

# Netrin-1- $\alpha3\beta1$ integrin interactions regulate the migration of interneurons through the cortical marginal zone

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**Cortical GABAergic interneurons, most of which originate in the ganglionic eminences, take distinct tangential migratory trajectories into the developing cerebral cortex. However, the ligand-receptor systems that modulate the tangential migration of distinct groups of interneurons into the emerging cerebral wall remain unclear. Here, we show that netrin-1, a diffusible guidance cue expressed along the migratory routes traversed by GABAergic interneurons, interacts with  $\alpha3\beta1$  integrin to promote interneuronal migration. In vivo analysis of interneuron-specific  $\alpha3\beta1$  integrin, netrin-1-deficient mice ( $\alpha3^{\text{lox/-}}$ -Dlx5/6-CIE, *netrin-1*<sup>-/-</sup>) reveals specific deficits in the patterns of interneuronal migration along the top of the developing cortical plate, resulting in aberrant interneuronal positioning throughout the cerebral cortex and hippocampus of conditional  $\alpha3^{\text{lox/-}}$ -Dlx5/6-CIE, *netrin-1*<sup>-/-</sup> mice. These results indicate that specific guidance mechanisms, such as netrin-1- $\alpha3\beta1$  integrin interactions, modulate distinct routes of interneuronal migration and the consequent positioning of groups of cortical interneurons in the developing cerebral cortex.**

The development of the six-layered cerebral cortex, composed predominantly of glutamatergic projection neurons and GABAergic interneurons, depends on the appropriate migration of neurons from ventricular zones of the dorsal and ventral telencephalon to their final position in the emerging cortical plate. Neuronal progenitors from the dorsal telencephalon migrate radially as cohorts along the radial glial scaffold, pass their predecessors in the developing cortical plate, and coalesce into layers, giving rise to glutamatergic projection neurons (1). In contrast, neuronal precursors from the ventral telencephalon, principally the medial ganglionic eminence (MGE), migrate tangentially through the cortical marginal zone and intermediate zone and enter the cortical plate, using the radial glial scaffold, before differentiating into GABAergic interneurons (2–4). A subset of these interneurons initially migrates radially inwards, toward the cortical ventricular zone, before turning back up toward the cortical plate (4). These different modes of neuronal migration are coordinated temporally to achieve the “inside-out” laminar organization of neurons in the neocortex.

Specific cell adhesion interactions between neurons, glia, and the surrounding ECM are critical for the appropriate migration and placement of cortical neurons. These adhesive interactions are mediated in large part by integrins, heterodimeric cell-surface ECM receptors, which serve as structural links between extracellular ligands and the internal cytoskeleton. The functional significance of integrin signaling for cerebral cortical development is demonstrated by the distinct types of cortical malformations exhibited by mice deficient in  $\alpha_v$ ,  $\alpha_3$ ,  $\alpha_6$ ,  $\beta_1$ , and  $\beta_4$  integrins (1, 5–8). However, the selective adhesive interactions needed to coordinate the migration of distinct groups of interneurons and projection neurons into the developing cerebral cortex remain poorly understood.

The prominent expression of netrin-1 in the ganglionic eminence (GE), where interneurons originate (9), as well as the expression of  $\alpha3\beta1$  integrin in migrating cortical neurons (5), suggests that netrin-1- $\alpha3\beta1$  integrin interactions may influence specific aspects of the tangential migration of GABAergic interneurons. Recent evidence that  $\alpha3\beta1$  integrin serves as a receptor for netrin-1 in epithelial cells and evidence for cross-talk between integrins and classical netrin receptors have opened an avenue for investigating this possibility (10). Netrins serve as secreted guidance cues, regulating axonal outgrowth and neuronal migration by binding to Deleted in Colon Cancer (DCC), Down's Syndrome Cell Adhesion Molecule (DSCAM), or UNC5 receptors (11, 12). Netrin-1 interacts with DCC and DSCAM to induce growth cone attraction (12, 13), whereas its association with UNC5 receptors promotes axonal repulsion (14). Recently, Yebrá and colleagues (10) have demonstrated that  $\alpha6\beta4$  and  $\alpha3\beta1$  integrins regulate the migration of epithelial cells on netrin-1 through a direct interaction of these integrins with the C-terminal domain of netrin-1, although the significance of these interactions is yet to be determined in vivo. Here, we have investigated the contribution of netrin-1- $\alpha3\beta1$  integrin-mediated signal transduction to the migration of GABAergic interneurons from the MGE into the developing cerebral wall.

