Connexin 43 controls the multipolar phase of neuronal migration to the cerebral cortex

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The prospective pyramidal neurons, migrating from the proliferative ventricular zone to the overlying cortical plate, assume multipolar morphology while passing through the transient subventricular zone. Here, we show that this morphogenetic transformation, from the bipolar to the multipolar and then back to bipolar again, is associated with expression of connexin 43 (Cx43) and, that knockdown of Cx43 retards, whereas its overexpression enhances, this morphogenetic process. In addition, we have observed that knockdown of Cx43 reduces expression of p27, whereas overexpression of p27 rescues the effect of Cx43 knockdown in the multipolar neurons. Furthermore, functional gap junction/hemichannel domain, and the C-terminal domain of Cx43, independently enhance the expression of p27 and promote the morphological transformation and migration of the multipolar neurons in the SVZ/IZ. Collectively, these results indicate that Cx43 regulates the passage of migrating neurons through their multipolar stage via p27 signaling and that interference with this process, by either genetic and/or environmental factors, may cause cortical malformations.

embryonic neocortex | intermediate zone | radial migration

The laminated structure of the mammalian cerebral cortex is an end product of coordinated generation, migration, and deposition of neurons to their final locations during the embryonic period (1). All prospective cortical pyramidal neurons are generated in either the ventricular (VZ) or subventricular (SVZ) zones and, after their final division, migrate radially to the cortical plate (CP) situated beneath the pial surface (2). Although it has been observed long ago that cells in the SVZ and intermediate zone (IZ) display multiple processes as they choose and translocate between adjacent radial glial fibers (3, 4), it has been only until recently possible to study this transient process by live imaging combined with genetic introduction of GFP (5–9). The newly generated neurons usually undergo transient multipolar transformation before assuming radial migration (8, 10). Based on these observations, it has been proposed that many developmental disorders, such as periventricular nodular heterotopia, subcortical band heterotopia, and doublecortex syndrome are related to migration abnormalities including its multipolar stage at SVZ/VZ (11–13).

The molecular mechanisms controlling directly and/or indirectly the multipolar stage of neuronal migration have just begun to be recognized (7, 14–17). For example, knockdown or inactivation of Filamin A or LIS1 accumulated the multipolar neurons in the VZ and SVZ, whereas knockdown or inactivation of Doublecortin (DCX) accumulated these cells in the IZ (18, 19). Conversely, increasing filamin A activity by siRNA of Filamin A-interacting protein accelerated the transition to a bipolar shape in the SVZ, and overexpression of DCX increased the number of bipolar cells in the IZ (20, 21). In addition, Cdk5 has also been shown to control the multipolar-to-bipolar transition during radial migration (7, 18, 20). Recently, it has been reported that the p27 protein, a Cdk inhibitor, not only inhibits cell cycle progression (22, 23), but also regulates cell motility (24) and promotes

migration and differentiation of cortical projection neurons (25–27). However, it is not clear whether p27 also plays a role in the neuronal translocation and multipolar morphological transformation in the SVZ/IZ.

Application of immunohistochemistry has revealed that gap junction connexins are expressed in both radial glial cells and the migrating postmitotic neurons and may play a role in both cell proliferation and radial migration (28–30). Use of shRNA techniques has shown that gap junction adhesion, rather than channels or C terminus of Cx43, is responsible for the radial migration (29). Recently, studies using Cx43cKO and Cx43k258stop mice have indicated that the C terminus is required for cortical neuronal migration (30). In addition, independent regulation of the gap junction channel or C-terminal domain in neurite outgrowth (31, 32) and cellular motility (33–37) have been demonstrated in various cell types, indicating that they may affect the neocortical development (38).

Results

Cx43 Is Expressed in the Postmitotic Multipolar Neurons in the SVZ/IZ.

To confirm the expression of Cx43 in postmitotic multipolar neurons in the upper portion of the SVZ and the IZ, we labeled the VZ precursors with GFP via in utero electroporation at embryonic day (E)15 and examined the expression of Cx43 in GFP⁺ cells at E16.5. As illustrated in Fig. 1A, GFP⁺ cells were observed in both the SVZ and IZ, which had been confirmed by Tuj-1 immunostaining to be postmitotic neurons. The VZ/SVZ GFP⁺ cells usually show an elongated cell body with a leading process directed toward the pial surface, indicating that they are migrating postmitotic neurons. From the upper SVZ to the IZ, many GFP⁺ cells display multiple processes. Colocalization of Cx43 staining with the GFP⁺ cells confirmed that both the postmitotic bipolar and multipolar neurons in the SVZ/IZ express Cx43 (Fig. 1A) and suggest that it may be engaged in the neuronal translocation and morphological transformation in the SVZ/IZ.

