Neonatal CX26 removal impairs neocortical development and leads to elevated anxiety

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Electrical coupling between excitatory neurons in the neocortex is developmentally regulated. It is initially prominent but eliminated at later developmental stages when chemical synapses emerge. However, it remains largely unclear whether early electrical coupling networks broadly contribute to neocortical circuit formation and animal behavior. Here, we report that neonatal electrical coupling between neocortical excitatory neurons is critical for proper neuronal development, synapse formation, and animal behavior. Conditional deletion of Connexin 26 (CX26) in the superficial layer excitatory neurons of the mouse neocortex around birth significantly reduces spontaneous firing activity and the frequency and size of spontaneous network oscillations at postnatal day 5–6. Moreover, CX26-conditional knockout (CX26-cKO) neurons tend to have simpler dendritic trees and lower spine density compared with wild-type neurons. Importantly, early, but not late, postnatal deletion of CX26 decreases the frequency of miniature excitatory postsynaptic currents (mEPSCs) in both young and adult mice, whereas miniature inhibitory postsynaptic currents (mIPSCs) were unaffected. Furthermore, CX26-cKO mice exhibit increased anxiety-related behavior. These results suggest that electrical coupling between excitatory neurons at early postnatal stages is a critical step for neocortical development and function.

CX26 | development | anxiety | neocortex

During neocortical development, gap junction-mediated electrical coupling plays a critical role in various developmental processes, including neuronal migration (1, 2), synaptogenesis (3), and synchronous firing (4, 5). It is generally believed that gap junction-mediated communication between excitatory neurons during development is required for the formation of chemical synapses (6). For example, we previously showed that sister excitatory neurons in the neocortex are initially electrically coupled, and blockade of this electronic communication impairs the subsequent formation of specific chemical synapses between them in ontogenetic neuronal clones (7), as well as the functional similarity (8). Whereas these studies have provided crucial insights into the role of gap junctions in the precise microcircuit assembly of excitatory neurons and functional organization of the neocortex, whether electrical coupling-mediated networks contribute to the overall development of the neocortex and animal behavior remains largely unknown.

Electrical coupling networks among excitatory neurons in the neocortex are developmentally regulated. Electrical couplings are the morphological correlates of dye coupling and low resistance intercellular pathways (9). Functional electrical coupling between excitatory neurons has been abundantly observed at late embryonic and early postnatal stages (10, 11). As chemical synapses approach the time point of their elimination, chemical connections between excitatory neurons begin to emerge, illustrating a sequential developmental time course for the two types of connections among excitatory neurons (6, 11).

Electrical coupling is mediated by the gap junction channel, formed by two sets of hexameric hemichannels, each composed of six connexin (CX) proteins. The gap junction channel directly connects the cytoplasm of two neighboring cells and allows the nonselective exchange of ions and small organic molecules (12). The types of connexins in a channel can influence channel properties such as conductance and selectivity (13). From a family of over 20 genes in mammals, only a handful of connexins have been found to be expressed in vertebrate neurons, including CX26, CX36, CX45, CX50, CX30.2, and CX31.1 (14). Converging evidence suggests that electrical coupling between the developing excitatory neurons in the neocortex is primarily mediated by CX26 proteins (1, 7, 15). However, whether CX26-mediated electrical coupling regulates the development and synapse formation of excitatory neurons in vivo remains unclear. In addition, although functional electrical coupling between excitatory neurons is largely eliminated after the first postnatal week, CX26 proteins remain abundantly present in excitatory neurons even at the mature stage (13, 16). Therefore, the precise role of CX26 proteins in excitatory neurons after the disappearance of electrical coupling is unclear. Moreover, whereas it has long been hypothesized that electrical couplings among excitatory neurons contribute to synchronized oscillatory activity during the developing neocortex, which may facilitate

Significance

It has long been postulated that the formation of electrically coupled neuronal domains guides the emergence of chemically transmitting neuronal connections in the developing neocortex. However, whether electrical coupling broadly contributes to in vivo neocortical circuit development and animal behavior remains largely unknown. Here, we demonstrate that specific deletion of Connexin 26 (CX26) in neocortical excitatory neurons around birth significantly reduces the frequency and size of spontaneous network oscillations. CX26 deletion at early, but not late, postnatal stages, impairs dendritic morphology and excitatory synapse development. Moreover, CX26-conditional knockout (CX26-cKO) mice exhibit increased anxiety-like behavior. Our data suggest that transient electrical coupling between excitatory neurons is critical for proper development and function of the neocortex.