## Results

**Netrin-1 Is Localized Along the Migratory Route of Cortical GABAergic Interneurons.** To evaluate the potential involvement of netrin-1- $\alpha3\beta1$  integrin signaling in interneuronal migration, we mapped the expression pattern of netrin-1 and  $\alpha3\beta1$  integrin in embryonic cortex during interneuronal migration (Fig. 1 and Fig. S1). Sections from netrin-1 gene trap indicator mice (Netrin-1<sup>LacZ/+</sup>), in which  $\beta$ -galactosidase expression faithfully indicates endogenous netrin-1 gene expression (15), were co-labeled with  $\beta$ -galactosidase and  $\alpha3$  integrin antibodies. Prominent netrin-1-LacZ expression is evident in the ventricular zone of the GE and in the marginal zone of the developing cortex (Fig. 1B, E, and H).  $\alpha3$  integrin is present in radially and tangentially oriented neurons in the cerebral wall (Fig. 1A, J, and L and Fig. S1). Both  $\alpha3$  integrin and netrin-1 are prominently co-expressed in the marginal zone region (Fig. 1C, F, and I).

Although LacZ expression is indicative of netrin-1 gene expression patterns, it may not reveal fully the localization of secreted netrin-1 in the developing cortex. To examine the expression pattern

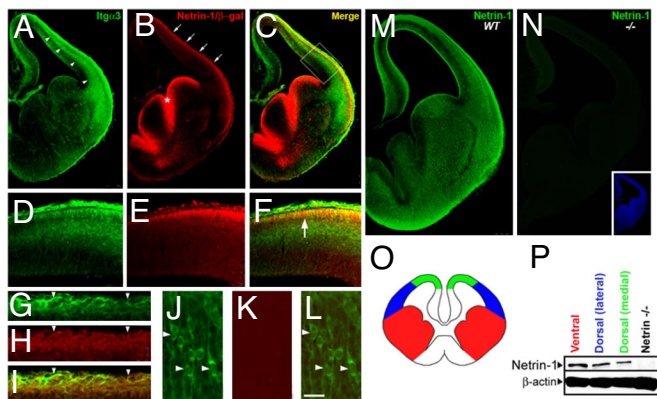
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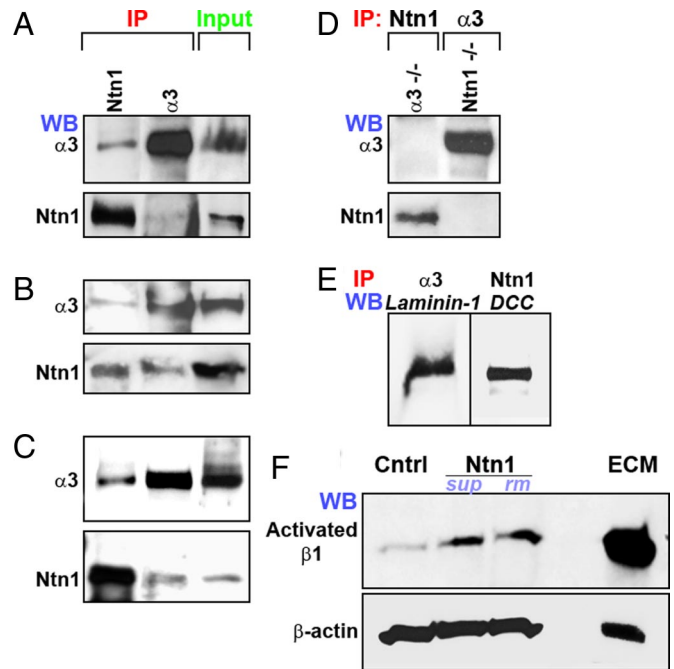
This article contains supporting information online at [www.pnas.org/cgi/content/full/0811343106/DCSupplemental](http://www.pnas.org/cgi/content/full/0811343106/DCSupplemental).