Knockdown of Cx43 Impairs Migration and Transformation. To test whether Cx43 affects the neuronal translocation and morphological transformation in the SVZ/IZ, we labeled VZ precursors by electroporation with GFP plasmid at E15 and examined the position of GFP⁺ postmitotic neurons in the SVZ/IZ at E17. As shown in Fig. 1B, the GFP⁺ neurons migrated into the IZ in the control brain. In contrast, in the brain coelectroporated with Cx43 shRNA ACT2, the GFP⁺ cells usually accumulate at the interface of the SVZ/IZ, and few GFP⁺ cells migrated into the middle and upper portions of the IZ (Fig. 1B and C), which

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Fig. 1. Distribution of Cx43 in the SVZ/IZ. (A) The VZ precursors were electroporated with GFP plasmid at E15, and immunostaining for Cx43 was performed at E16.5. The arrowheads indicate the colocalization of Cx43 staining (red) and GFP fluorescence (green) in the postmitotic bipolar and multipolar neurons. (B) The position of GFP⁺ cells within the cerebral cortex in control, Cx43 shRNA ACT2, and ACT2 plus Cx43 plasmids in the brain electroporated at E15 and examination at E17. (C) Distribution of GFP⁺ cells across the cerebral cortex from the VZ to the IZ in control, ACT2, and ACT2 plus Cx43 plasmids electroporated brain. **P* < 0.05, ***P* < 0.01, vs. control, ****P* < 0.01, vs. ACT2, *n* = 10 and 6, respectively. (D) The morphology of GFP⁺ cells in control, and the brains electroporated with ACT2 and ACT2 plus Cx43 plasmids at E15 and examined at E16.5. (Scale bars: A, 20 μm; B, 60 μm; D, 10 μm.)

indicates that knockdown of Cx43 impairs neuronal translocation to the SVZ/IZ. We then examined the morphology of the postmitotic neurons after knockdown of Cx43. The electroporation was performed at E15, and examination was made at E16.5. In the control brain, the GFP⁺ postmitotic neurons that migrate into the IZ usually have irregular shape with multiple processes emanating from the soma (Fig. 1D). In the ACT2-electroporated brain, the GFP⁺ cells are more compacted and have relatively smaller soma with fewer and thinner processes (Fig. 1D), suggesting that knockdown of Cx43 also impairs the morphological transformation of neurons in the SVZ/IZ.

We further tested whether expression of Cx43 could rescue the neuronal translocation and morphological transformation defects induced by knockdown of Cx43 in the SVZ/IZ postmitotic neurons. As expected, compared with the brain electroporated with ACT2, coexpression of Cx43 significantly increased the number of GFP⁺ cells that migrated into the middle and upper portion of the IZ and reduced the number of GFP⁺ cells at the interface of the SVZ/IZ (Fig. 1 *B* and *C*). Furthermore, coexpression of Cx43 also increased the number and length of the processes of the multipolar neurons in the SVZ/IZ (Fig. 1*D*).

Cx43 Affects the Neuronal Migration and Transformation via p27. As shown in Fig. 24, p27 expression in the VZ at E16 is generally lower, except in some cells at the ventricular surface. However,

Fig. 2. Cx43 attributes to the expression of p27 in the SVZ/IZ. (A) P27 immunostaining (green) in the cerebral cortex at E16. (B) Colocalization of p27 staining (red) with GFP⁺ multipolar neurons in the IZ. Electroporation was performed at E15 and examination at E17. (C) Expression of p27 depends on Cx43 in the SVZ/IZ postmitotic neurons. Electroporation was performed at E15, and immunostaining for p27 was performed at E16.5. Knockdown of Cx43 with ACT2 reduced the expression of p27 in the postmitotic neurons. (D) The multipolar GFP⁺ cells in the IZ displayed down-regulated expression of p27 after knockdown of Cx43. (E) Coelectroporation with Cx43 enhanced the expression of p27 in the SVZ/IZ. (F) Coelectroporation with Cx43 rescued the expression of p27 in the GFP⁺ postmitotic neurons in the IZ. (Scale bars: A, 60 μ m; B, D, and F, 10 μ m; C and E, 40 μ m.)