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chemical synapse formation and neuronal circuit assembly (5, 17), this hypothesis has never been experimentally validated.

It has been proposed that transient electrical coupling at early postnatal stages critically influences the functional organization of the adult cortex (18). For example, disruption of gap junction coupling by viral expression of a dominant-negative mutant of CX26 or by daily administration of a gap junction blocker, carbamoyxolone, during the first postnatal week greatly diminished the orientation of sister excitatory neurons in the adult mouse visual cortex, suggesting that the ontogenetic-to-functional column maturation requires intercellular communication through gap junctions (8). Furthermore, the questions remain as to whether transient electrical coupling-mediated networks impact adult behavior.

In this study, we used an inducible conditional knockout (cKO) strategy to delete CX26 specifically in excitatory neurons of the mouse cortex at early embryonic day 18 to postnatal day 2 (E18–22) or late (P7–8) postnatal stages, and investigated whether transient electrical coupling regulates circuit formation in excitatory neurons and affects adult mouse behavior.

**Results**

**Conditional Deletion of CX26 in Excitatory Neurons at Early Postnatal Stages.** Previous studies including ours showed that CX26 is a major connexin isomorph that mediates electrical coupling between excitatory neurons in the developing neocortex (1, 7, 15). Moreover, previous study demonstrated CX26-mediated gap junction adhesion is necessary for radial migration in embryonic neocortex (1). To specifically remove CX26 from excitatory neurons of the cortex at early postnatal stages, we generated the inducible and conditional CX26 knockout mouse line (CX26-cKO mice, Emx1-CreER<sup>++</sup>; CX26<sup>fl/fl</sup>) by crossing the floxed CX26 allele-bearing mouse strain (CX26<sup>fl/fl</sup>) to a mouse line expressing the tamoxifen-inducible form of Cre recombinase (CreER) driven by the en-<sup>1.2%</sup><sup>-</sup> promoter (Emx1-CreER<sup>++</sup>) (Fig. 1 and Table S2 was conditionally de-

Towards spontaneous activity has been observed in the neocortex of P6 CX26-cKO mice was first examined by Western blot and found to be significantly reduced compared with that in CX26-WT littermates (Emx1-CreER<sup>++</sup>; CX26<sup>fl/fl</sup>) (Fig. 1 C and D and SI Appen-

**Synchronous spontaneous activity has been observed in the neocortex of P6 CX26-cKO mice was first examined by Western blot and found to be significantly reduced compared with that in CX26-WT littermates (Emx1-CreER<sup>++</sup>; CX26<sup>fl/fl</sup>) (Fig. 1 C and D and SI Appendix, Table S1). These data demonstrate that tamoxifen treatment around birth is able to effectively reduce endogenous CX26 protein expression in the neocortex at early postnatal stages. To specifically label the cells in which CX26 was conditionally deleted, we generated Emx1-CreER<sup>++</sup>; CX26<sup>fl/+</sup> Rosa26-ttdTomato (CX26-WT) mice and Emx1-CreER<sup>++</sup>; CX26<sup>fl/fl</sup>; Rosa26-ttdTomato littermates (CX26-cKO) (SI Appendix, Fig. S1A). This breeding strategy permits cells that undergo CX26 deletion to express tdTomato (SI Appendix, Fig. S1B). To further test the functional impairment of gap junction communication after CX26-cKO, we injected neurobiotin dye into tdTomato-positive (ttdTomato<sup>+</sup>) cells and then counted the number of neighboring dye-coupled cells at P4. We found that the average number of cells coupled to one ttdTomato<sup>+</sup> cell was significantly lower in CX26-cKO mice than in CX26-WT mice (Fig. 1 E–G and SI Appendix, Table S1), suggesting that deletion of CX26 impaired functional gap junction communication at early postnatal stages.