**Fig. 1.** Expression of netrin-1 and  $\alpha 3\beta 1$  integrin in the developing cortex. (A–C) Coronal sections of the forebrain at E13.5 labeled with  $\alpha 3$  integrin (A) and  $\beta$ -galactosidase (B) antibodies demonstrate that tangentially migrating interneurons express  $\alpha 3\beta 1$  integrin (arrowheads in A) and indicate that netrin-1 is present at the cortical marginal zone (arrows in B) and ventricular zone of the GE (asterisk in B). C is a merge of A and B. (D–F) High magnification of the developing cerebral wall boxed in C shows colocalization (arrow in F) of netrin-1 (E) and  $\alpha 3\beta 1$  integrin (D) at the marginal zone. (G–I) Tangentially migrating interneurons at the marginal zone express  $\alpha 3\beta 1$  integrin (arrowheads in G and I) and netrin-1 (arrowheads in H and I). (J–L) Radially migrating neurons in the intermediate zone express  $\alpha 3$  integrin (arrowheads in J and L), but not netrin-1 (K and arrowheads in L). (M) Coronal section of E13.5 cortex labeled with netrin-1 antibodies demonstrates that secreted netrin-1 is expressed in the developing forebrain during tangential neuronal migration. (N) Netrin-1 expression was not detected in the forebrain of E13.5 netrin-1-null mice by immunohistochemistry. (O–P) Immunoblot of netrin-1 in the GE, lateral pallium, and dorsal pallium of E14.5 forebrain (P) indicate that netrin-1 is expressed in each of these regions (schematized in O). (Scale bar, 15  $\mu$ m).

of functional, secreted netrin-1 in the developing telencephalon during tangential migration, embryonic day 13.5 (E13.5) cortices were immunolabeled with netrin-1 antibodies. Expression of secreted protein was detected in marginal zone and intermediate zone regions traversed by tangentially migrating interneurons (Fig. 1M). This immunolocalization pattern was not detected in netrin-1-null mice (Fig. 1N and P). The presence of netrin-1 protein in the ventral and dorsal telencephalon was verified further by immunoblotting performed on microdissected portions of the telencephalon (Fig. 1P). Together, these results reveal  $\alpha 3\beta 1$  integrin expression in interneurons and the presence of netrin-1 in the migratory route of these interneurons in the developing cerebral cortex.

**Netrin-1- $\alpha 3$  Integrin Interactions.** To determine if  $\alpha 3$  integrin and netrin-1 interact with each other in the developing cerebral cortex, we first performed co-immunoprecipitations of netrin-1 and  $\alpha 3$  integrin. Immunoprecipitation of E13.5 forebrain lysates with anti-netrin-1 antibodies and immunoblotting with anti- $\alpha 3$  integrin antibodies or immunoprecipitation of lysates with anti- $\alpha 3$  integrin antibodies and immunoblotting with anti-netrin-1 antibodies demonstrates that  $\alpha 3$  integrin co-immunoprecipitates with netrin-1 in vivo (Fig. 2A). Previously known ligands for  $\alpha 3$  integrin (i.e., laminin-1) or other known receptors for netrin-1 (i.e., DCC) were co-immunoprecipitated in these assays and served as positive controls (Fig. 2E). Netrin-1 or  $\alpha 3$  integrin was not co-immunoprecipitated when  $\alpha 3$  integrin-deficient or netrin-1-deficient forebrain extracts were used to co-immunoprecipitate netrin-1 or  $\alpha 3$  integrin, respectively (Fig. 2D). To verify further the interaction between  $\alpha 3$  integrin and netrin-1, we performed co-immunoprecipitations of netrin-1 and  $\alpha 3$  integrin using netrin-1-expressing 293 cells transfected with  $\alpha 3$  integrin. Immunoprecipitation of these cell lysates with anti-netrin-1 antibodies and immunoblotting with anti- $\alpha 3$  integrin antibodies or immunoprecipitation of lysates with anti- $\alpha 3$  integrin antibodies and immuno-