expression of p27 begins to increase in cells from the lower portion of the SVZ and reaches a maximum in the cells passing through the IZ. Colocalization of p27 with the GFP⁺ multipolar cells in the SVZ/IZ (GFP plasmid electroporation at E15 and examined at E16.5) confirmed that the VZ/SVZ-generated postmitotic multipolar neurons express p27 (Fig. 2*B*). Because the expression pattern of p27 is synchronized with the morphological transformation of the postmitotic multipolar neurons in the SVZ/IZ, we asked whether Cx43 knockdown induced impairments of neuronal translocation, and morphological transformation, is accompanied with p27 expression changes. As shown in Fig. 2*C*, p27 expression in GFP⁺ cells in the SVZ/IZ was significantly reduced in brain slices after knockdown of Cx43 with ACT2. At higher magnification, the individual and clustered GFP⁺ cells lack expression of p27 in the IZ (Fig. 2*D*). In addition, coelectroporation of Cx43 significantly increased the expression of p27 in the SVZ/IZ postmitotic neurons (Fig. 2*E*). Specifically, more individual GFP⁺ cells express p27 in the IZ (Fig. 2*F*).

Fig. 3. p27 is involved in Cx43-induced neuronal translocation and morphological transformation in the SVZ/IZ. (A) Electroporation of p27 was performed at E15 and examination at E18. (B) Distribution of GFP⁺ cells across the cerebral cortex from the VZ to the CP in control and p27 shRNA electroporated brain (***P* < 0.01, *n* = 6). (C) Coelectroporation with p27 rescued the neuronal translocation and morphological transformation defects induced by ACT2 in the SVZ/IZ. Electroporation was performed at E15 and examination at E17. (D) Distribution of GFP⁺ cells across the cerebral cortex from the VZ to the IZ in ACT2 and ACT2 plus p27 electroporated brain (**P* < 0.5, ***P* < 0.01, *n* = 6). (E) Coelectroporation with p27 enhanced the number and length of the multipolar processes in the IZ postmitotic neurons. Much longer and thicker processes were observed in the p27 coelectroporated brain. (Scale bars, A and C, 40 μ m; E, 10 μ m.)

few of them enter into the upper portion of the IZ. In addition, the GFP⁺ cells in the SVZ/IZ displayed an immature phenotype with a smaller cell body and reduced the number and length of the processes (Fig. 3 A and B).

As a next step, we tested the effect of p27 overexpression in the brain electroporated with Cx43 shRNA (ACT2) at E15 and the position of the GFP⁺ cells examined at E17. After knockdown of Cx43, most GFP⁺ cells still stayed at the interface of the SVZ/IZ and the lower portion of the IZ, and a few migrated into the middle and upper portion of the IZ (Fig. 3 C and D). In contrast, coelectroporation with p27 significantly increased the number of GFP⁺ cells entry into the middle and upper portion of the IZ (Fig. 3 C and D). In addition, the GFP⁺ cells in the IZ display much longer and thicker processes in the brain coelectroporated with p27 than those in the brain electroporated only with ACT2 (Fig. 3E). These results indicate that Cx43 might affect the translocation and morphological transformation of the SVZ/IZ neurons via regulation of p27.

Both Gap Junction/Hemichannel and C-Terminal Domain of Cx43 Up-Regulate p27 Expression. Previous studies have shown that gap junctions/connexins control the neuronal migration via docking adhesion, but not via the C terminus or their channel property (29). In contrast, a more recent study demonstrated that C terminus is involved in the radial neuronal migration (30). These results imply that connexins may affect cortical development via diverse mechanisms at different stages. To dissect the role of functional gap junction/hemichannel and the C terminus of Cx43, we introduced Cx43 mutant plasmids expressing truncated peptides