To analyze the distribution of cKO cells, we crossed the Emx1-CreER<sup>++</sup> line with a loxp-flanked Rosa26tdTomato (tdTomato) line (19). After tamoxifen treatment (as described above), the distribution of ttdTomato<sup>+</sup> cells was examined at P10, with a majority being found in the cortex as well as the hippocampus (SI Appendix, Fig. S2A). In this study, we focused on the cortex. We observed ttdTomato<sup>+</sup> cells mainly in the superficial layers (layer 2/3) of the neocortex (SI Appendix, Fig. S2 B and C). To characterize the ttdTomato<sup>+</sup> cell type, we performed immunohistochemical staining against neuronal nuclei (NeuN), a marker for neurons in the cortex, and γ-aminobutyric acid (GABA), a marker for inhibitory neurons (20). We found that almost all ttdTomato<sup>+</sup> cells expressed NeuN, but none expressed GABA (SI Appendix, Fig. S2 D and E), indicating that ttdTomato<sup>+</sup> cells are mainly glutamatergic excitatory neurons in the cortex. Moreover, ~5% of ttdTomato<sup>+</sup> cells were astrocytes (2.9 ± 1.2%, n = 3 mice), indicating that CX26 genes were removed in very few astrocytes by this knockout strategy. To determine the proportion of ttdTomato<sup>+</sup> excitatory neurons (ttdTomato<sup>+</sup>/NeuN<sup>+</sup> cells) among total excitatory neurons (NeuN<sup>+</sup>/GABA<sup>−</sup> cells), we systematically analyzed the fraction of ttdTomato<sup>+</sup>/NeuN<sup>+</sup> cells among NeuN<sup>+</sup>/GABA<sup>−</sup> cells in the cortex and found that ~50% of total excitatory neurons in layer 2/3 of the neocortex (NeuN<sup>+</sup>/GABA<sup>−</sup>) were labeled by ttdTomato (SI Appendix, Fig. S2F and Table S2). It is generally believed that the migration of excitatory neurons in the neocortex stops at P7 (21). We found that deletion of CX26 did not affect the laminar distribution of ttdTomato<sup>+</sup> CX26-cKO neurons compared with ttdTomato<sup>+</sup> CX26-WT neurons in the neocortex at P6 (SI Appendix, Fig. S1 B and C), indicating that deletion of CX26 around birth did not impair the migration of excitatory neurons.

**Deletion of CX26 Reduces Synchronous Network Oscillations at Early Postnatal Stages.** Synchronous spontaneous activity has been found throughout the developing neocortex and suggested to play a critical role in neocortical development (3, 5, 22). Thus, we speculated that gap junction-mediated electrical coupling between neocortical excitatory neurons at early postnatal stages promotes synchronous activity, which in turn facilitates neuronal development and synapse formation (Hebbian theory). To test this hypothesis, we examined spontaneous calcium activities and their synchronization in layer 2/3 neurons of the somatosensory cortex with large-scale calcium imaging (about 300 cells per movie on average) at P5–6. A highly sensitive fluorescent calcium indicator dye, Cal-520 acetylated methyl ester (Cal-520 AM) (23), was loaded into acute brain slices, and calcium transients in
response to action potentials (APs) were recorded at individual cell resolution (SI Appendix, Fig. S3 and Movie S1). Spontaneous activities and their synchronization were detected and analyzed with custom-made software (Materials and Methods and SI Appendix, Fig. S3), which automatically generated the raster plots of the activity and the histogram plots of the fraction of active cells (Fig. 2A and B). Active cells are neurons exhibiting at least one calcium transient within the period of recording (24). We analyzed the proportion of active cells, which was defined as the percentage of active cells among all calcium dye-loaded cells in one field, and the frequency of spontaneous activity of active cells, which was defined as the number of calcium transients per second, as previously reported (25). We found that the mean proportion of active cells in CX26-cKO mice was significantly lower than in CX26-WT mice (Fig. 2C and SI Appendix, Table S3). In addition, the mean frequency of spontaneous activity of active cells in CX26-cKO mice was significantly lower than that in CX26-WT mice (Fig. 2D and SI Appendix, Table S3). These data suggested that CX26-mediated electrical coupling between excitatory neurons promotes the spontaneous activity of neocortical cells at early postnatal stages.