**Fig. 2.** Netrin-1- $\alpha 3\beta 1$  integrin interactions. To verify netrin-1- $\alpha 3\beta 1$  integrin interactions, E13.5 forebrain extracts (A), netrin-1-expressing 293 cell lysates (B), or a mixture of purified netrin-1 (ntn1) and  $\alpha 3\beta 1$  integrin proteins (C) were immunoprecipitated (IP) with  $\alpha 3$  integrin or netrin-1 antibodies and Western blotted (WB) with antibodies to  $\alpha 3$  integrin or netrin-1. (A–C) In each assay, netrin-1 co-immunoprecipitated with  $\alpha 3$  integrin and vice versa. (D) Netrin-1 or  $\alpha 3$  integrin was not detected when  $\alpha 3$  integrin-deficient or netrin-1-deficient forebrain extracts were used to co-immunoprecipitate netrin-1 or  $\alpha 3$  integrin. (E) The co-immunoprecipitation of  $\alpha 3$  integrin and laminin-1, a known  $\alpha 3$  integrin ligand, and of netrin-1 and DCC, a known netrin-1 receptor, from E14.5 forebrain lysates serves as positive controls. (F) Robust  $\beta 1$  integrin receptor activation is observed following netrin-1 supernatant (sup), recombinant mouse netrin-1 (rm), and ECM stimulation of MGE neurons.

blotting with anti-netrin-1 antibodies shows that  $\alpha 3$  integrin co-immunoprecipitates with netrin-1 in vitro (Fig. 2B). To establish whether netrin-1 and  $\alpha 3\beta 1$  integrin associate directly, netrin-1-containing supernatants from stably transfected 293 cells were incubated with purified  $\alpha 3\beta 1$  integrin protein. Netrin-1 or  $\alpha 3$  integrin bound to each other was analyzed following immunoprecipitation with either anti- $\alpha 3$  integrin or netrin-1 antibodies, respectively.  $\alpha 3$  integrin co-immunoprecipitates with netrin-1 in these assays, thus further indicating a direct association between  $\alpha 3$  integrin and netrin-1 (Fig. 2C).

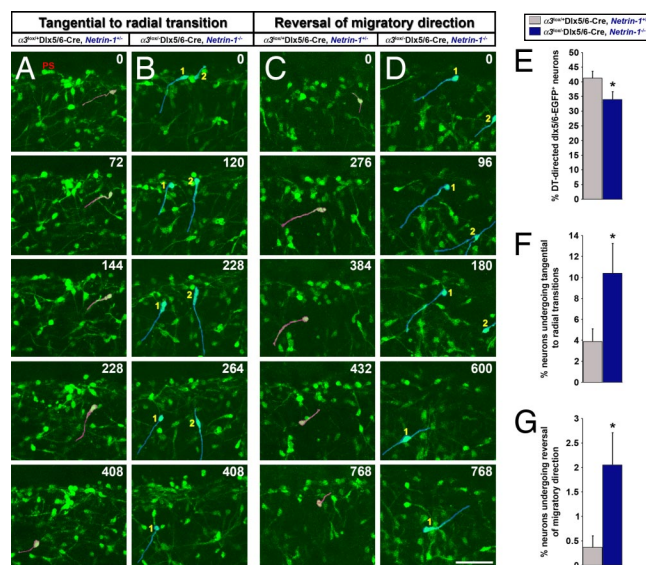
**Netrin-1 Activates  $\beta 1$  Integrin Receptors.** To establish whether the physical interaction between netrin-1 and  $\alpha 3\beta 1$  integrin has functional relevance, we examined the effects of netrin-1 on  $\beta 1$  integrin receptor activation. E14.5 MGE cells were incubated with netrin-1-containing supernatant, recombinant mouse netrin-1 (50 ng/ml), medium alone, or ECM (10 mg/ml). The medium-alone condition serves as a negative control, and ECM stimulation functions as a positive control for  $\beta 1$  integrin receptor activation. The activation of  $\beta 1$  integrin in netrin-1-treated MGE cells was assessed by application of a  $\beta 1$  integrin antibody (9EG7), which specifically recognizes its active conformation. Supernatants containing secreted netrin-1 or mouse recombinant netrin-1 markedly increased  $\beta 1$  integrin receptor activation in MGE cells when compared with controls (Fig. 2F), thus demonstrating a functional, physiologically relevant interaction between netrin-1 and  $\alpha 3\beta 1$  integrin.