corresponding to the gap junction/hemichannel (Cx43-m257) and the C terminus (Cx43-t257), respectively, which had been identified by DNA sequencing and protein expression in N2a cells. Transfection with Cx43-t257, but not Cx43-m257, can be recognized by an antibody against the C-terminal domain, whereas transfection with Cx43-m257 but not Cx43-t257 can be identified by an antibody targeting the N-terminal domain (Fig. S1A). We also tested the knockdown efficiency of ACT2 on those mutant plasmids. ACT2 effectively knocked down the expression of Cx43 and Cx43-m257 but showed little effect on the expression of Cx43-t257 (Fig. S1A and B). The functional gap junction/hemichannel was demonstrated by Lucifer yellow (LY) uptake in N2a cells. As shown in Fig. 4A, no LY uptake was detected in N2a cells transfected with vehicle vector (pcDNA3) in 0 mM Ca²⁺ medium. In contrast, LY uptake was observed in N2a cells transfected with Cx43 full length and with Cx43-m257, suggesting that Cx43-m257 retains the gap junction/hemichannel property after truncated by the C terminus. While in N2a cells transfected with Cx43-t257, no LY uptake was detected (Fig. 4A). In addition, punctate and plank-like immunostaining in the membrane was observed in N2a cells transfected with Cx43-m257 (Fig. 4A), which is typical for functional gap junctions/hemichannels, whereas transfection with Cx43-t257 revealed a cytoplasmic staining (Fig. 4A). One characteristic of functional gap junctions/hemichannels is their extracellular Ca²⁺-dependent gating property. As expected, in 2 mM Ca²⁺ medium, the LY uptake was significantly reduced in N2a cells transfected with Cx43-m257 (Fig. 4B) and with Cx43 full length. In addition, the LY uptake in N2a cells transfected with Cx43 full length and Cx43-m257 was totally abolished by gap junction blocker MFA (Fig. 4B).

To determine the role of different Cx43 components (Cx43 full length, C terminus, and the channel portion), we tested their effect on p27 expression. As shown in Fig. 4C, transfection with Cx43, Cx43-m257, or Cx43-t257 increases the expression of P27 in N2a cell compared with the control. Furthermore, as expected, knockdown of Cx43 or Cx43-m257 with ACT2 significantly reduced the expression of P27, whereas ACT2 had no effect on p27 expression in Cx43-t257 transfected N2a cells (Fig. 4D), which is consistent with our observation that ACT2 effectively knocks down expression of Cx43 and Cx43-m257, but has little effect on the expression of Cx43-t257 in N2a cells (Fig. S1). Our results suggest that the terminus of Cx43 (Cx43-t257) may induce p27 expression via a different mechanism from that of functional gap junctions/hemichannels.

Ion Channel Function as well as C-Terminal Domains of Gap Junctions/Hemichannels Play a Role in Neuronal Translocation and Their Multipolar Transformation. To confirm the rescuing capacity of Cx43-m257 and Cx43-t257 on migration and morphological transformation defects induced by knockdown of Cx43, electroporation was made at E15, and the position of GFP⁺ cells was assessed at E17. After knockdown of Cx43 with ACT2, many GFP⁺ cells remain in the SVZ and/or accumulate at the interface of the SVZ/IZ (Fig. 5A). Coelectroporation with Cx43-m257 significantly increased the number of GFP⁺ cells that migrated to the upper portion of the IZ (Fig. 5A). Interestingly, coelectroporation with Cx43-t257 showed a similar effect (Fig. 5A). The distribution of GFP⁺ cells across the VZ/SVZ/IZ was displayed in Fig. 5B. In addition, coelectroporation with Cx43-m257 or Cx43-t257 significantly increased the number and length of the processes in GFP⁺ cells in the SVZ/IZ (Fig. 5C). Thus, our results suggest that the trophic effect of Cx43 on neuronal translocation and morphological transformation in the SVZ/IZ postmitotic neurons depend on both its functional gap junction/channels and C terminus.