We next analyzed spontaneous network oscillations at P5–6. The spontaneous network oscillations were defined as at least 10% of active cells having calcium transients within a 500-ms time window (Materials and Methods) (26, 27). We found that spontaneous network oscillations in CX26-WT brain slices occurred at ∼0.1 Hz frequency and the synchronicity duration was less than 200 ms (Fig. 2A, Lower). Interestingly, the proportion of cells involved in the spontaneous network oscillations (oscillating cells) among the total active cells in CX26-cKO mice was significantly lower than that in CX26-WT mice (Fig. 2E and SI Appendix, Table S3), indicating that deletion of CX26 reduces the size of network oscillations. In addition, the frequency of spontaneous network oscillations was significantly reduced in CX26-cKO mice compared with CX26-WT mice (Fig. 2F and SI Appendix, Table S3). Together, these results suggest that CX26-mediated electrical coupling between excitatory neurons strongly contributes to spontaneous neocortical network oscillations at early postnatal stages.

**Deletion of CX26 Affects Dendritic Morphology and Spine Density of Excitatory Neurons.** To investigate whether CX26 proteins play a role in dendritogenesis in excitatory neurons, we systematically analyzed the dendritic morphology of individual layer 2/3 tdTomato+ neurons (the distance from the cell body to the bottom of cortical layer 1 is ∼150 μm) in the somatosensory cortex of CX26-WT and CX26-cKO mice at P15–17 and P42–45 by intracellular filling with lucifer yellow (LY). The total dendritic length (TDL), the number of terminals, the number of bifurcations, and Sholl analysis of the dendritic branch intersections with concentric circles of increasing radii from the soma were quantified after 3D reconstruction of layer 2/3 tdTomato+ excitatory neurons in CX26-WT and CX26-cKO neocortex at P15 and P45. (Scale bars, 50 μm.) (B–D) Quantification of total dendritic length (TDL) (B), the number of dendritic terminals (C), and the number of bifurcations (D). (E and F) Sholl analysis of the number of dendritic intersections in P15–17 (E) and P42–45 (F) mice. (G) Confocal images showing dendritic spines of layer 2/3 excitatory neurons. (Scale bars, 2 μm.) (H and I) Quantitative analysis of average dendritic spine density (H) and spine length (I). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed unpaired t test.

**Fig. 3.** CX26 is required for normal dendritogenesis and spinogenesis in layer 2/3 excitatory neurons in young and adult mice. (A) Neuro lucida 3D reconstruction of layer 2/3 tdTomato+ excitatory neurons in CX26-WT and CX26-cKO neocortex at P15 and P45. (Scale bars, 50 μm.) (B–D) Quantification of total dendritic length (TDL) (B), the number of dendritic terminals (C), and the number of bifurcations (D). (E and F) Sholl analysis of the number of dendritic intersections in P15–17 (E) and P42–45 (F) mice. (G) Confocal images showing dendritic spines of layer 2/3 excitatory neurons. (Scale bars, 2 μm.) (H and I) Quantitative analysis of average dendritic spine density (H) and spine length (I). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed unpaired t test.
P15–17 and P42–45 (Fig. 3 E and F). Furthermore, the density of dendritic spines on the segment of the second branches (about 50 μm away from the cell body) of CX26-cKO neurons was significantly lower than that of CX26-WT neurons at P15–17 and P42–45 (Fig. 3 G and H and SI Appendix, Table S4), whereas the spine length exhibited no significant difference (Fig. 3 I and SI Appendix, Table S4). Together, these data suggest that CX26 proteins at early postnatal stages are crucial for normal dendritogenesis and synaptogenesis in neocortical excitatory neurons.

