiments ($N = 6$). Scale bars, 50 μm. ***$P < 0.0005$. Error bars, mean ± SEM.

Glutamate and *N*-methyl-D-aspartate receptors (NMDARs) are involved in radial migration (*11*–*14*). In these studies, glutamate in the CP has been considered to act as a chemoattractant for migrating neurons via nonsynaptic NMDARs. On the other hand, if glutamatergic synaptic transmission induces the change of migration mode, local application of glutamate should enhance the initiation of locomotion. Thus, we performed experiments using caged glutamate (Fig. 4A and movie S8). MpNs in the uncaged area, which were marked by the red fluorescence of Kikume green-red, rapidly initiated locomotion and passed through the SP without a stationary phase. The displacement of neurons from the uncaged areas and the migration toward the MZ cannot be explained by simple chemotactic effects of glutamate.

To estimate the glutamate receptors involved in the synaptic transmission between SpNs and MpNs, we first performed a gene expression profiling of newborn excitatory neurons (fig. S8). After in utero electroporation of GFP expression plasmid into E14 cortices, RNAs were purified from GFP-labeled cells collected at E15, E16, and E17 by fluorescence-activated cell sorting (FACS). Because GFP-labeled neurons passed through the multipolar to bipolar stages during these 3 days, we could show changes in gene expression that occurred as the migration stages proceeded. Enrichment analyses revealed that the expression of genes associated with gene ontology terms containing "synaptic contact" increased during migration. In particular, gene expressions of NR1 and NR2B subunits of NMDARs and postsynaptic density proteins such as PSD95 increased (fig. S9, A and B). Furthermore, NMDAR antagonists AP5 and MK801 inhibited radial migration in the cultured cortical slices, as reported previously using rat tissues (*12*) (fig. S9C).

We thus investigated the postsynaptic mechanism of MpNs focusing on PSD95 and NMDAR. Knockdown of PSD95 resulted in impaired radial migration, which was rescued by the short hairpin RNA (shRNA)–resistant expression construct of PSD95 (Fig. 4B and fig. S10, A and B). Observation of the morphology of migrating neurons revealed that multipolar-to-bipolar transition was impaired by knockdown of PSD95 (fig. S10, C to E). We next examined the involvement of NMDARs using $NR1^{flox/flox}$ mice (*15*). In utero electroporation of Cre expression plasmid resulted in accumulation of NR1 knockout neurons just under the SP (Fig. 4C). Because NR1 and PSD95 are essential components for NMDAR-mediated synaptic transmission, these results support the idea that glutamatergic synaptic transmission from SpNs to MpNs via NMDARs induces multipolar-to-bipolar transition.

Finally, we performed Ca^{2+} imaging of migrating neurons. We observed that the Ca^{2+} concentration in MpNs transiently increased just before initiation of locomotion, suggesting that Ca^{2+} entry through NMDARs plays roles in multipolar-to-bipolar transition (fig. S11 and movies S9 and