**Disruption of Netrin-1- $\alpha 3\beta 1$  Integrin Interactions Reduces the Number of Interneurons Migrating Through the Cortical Marginal Zone.** In vitro observations suggest that netrin-1-modulated interneuro-



nal migration from the MGE involves  $\alpha 3$  integrin function (see Fig. S2 and *SI Text*). To evaluate the significance of netrin-1- $\alpha 3$  integrin interactions during interneuronal migration in vivo, we generated interneuron-specific  $\alpha 3$  integrin-deficient mice in a netrin-1-null background. *Dlx5/6-Cre-IRES-EGFP* (*Dlx5/6-CIE*) mice, in which interneurons generated from the ganglionic eminence express Cre and EGFP, were used to inactivate  $\alpha 3$  integrin in interneurons. Generation of mice with a floxed and null *Itga3* allele ( $\alpha 3^{lox/-}$ ) in a netrin-1-null background, which also contain the *Dlx5/6-Cre-IRES-EGFP* transgene, enables the inactivation of netrin-1- $\alpha 3$  integrin interactions in interneurons. *Dlx5/6-Cre* induces recombination throughout the GE and ventral telencephalon from E12.5 onwards (16), and loss of  $\alpha 3$  integrin is evident in the interneurons of  $\alpha 3^{lox/-}$ -*Dlx5/6-CIE* mice (Fig. S1). We compared the patterns of tangential interneuronal migration in double-mutant mice with  $\alpha 3$  integrin ( $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>+/+</sup>) or netrin-1 ( $\alpha 3^{+/+}$ -*Dlx5/6-CIE*, *netrin-1*<sup>-/-</sup>) single-mutant mice and in control ( $\alpha 3^{lox/+}$ -*Dlx5/6-CIE*, *netrin-1*<sup>+/+</sup>) mice. Migration patterns were evaluated first at E13.5, during early stages of cortical tangential migration, immediately following *Dlx5/6-Cre*-mediated inactivation of  $\alpha 3$  integrin. We determined the extent of interneuronal migration in the dorsal telencephalon. Because tangential migration through the marginal zone precedes migration through the intermediate zone or subventricular zone, we measured the extent of migration as the distance from the corticostriatal junction to the tip of the leading process of the foremost tangentially migrating neuron in the cortical marginal zone.  $\alpha 3$  integrin ( $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>+/+</sup>) and netrin-1 ( $\alpha 3^{+/+}$ -*Dlx5/6-Cre*, *netrin-1*<sup>-/-</sup>) single mutants as well as double-mutant ( $\alpha 3^{lox/-}$ -*Dlx5/6-Cre*, *netrin-1*<sup>-/-</sup>) mice displayed an 8% (SD  $\pm 1.6$ ), 9% (SD  $\pm 1.7$ ), and 10% (SD  $\pm 1.3$ ) reduction in the extent of migration through the marginal zone, respectively, compared with controls (Fig. S3 A-E). Further, binning analysis of interneurons across the developing cerebral wall (i.e., from pial to ventricular surface) indicates a 50% (SD  $\pm 0.02$ ) reduction in interneurons in the marginal zone of double mutants (Fig. S3 A-D and F-I). We also examined the number of interneurons migrating into the cortex by dividing the dorsal telencephalon, from the corticostriatal junction to the top of the dorsal telencephalon, into 10 equal bins and quantifying interneurons in each of these bins. A significant decrease in the number of EGFP<sup>+</sup> interneurons migrating into the dorsal telencephalon in single- and double-mutant mice was evident (data not shown). These results suggest that netrin-1 and  $\alpha 3\beta 1$  integrin may regulate very early stages of tangential neuronal migration through the cortical marginal zone.