**Deletion of CX26 During the Period of Active Electrical Coupling Impairs Excitatory Synapse Formation.** To examine whether CX26 deletion affects synapse formation, we performed whole-cell patch-clamp recordings from layer 2/3 tdTomato+ neurons in the somatosensory cortex of CX26-WT and CX26-cKO mice at P15–16 (Fig. 4 B). We examined both miniature excitatory post-synaptic currents (mEPSCs, ~60 mV holding potential) and miniature inhibitory post-synaptic currents (mIPSCs, +10 mV holding potential) in the same neurons in the presence of tetrodotoxin (TTX, 5 μM) (28) (Fig. 4 C). We found that the frequency of mEPSCs of CX26-cKO neurons was significantly lower than that of CX26-WT neurons (Fig. 4 F and SI Appendix, Table S5), whereas the peak amplitude of mEPSCs was indistinguishable between CX26-WT and CX26-cKO neurons (Fig. 4 G and SI Appendix, Table S5). We did not observe any significant change in mIPSCs (Fig. 4 H and J and SI Appendix, Table S5). Notably, CX26-cKO neurons exhibited normal intrinsic electrophysiological properties at P15–16 and P42–45, including input resistance (Rinp), resting membrane potential (RMP), AP threshold, AP amplitude, and AP spiking width (SI Appendix, Fig. S4). To test whether deletion of CX26 affects synapse formation of WT neurons in CX26-cKO mice, we examined both mEPSCs and mIPSCs of tdTomato+ pyramidal cells in the vicinity of the tdTomato+ neurons from CX26-cKO mice (Fig. 4 D and E). We did not observe any obvious difference in the frequency or amplitude of mEPSCs and mIPSCs between tdtTomato+ neurons in CX26-cKO brain slices and tdtTomato+ neurons in CX26-WT brain slices (Fig. 4 F–I and SI Appendix, Table S5). These results suggest that CX26 deletion leads to cell-autonomous defects in excitatory synapse formation.

Because extensive synaptic and dendritic pruning occurs even after P15–16 (29), we further examined mEPSCs/mIPSCs in CX26-WT and CX26-cKO neurons at P42–45 (Fig. 4 A, Upper and SI Appendix, Fig. S5.A). We found that both the frequency and the peak amplitude of mEPSCs were significantly lower in CX26-cKO neurons than in CX26-WT neurons at P42–45 (Fig. 4 J and K and SI Appendix, Table S5). Moreover, there was not any significant change in mIPSCs at P42–45 (Fig. 4 J and K and SI Appendix, Table S5). These results suggest that CX26 deletion at early postnatal stages impaired excitatory synaptic transmission in both young and adult mice.

Although functional electrical coupling between excitatory neurons was only detected from the late embryonic stages to the first postnatal week (7, 10, 11), accumulated evidence indicates that CX26 proteins are present in excitatory neurons throughout neocortical development (13, 15, 16). To determine whether CX26 proteins in excitatory neurons, after the period of electrical coupling, modulate synaptic transmission, we deleted CX26 from excitatory neurons after the first postnatal week (late cKO, Fig. 4 A). Tamoxifen was administered to mice during P7–8 (Fig. 4 A). Similar to the results from tamoxifen administration during E18.5–P2 (early cKO), almost all tdTomato+ cells expressed NeuN but not GABA (SI Appendix, Fig. S6), indicating that genetic deletion predominantly occurred in excitatory neurons. We recorded mEPSCs and mIPSCs of tdTomato+ neurons in neocortical layer 2/3 of CX26-WT and CX26 late-cKO mice at P15–16 (SI Appendix, Fig. S5B). Interestingly, CX26 deletion after P7 did not affect the frequency or amplitude of mEPSCs and mIPSCs (Fig. 4 L and M and SI Appendix, Table S5). These results suggest that deletion of CX26 after the first postnatal week—the period of active electrical coupling—does not affect synaptic transmission of excitatory neurons.

**Neocortical Deletion of CX26 Leads to Increased Anxiety-Like Behavior.** Our results showed that CX26-mediated gap junction regulates dendrite development and synapse formation in layer 2/3 excitatory neurons. We next examined whether CX26 deletion at early postnatal stages affects adult mouse behavior. No obvious difference in animal growth was observed between the WT and cKO mice, as reflected by the body weight of P40–50 mice (SI Appendix, Fig. S7). We evaluated the anxiety-like behavior in the open field test (OF) and the elevated plus maze test (EPM) (30). In the OF test, mice were exposed to a flat arena during 15 min of spontaneous movement, and the movement path was simultaneously tracked (Fig. 5 A). We found that CX26-WT and CX26-cKO mice showed no significant difference in the total distance traveled in the field (Fig. 5 B and SI Appendix, Table S6), indicating CX26 deletion did not impair locomotor activity of mice. However, the distance (Fig. 5 C and...
and mice to deletion significantly reduced the frequency of spontaneous calcium activity of excitatory neurons during P5–6. This result is consistent with the previous studies showing that gap junction and hemichannel inhibitors significantly block spontaneous activity in the rodent neonatal neocortex (3) and the human fetal neocortex (33). Given the critical roles of spontaneous calcium activity in the formation and reinforcement of neocortical synapses at the early postnatal days (34), we speculated that CX26 cKO-induced reduction of spontaneous activity affects neocortical development.