The rescuing effect of Cx43-m257 or Cx43-t257 on the neuronal migration defect induced by ACT2 was further examined by electroporation at E15 and assayed at E18. As shown in Fig. 5D, knockdown of Cx43 with ACT2 retarded most of the GFP⁺

Fig. 4. Expression of functional gap junction/hemichannel domain and C-terminal domain of Cx43 increase P27 expression in N2a cells. (A) Lucifer yellow uptake in N2a cells transfected with Cx43-m257 and Cx43-t257 in 0 mM Ca^{2+} medium. TPCxs refer to truncated peptides of connexins. (B) LY uptake in N2a cells transfected with Cx43-m257 was extracellular Ca^{2+} sensitive and was blocked by gap junction/hemichannel blocker MFA. (C) Expression of p27 in N2a cells transfected with Cx43, Cx43-m257, or Cx43-t257 and the knockdown effects of ACT2. Cx43 full length, functional gap junctions/hemichannels, and, to a lesser extent, the C terminus increased the expression of p27 in N2a cells. ACT2 effectively reduced the expression of p27 in Cx43 and Cx43-m257 transfected cells but has little effect on expression of p27 in Cx43-t257 transfected cells. (D) Quantification of normalized blot density. $^{**}P < 0.01$, $n = 6$. (Scale bars: A and B, 40 μm .)

cells in the SVZ/IZ, and very few cells migrated into the CP. In contrast, coelectroporation with Cx43 or Cx43-m257 significantly increased the number of GFP⁺ cells in the CP and reduced the number of GFP⁺ cells retarded in the IZ, whereas coelectroporation with Cx43-t257 had little effect on neuronal migration into CP (Fig. 5 *D* and *E*). Our results suggest that gap junctions/hemichannels (including the adhesion property) are essential for the cell entrance into the CP. However, the rescuing effect of Cx43 full length on neuronal migration into the CP is much more obvious than that of the Cx43-m257, suggesting an auxiliary role of the C terminus in neuronal migration. These results indicate that both functional gap junctions/hemichannel and C terminus of Cx43 affects neuronal migration by controlling multipolar transformation in the SVZ/IZ via p27 signaling.

Discussion

The involvement of plasma membrane channels and Ca^{2+} fluctuations in neuronal migration through effect on the cytoskeleton controlling nuclear translocation was discovered two decades ago (39, 40), but the role of specific gap junctions/hemichannels on the distinct phases of neuronal migration is only beginning to be elucidated (37, 38). One of these phases is transient morphological transformation of postmitotic neurons from the bipolar to multipolar shape in the SVZ/IZ and then back to bipolar shape. Although, it was initially assumed that newly generated neurons migrate to the CP guided mainly by a leading process (2), subsequent examinations revealed that many migrating cells form transitionally multiple protoplasmic processes (4). This finding was confirmed by the studies using modern methods, indicating that this stage may be an essential step for the proper neuronal migration to the CP, particularly at the later stages (8, 10).

In the present study, we observed that knockdown of Cx43 prevents formation of the multipolar stage in the SVZ/IZ and

delays neuronal migration to the CP. Furthermore, we provided evidence that p27 is engaged in the regulation of Cx43 in neuronal translocation and morphological transformation in the SVZ/IZ. We found that expression of Cx43 markedly increases the level of p27 and, in addition, both the gap junction channel domain and the C-terminal domain enhance the level of p27. Considering the extensive effect of p27 on neuronal differentiation and radial migration (22, 24, 26, 27), our data support the concept that Cx43 may act as multifaceted regulator for neuronal migration, in which the gap junction channel and C terminus independently control neuronal translocation from the SVZ to IZ and their morphological transformation via p27.

Earlier studies have shown that gap junction channels affect the proliferation of the VZ/SVZ precursors by releasing ATP (41) and that gap junctions/hemichannels also modulate the interkinetic nuclear migration and intermediate neuronal progenitor migration (42–44). Recent evidence indicates that gap junction connexins also play a role in radial migration (28–30). Using siRNA techniques, it has been shown that gap junction adhesion, but not channels or C terminus, is responsible for the radial migration (29), whereas examination of the Cx43cKO and Cx43k258stop mice shows that the C terminus of Cx43 is also a player (30). These seemingly conflicting results suggest that gap junction connexins may affect cerebral cortex development through multifaceted mechanisms (38). Our findings highlight the multiple effects of gap junction connexins at multiple stages during cortical genesis. In addition, we have observed that overexpression of gap junction domain (adhesion and channel) but not the C-terminal domain or the p27 rescue neuronal migration to the CP, although the latter two can rescue neuronal translocation and growth of multiple processes on the post-mitotic neurons. Thus, our results support the previous observations that gap junction adhesion guides the neuronal migration