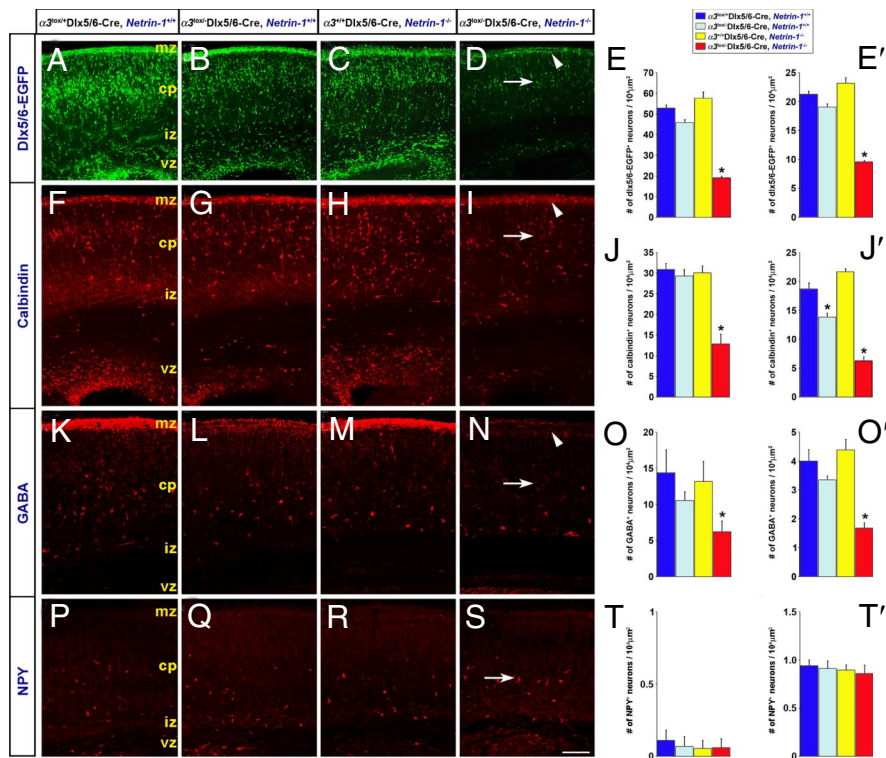
To explore further the relevance of netrin-1- $\alpha 3$  integrin interactions to cortical tangential migration, we examined migration patterns at E16.5, at the height of interneuronal migration into the developing cerebral wall. Quantification of interneuron distribution within distinct compartments of the cerebral wall indicates that, compared with control mice,  $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>-/-</sup> double-mutant mice exhibit a substantial reduction in EGFP<sup>+</sup> interneurons migrating through the marginal zone region of the cortical plate (control, 27% [SD  $\pm 1.06$ ];  $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>-/-</sup>, 19% [SD  $\pm 0.52$ ]) (Fig. S4 A and C). Correspondingly, double mutants display an increased number of EGFP<sup>+</sup> cells ectopically migrating through the ventricular zone (control, 28% [SD  $\pm 1.04$ ];  $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>-/-</sup>, 37% [SD  $\pm 0.47$ ]) (Fig. S4 B and D). Both  $\alpha 3$  integrin and netrin-1 single-mutant mice exhibited an increase in EGFP<sup>+</sup> interneurons ectopically placed in the ventricular zone (control, 28% [SD  $\pm 1.04$ ];  $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>+/+</sup>, 32% [SD  $\pm 0.58$ ];  $\alpha 3^{+/+}$ -*Dlx5/6-CIE*, *netrin-1*<sup>-/-</sup>, 34% [SD  $\pm 0.91$ ]) (Fig. S4 B and D). The ratio of interneurons in the marginal zone to those in the ventricular zone indicates the significance of this misplacement and reveals that a substantial number of interneurons in netrin-1- $\alpha 3$  integrin-deficient mice are misrouted (Fig. S4E). These data suggest that netrin-1- $\alpha 3$  integrin interactions may