More importantly, we found that deletion of CX26 significantly reduced the frequency and size of spontaneous network oscillations. Two distinct network oscillations, cortical early network oscillations (cENO) and cortical giant depolarizing potentials (cGDP), were observed in the developing rat cortex (24). The cENOs peak around birth (P0–3) and are no longer present when cGDPs dominate the network (P6–P8) (24), although there is a transition period (P4–5) during which both patterns can be simultaneously recorded in the rat neocortex (24). We found that the network oscillations in the superficial neocortical layers of mice during P5–6 display two features: (i) the frequency is ~0.1 Hz, and (ii) the synchronicity duration is less than 200 ms. These data suggest that the network oscillations in the superficial neocortical layers of mice during P5–6 are mainly cGDPs (24). cGDPs appear to be strongly dependent on the actions of GABA as they are almost completely blocked by the GABA_A receptor antagonist (24). Our study indicates that, besides GABAergic transmission, CX26-mediated electrical coupling between excitatory neurons contributes to the expression of cGDPs at early postnatal stages.

To explicitly examine CX26-cKO versus CX26-WT neurons, we engineered them to express tdTomato fluorescent protein. We found that tdTomato+ neurons in CX26-cKO mice exhibit normal intrinsic electrophysiological properties at both P15–16 and P42–45 (SI Appendix, Fig. S4). Their distribution pattern in the neocortex is similar to that of tdTomato+ neurons in CX26-WT mice. These data suggest that deletion of CX26 at birth does not affect intrinsic electrophysiological maturation and neocortical excitatory neuron migration. However, deletion of CX26 significantly reduces the complexity of dendritic arbor and spine density in both young and adult mice, indicating that CX26-mediated gap junction between excitatory neurons at the early postnatal stage is required for normal dendritogenesis and spino genesis in excitatory neurons. Furthermore, CX26-cKO neurons exhibit a significant decrease in the frequency of mEPSCs at P15–16 and in both the frequency and the peak amplitude of mEPSCs at P42–45, suggesting that deletion of CX26 at early postnatal stages can prevent, but not delay, excitatory synapse formation. Notably, nearby tdTomato+ excitatory neurons (tdTomato+) in CX26-cKO mice show normal synaptic transmission. These data suggest that deletion of CX26 largely impairs the postsynaptic development (i.e., receiving excitatory synaptic inputs) but not presynaptic activity (i.e., sending excitatory synaptic outputs). Interestingly, removal of CX26 after the first postnatal week did not affect synapse formation in neocortical excitatory neurons (Fig. 4 L and M). Together, these findings provide clear evidence of the crucial role of electrical transmission between excitatory neurons at early postnatal stages in regulating neuronal development and synapse formation in the neocortex.

To further explore the role of global electrical coupling in the functional development of the neocortex, we examined potential behavioral changes associated with the observed dendritic and synaptic alterations in CX26-cKO mice. Interestingly, we found that CX26-cKO mice exhibit an increased level of anxiety-like behaviors at early postnatal stages in depression-like behavioral tests, including the tail suspension test (SI Appendix, Fig. S8B); the forced swimming test (SI Appendix, Fig. S8A); learning and memory-associated behavioral tests, including Morris water maze test (SI Appendix, Fig. S9 A–D), Y maze test (SI Appendix, Fig. S9 E and F), and fear conditioning test (SI Appendix, Fig. S9 G and H); and motor coordination-associated rotarod test (SI Appendix, Fig. S10).