Fig. 5. Gap junction/hemichannel domain and C-terminal domain of Cx43 affects neuronal translocation and morphological transformation in the SVZ/IZ. (A) The position of GFP⁺ cells in ACT2, ACT2+Cx43m257, and ACT2+Cx43t257 in the brain electroporated at E15 and examined at E17. (B) Distribution of GFP⁺ cells across the cerebral cortex from the VZ to the IZ in ACT2, ACT2+Cx43m257, and ACT2+Cx43t257 electroporated brain. * $P < 0.05$, ** $P < 0.01$, vs. control, $n = 6$. (C) The morphology of GFP⁺ cells in ACT2 and ACT2+m257, ACT2+t257 electroporated brain. (D) Gap junction channel domain plays an essential role for neuronal migration to the CP. The brains were electroporated at E15 and examined at E18. The position of GFP⁺ cells in ACT2, ACT2+Cx43, ACT2+Cx43-m257, and ACT2+Cx43t257 electroporated brain. (E) Distribution of GFP⁺ cells in the cerebral cortex in ACT2, ACT2+Cx43, ACT2+Cx43-m257, and ACT2+Cx43-t257 electroporated brain. ** $P < 0.01$, vs. ACT2, $n = 6$; ## $P < 0.01$, vs. ACT2+Cx43, $n = 6$; \$ $P < 0.05$, \$\$ $P < 0.01$, vs. ACT2+Cx43-m257, $n = 6$. (Scale bars: A, 50 μ m; C, 15 μ m; D, 80 μ m.)

to the CP (38). However, if adhesion is the only mechanism by which Cx43 promotes neuronal migration, the accumulated neurons in the IZ would display extensive processes, as demonstrated by functional disruption of DCX with siRNA (21). Instead, we observed that the retarded neurons display much shorter and thinner processes, suggesting that Cx43 plays additional roles during the multipolar stage. Indeed, we observed that the rescuing effect of Cx43 full length is much stronger than that of gap junction channel domain, suggesting an auxiliary role of C terminus (30), even though it is not clear whether the C terminus needs to anchor to the membrane. Because C terminus interacts with a variety of proteins related to the cytoskeleton system, such as zona occludens-1, V-Src, and tubulin (45–47), it would be interesting to identify whether these interactions are involved in the transformation of the multipolar neurons.

Apart from involvement in cell cycle progression, Cdk inhibitor p27 also promotes neuronal differentiation (by stabilizing Neurogenin2, carried by the N-terminal half) and neuronal migration (by blocking RhoA signaling, in the C-terminal half) (26). These multiple activities make p27 a candidate for the regulation of Cx43 during the multipolar stage. In harmony with a previous report (48), we found that gap junction/channel domain and C-terminal domain up-regulate the expression of p27. Extensive studies have revealed that gap junction channel increases the synthesis of p27 via intracellular cAMP mechanism, whereas C

terminus reduces the degradation of p27 via inhibition of skp2 (S phase kinase-associated protein 2), the human F-box protein that regulates the ubiquitination of p27 (48, 49). Considering the effect of p27 on neuronal differentiation and radial migration (22, 24, 26, 27), our findings support the concept that Cx43 serves as multifaceted regulator for the neuronal migration.

Because the dynamic motility of the multiple processes requires the rearrangement of the cytoskeletal system, the multipolar transformation would be a sensitivity stage for disruption of neuronal migration to the CP (11). For example, mutations in the human X-linked gene encoding filamin A cause periventricular nodular heterotopia, a malformation type that has been associated with epilepsy and other mental disorders (12, 13). In addition, mutations in the DCX gene are the most common cause of subcortical band heterotopia and double cortex syndrome (13). Multiple neurites, which developed transiently in the SVZ/IZ, may facilitate lateral displacement of radially migrating neurons to the adjacent radial glial fascicles (3). A recent study showed that this displacement depends on EphA/ephrin-A signaling and serves as a mechanism for proper intermixing of neuronal subtypes in the overlying radial columns (50). The present data suggest that this process may be further enhanced and stabilized by Cx43–p27 signaling, and that interference with this phase of neuronal migration can cause silent abnormality of neuronal composition of the functional cortical columns that are not detectable with routine morphological methods.

In summary, our results provide evidence for a unified mechanism in which the C terminus, adhesion, and channel of Cx43 play a coordinated role in cortical development, i.e., functional channels and C terminus participate in the neuronal translocation and morphological transformation in the SVZ/IZ via the p27 signaling, whereas gap junction stabilize the leading process and guide radial migration into the CP (29).