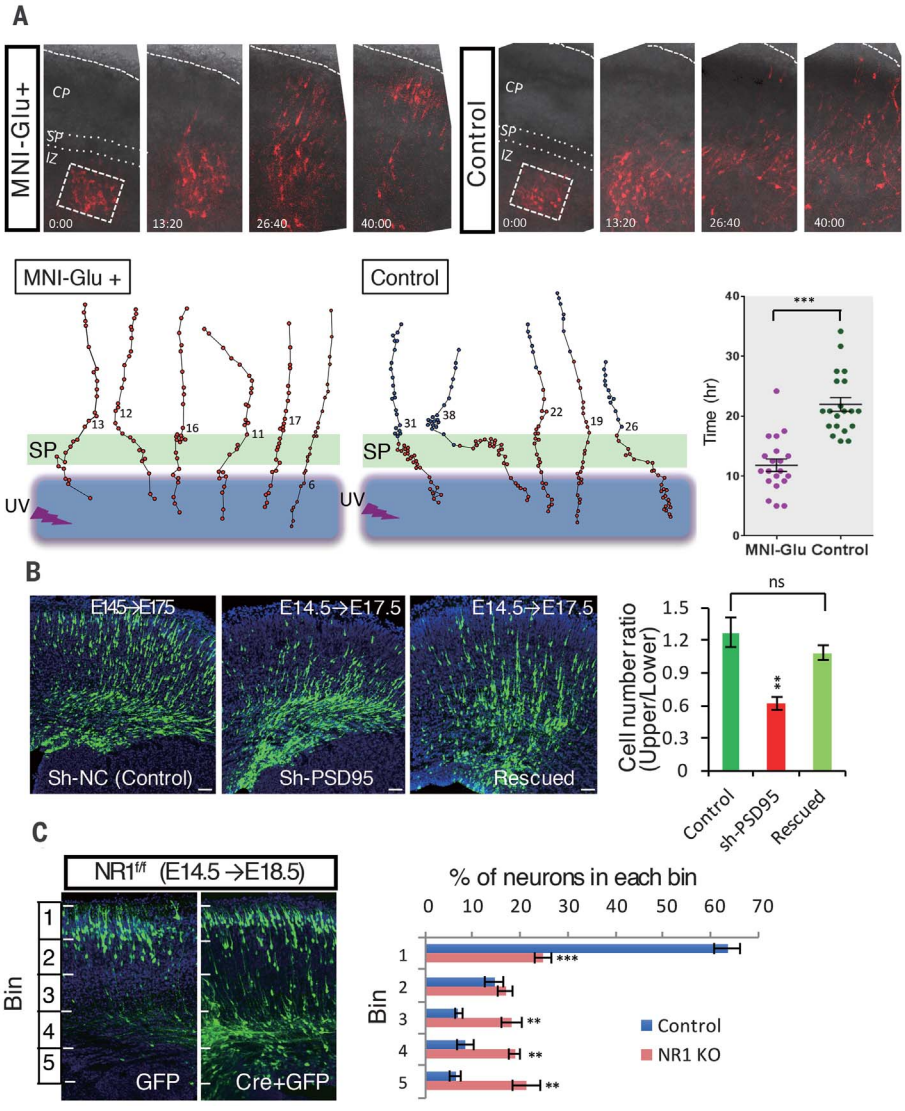

Fig. 4. NMDAR and PSD95 in MpNs are involved in radial migration. (**A**) The cortical slices were cultured in the presence (MNI-Glu^{+}) or absence (control) of caged glutamate, and the areas indicated by rectangles were laser-irradiated. Tracking of neuronal migration indicated that neurons in the uncaged area rapidly initiated locomotion. The time it took for red neurons to initiate locomotion after laser irradiation was plotted (N = 20). (**B**) Knockdown of PSD95 resulted in accumulation of MpNs below the SP. Three brains of independent experiments were used, and three sections from each brain were analyzed (N = 9). (**C**) The cortices of NR1$^{flox/flox}$ mice were electroporated with Cre and GFP expression plasmids at E14. Knockout of NR1 resulted in delay of neuronal migration. Three sections from one embryo and four sections from another embryo of independent experiments (N = 7) were used for quantification. Scale bars, 50 μm. $^{**}P < 0.005$. $^{***}P < 0.0005$. Error bars, mean ± SEM.

S10). However, we cannot exclude the possibility that AMPA receptors are also involved in Ca^{2+} signaling because their expressions increased during migration (fig. S9B). Increased Ca^{2+} transients in migrating neurons in the CP, which may be induced by radial glial fibers (*16*), cause migration arrest and maturation (*17*), suggesting that the roles of Ca^{2+} signaling change depending on the stage of migration.

Our data suggested that subplate neurons facilitate multipolar-to-bipolar transition of migrating excitatory neurons by NMDAR-mediated synaptic transmission, although the possibility that there are also contributions from deep layer neurons cannot be excluded (fig. S12). Impairment of multipolar-to-bipolar transition and accumulation of multipolar neurons below the subplate are observed in mice mutant for a variety of genes, including RP58 (*2*, *18*); some of these genes are involved in human neurodevelopmental disorders such as fragile X syndrome (*19*). Subplate neurons might regulate the expression and function of these genes in multipolar neurons through NMDAR-mediated Ca^{2+} signaling. The subplate has been associated with autism and schizophrenia by gene expression profiling (*6*). Disruption of these transient synapses during development may contribute to these and other psychiatric disorders.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/360/6386/313/suppl/DC1
Materials and Methods
Figs. S1 to S12
Movies S1 to S10
References (*20*, *21*)

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短暂的指令

Transient instruction changes migration

The brain neocortex is built by waves of neurons migrating from deep within the brain to the surface layers. Ohtaka-Maruyama *et al.* found that a layer of neurons that multipolar neurons encounter on their travels instructs the migrating neurons to change phenotype and direction (see the Perspective by Schinder and Lanuza). These subplate neurons form transient glutamatergic synapses with the immature migrants. This results in the migrating multipolar neurons becoming bipolar, more directed, and faster in their final migrations.

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