Discussion

In this study, we took advantage of the Emx1-CreER mouse to specifically delete the CX26 gene—a major connexin gene expressed in neocortical excitatory neurons (1, 7, 15)—starting at E18.5. To avoid impairing radial migration of excitatory neurons at embryonic stages (1), we deleted CX26 around birth. Emx1 is selectively expressed in glutamatergic excitatory neurons during late embryonic and early postnatal stages, including the neocortex and hippocampus (31). Indeed, by crossing the Emx1-CreER mice with Ai14 tdTomato-reporter mice, we found that the majority of neocortical tdTomato+ neurons were excitatory neurons, mainly located in the superficial cortical layers. Previous dye-injection experiments in the guinea pig neocortical slices suggested that electrical coupling during the early postnatal stage is mainly observed in the superficial layers (32). Therefore, our conditional knockout strategy provided a valuable approach to investigate the function of global electrical coupling on neuronal development, synapse formation, and animal behavior. Interestingly, we observed that CX26 deletion significantly reduced the frequency of spontaneous calcium activity of excitatory neurons during P5–6. This result is consistent with the previous studies showing that gap junction and hemichannel inhibitors significantly block spontaneous activity in the rodent neonatal neocortex (3) and the human fetal neocortex (33). Given the critical roles of spontaneous calcium activity in the formation and reinforcement of neocortical synapses at the early postnatal days (34), we speculated that CX26 cKO-induced reduction of spontaneous activity affects neocortical development.

Fig. 5. CX26-cKO induces anxiety-like behavior. (A) Schematic of zones in the open field arena and representative traces of mouse movement. (B) Total distance traveled in the open field test. (C and D) Distance traveled in the center (C) and corners (D) of the open field arena. (E and F) Time spent in the center (E) and corners (F) in the open field arena. (G) Schematic of different arms and representative traces of mouse movement in the elevated plus maze test. (H) Time spent in the open arms. (I) Entrances into the open arms during the elevated plus maze test. Data are presented as mean ± SEM. n.s. for P > 0.05, **P < 0.01, ***P < 0.001, two-tailed unpaired t test.
behavior. It is still unclear how CX26 cKO-induced defects in neocortical development result in anxiety-like behavior. Notably, in these animals, neurons with defective dendritic morphology and synapse development are largely restricted in the superficial layer 2/3. Should CX26 be deleted in all excitatory neurons in the neocortex, we would expect more behavior deficits. Moreover, although our anatomical and functional studies focus on the neocortex, it is possible that certain behavior defects may relate to the hippocampus, as in these animals CX26 is deleted in both the neocortex and hippocampus. The fact that CX26 deficiency triggers synaptic defects and cognitive dysfunction makes it an attractive candidate for further exploration in the context of neurodevelopmental disorders.

Although our study clearly demonstrates the importance of early electrical coupling in regulating neuronal development and synapse formation, the precise mechanisms underlying this regulation remain largely unknown. A variety of mechanisms could account for this regulation. For example, gap junction channels allow the permeation of inorganic ions (Na+, Ca2+, K+, etc.) and small molecules (cAMP and IP3, etc.) (12, 14). These chemical species have been implicated as second messengers and play a role in synapse formation among neocortical excitatory neurons (35). Another potential cellular mechanism could be that electrical coupling in early postnatal stages facilitates synchronized activity, which in turn promotes glutamatergic synapse formation between neocortical excitatory neurons (Hebbian theory). Indeed, we observed that deletion of CX26 in excitatory neurons around birth significantly reduces the frequency and size of network oscillations (Fig. 3 C and D) and subsequently the frequency of mEPSCs of neocortical excitatory neurons (Fig. 5 F). Future efforts along these lines will likely provide insights into the mechanistic link underlying how electrical coupling leads to structural plasticity and changes in synaptic connectivity in the developing neocortex.

Materials and Methods
Animals were maintained and handled followed the guidelines approved by Fudan University Shanghai Medical College. Tamoxifen was intraperitoneally administrated to E18.5 pregnant mice (0.025 mg/g body weight), and s.c. delivered to postnatal pups (0.05 mg/g body weight) by a 10-µL syringe. Detailed information about immunostaining, Western blotting, calcium imaging, electrophysiology, morphological analysis, and behavioral tests is included in SI Appendix. Data are presented as mean ± SEM and n refers to the number of independent experiments. Significant differences are detected when *P < 0.05, **P < 0.01, and ***P < 0.001.

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