Experimental Procedures

Animal and Tissue Preparation. All experimental procedures were in accordance with the animal welfare guidelines of Yale University on the ethical use of animals. In this study, we used timed pregnant CD-1 mice (Charles River) at E14 and E15.

Plasmids and siRNA. The plasmid DNAs included the following: Cx43 full-length cDNA (pCMV-Cx43) purchased from Origene (MC205621). The pCDNA3-m257 was provided by S. M. Taffet (Upstate Medical University College of Medicine, Syracuse, NY), pCDNA3-t257 was provided by E. Scemes (Albert Einstein College of Medicine, New York, NY), and pCS4-Myc-p27 was provided by Yukiko Gogoh (University of Tokyo, Tokyo, Japan). All constructs were verified by DNA sequencing at Yale University by the W. M. Keck facility. The following siRNAs were used in this study: pSIREN-Retro-QZsGreen Cx43 siRNACT2 provided by E. Scemes (Albert Einstein College of Medicine, New York, NY). pSIREN p27 siRNA was provided by Y. Gogoh (University of Tokyo, Tokyo, Japan). The targeting sequence is: 5'-GTGG-AATTCGACTTTCAG-3'. The extent of Cx43 or P27 knockdown elicited by their siRNA was compared with that of scrambled control siRNAs.

In Utero Electroporation. In utero electroporation was performed as described (43). For more details, see *SI Experimental Procedures*.

Immunofluorescence. Immunofluorescence was performed as described (43). The antibodies used include the following: Antibodies for wild-type Cx43 (1:200; epitope spanning amino acids 363–382 located at the C-terminal region of Cx43; Invitrogen), C-terminally truncated Cx43 (1:50; epitope spanning amino acids 120–140; Invitrogen), N-terminally truncated Cx43 (1:50; epitope spanning amino acids 120–140; Abgent), and p27 (1:100, BP Pharmingen). For more detailed process, see *SI Experimental Procedures*.

N2a Cell Culture and Western Blot. N2a cells were recovered and cultivated in DMEM/F12 (1:1) supplemented with FCS and penicillin/streptomycin (1%; Invitrogen). Efficiency of Cx43 knockdown with siRNA was tested on exogenous Cx43 by transiently cotransfecting N2a cells with 1 μ g of pCMV-Cx43 and 1 μ g of siRNA using Lipofectamine 2000 (Invitrogen). After 30 h, proteins were extracted with RIA lysis buffer, denatured with SDS lysis

buffer, fractionated on a SDS/PAGE gel, and transferred to a nitrocellulose membrane nylon (HybondECL; Amersham Biosciences) for immunoblotting. Primary antibodies were rabbit anti-Cx43 (Invitrogen; 1:500), and secondary antibodies were goat anti-rabbit IgG (H+L) HRP conjugate (Jackson Labs; 1:5,000). Signal was revealed by using ECL Western blotting detection reagents according to the manufacturer's instructions (Amersham Biosciences).

Lucifer Yellow Uptake. Cultured N2a cells transfected with Cx43, Cx43-m245, and Cx43-t257 were transferred to the 24-well plates with oxygenized ACSF. Before LY uptake experiments, ACSF was replaced with 0 mM Ca ACSF containing 1 mg/mL LY and incubated at room temperature for 5 min, and then changed back to the normal ACSF and washed three times. The cells were then fixed in 4% PFA for fluorescence detection.

Intensity Measurements of GFP Across Neocortical Layers. NIH Image J software (<http://rsb.info.nih.gov/ij>) was used to quantify GFP fluorescent intensity of neocortical layers. For each image, the outlines of the VZ, the IZ,

and the CP or other analyzed regions were visualized by DAPI staining channel, and then a threshold was set to isolate GFP labeling to match the size and distribution of cells perceived by eye in the original grayscale image. The average pixel intensity from each layer or regions corresponding to the GFP channel was measured and subtracted from the background intensity. Six sections from at least three brains per condition were used, and the results were expressed as the percentage of total GFP intensity, for each condition, within each cortical region.

Statistical Analysis. Statistical analysis was performed by using the Student *t* test or ANOVA test as stated in the appropriate experiments, where *P* < 0.05 is considered significant. Error bars are the SEM.

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