**Fig. 3.** Real-time assessment of interneuronal migration patterns through the cortical marginal zone reveals abnormal migration in  $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>-/-</sup> double mutants. (A-D) Live imaging of E15.5 cortices reveals an increase in the number of interneurons transitioning from tangential migration along the marginal zone to ventricular surface-directed radial migration in  $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>-/-</sup> double mutants (B, cells highlighted in blue) compared with  $\alpha 3^{lox/+}$ -*Dlx5/6-CIE*, *netrin-1*<sup>+/+</sup> controls (A, cells highlighted in red). The number of interneurons reversing their tangential migratory orientations in the marginal zone region is increased in  $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>-/-</sup> double-mutant cortices (D, cells highlighted in blue) compared with controls (C, cells highlighted in red). (E) Quantification of the number of interneurons migrating toward the dorsal telencephalon in controls and double-mutant mice. (F-G) Quantification of the number of interneurons transitioning from tangential to radial migration (F) and reversing tangential trajectories (G) through the marginal zone region. Time elapsed since the onset of observations is indicated in minutes. (Scale bar, 50  $\mu$ m.) Data shown are mean  $\pm$  SEM ( $n = 5$ ); \*, significant when compared with controls at  $P < 0.05$  (Student's *t* test). ps, pial surface.

modulate distinct patterns of interneuronal migration within the developing cerebral cortex, especially the interneuronal migration through the marginal zone region of the developing cerebral cortex.

**Real-Time Assessment of Interneuronal Migration Patterns Through the Cortical Marginal Zone Reveals Abnormal Migration in  $\alpha 3^{lox/-}$ -*Dlx5/6-Cre*, *Netrin-1*<sup>-/-</sup> Double Mutants.** To characterize further these altered patterns of interneuronal migration in vivo, real-time imaging of interneuron migration was performed on control and double-mutant E15.5 embryonic cortices. In the cerebral wall, the leading processes of migrating interneurons are oriented toward the direction of their movement. We found a significant decrease in the number of interneurons migrating toward the dorsal cortex, away from the GE, in  $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>-/-</sup> double mutants when compared with  $\alpha 3^{lox/+}$ -*Dlx5/6-CIE*, *netrin-1*<sup>+/+</sup> controls (control, 41% [SD  $\pm 2.3$ ];  $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>-/-</sup>, 34% [SD  $\pm 2.7$ ]) (Fig. 3E and Movies S1 and S2). Furthermore, compared with controls, a 2.7-fold increase in the number of interneurons switching from tangentially oriented migration along the marginal zone to ventricular surface-directed radial migration is evident in double-mutant mice (Fig. 3A, B, and F and Movies S3 and S4). This increase in ventricular surface-directed radial migration at the cortical marginal zone may underlie the reduction of interneurons at the marginal zone of  $\alpha 3^{lox/-}$ -*Dlx5/6-CIE*, *netrin-1*<sup>-/-</sup> double mutants. Last, double-mutant mice also displayed a 5.6-fold increase in the number of interneurons undergoing local reversals in the orientation of tangential migration (i.e., interneurons migrate tangentially toward the dorsal cortex, then turn around and move in the opposite direction) in the marginal zone region (Fig. 3C, D,



**Fig. 4.** Loss of netrin-1- $\alpha$ 3 $\beta$ 1 integrin interactions decreases the number and alters the positioning of interneurons in cortex at P0. (A–D, F–I, K–N) Compared with controls (A, F, K),  $\alpha$ 3 integrin mutants (B, G, L), or netrin-1 mutants (C, H, M),  $\alpha$ 3<sup>lox/-</sup>Dlx5/6-CIE, netrin-1<sup>-/-</sup> double-mutant mice (D, I, N), display a significant reduction in the number of EGFP<sup>+</sup>, calbindin<sup>+</sup>, and GABA<sup>+</sup> cells at the marginal zone (D, I, N, arrowhead) and throughout the P0 cortex (D, I, N, arrow). (E–E', J–J', O–O') Quantification of the number of EGFP<sup>+</sup>, calbindin<sup>+</sup>, and GABA<sup>+</sup> cells in the marginal zone (E, J, O) and cortex (E', J', O'). (P–S) No significant changes in the number or positioning of NPY<sup>+</sup> interneurons were noticed in the absence of netrin-1- $\alpha$ 3 $\beta$ 1 integrin interactions (S, arrow). (T–T') Quantification of the number of NPY<sup>+</sup> cells in the marginal zone (T) and cortex (T'). Data shown are mean  $\pm$  SEM; \*, significant when compared with controls at  $P < 0.05$  (one-way ANOVA, Tukey-Kramer posthoc test). cp, cortical plate; iz, intermediate zone; mz, marginal zone; vz, ventricular zone. (Scale bar, 170  $\mu$ m in A–D, 105  $\mu$ m in F–I, 65  $\mu$ m in K–N, and 100  $\mu$ m in P–S.)

and G; [Movies S5 and S6](#)). These results indicate that netrin-1- $\alpha$ 3 integrin interactions are required for interneurons to maintain appropriate, dorsally directed tangential migration along the cortical marginal zone region.

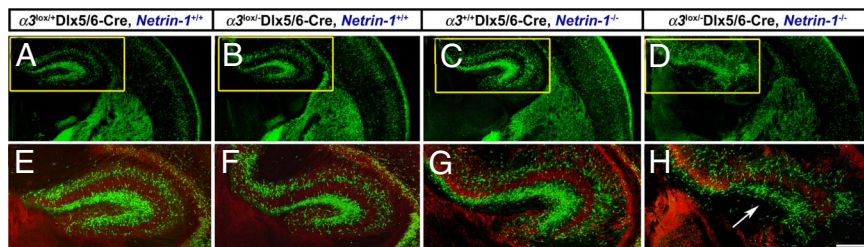
To investigate this deficit further, we performed a slice co-culture assay in which E14.5 MGE explants from control or conditional  $\alpha$ 3 integrin-mutant mice were placed over the ganglionic eminences of E14.5 wild-type or netrin-1-mutant coronal brain slices. Neuronal migration from the explants onto distinct domains of cortical substrates was assessed 72 h later.  $\alpha$ 3 integrin-deficient interneurons displayed significant deficits in their ability to migrate onto cortical slices in the absence of netrin-1 ([Fig. S5 A, D, E, H, and I](#)). A 25% (SD  $\pm$  0.06) reduction in interneurons traversing the upper cortical plate was noticed ([Fig. S5 A, D, E, H, and J](#)). Neuronal migration from wild-type explants onto netrin-1-deficient slices was not affected ([Fig. S5 A, C, E, G, and I](#)). Together, these real-time interneuronal migration analyses and slice co-culture assays suggest that netrin-1- $\alpha$ 3 integrin signaling facilitates interneuronal migration in the developing cerebral cortex, specifically the subset of interneurons migrating through the top of the cortical plate.

**Loss of Netrin-1- $\alpha$ 3 $\beta$ 1 Integrin Interactions Decrease the Number and Alter the Positioning of Interneurons in Cerebral Cortex.** To evaluate the outcome of disrupted netrin-1- $\alpha$ 3 integrin interactions on interneuronal organization in vivo, we analyzed the positioning of interneurons in the postnatal day 0 (P0) cerebral cortex, when active interneuronal migration comes to an end. Netrin-1- $\alpha$ 3 integrin double-null mice do not survive beyond P0. The migration deficits observed in  $\alpha$ 3<sup>lox/-</sup>Dlx5/6-CIE, netrin-1<sup>-/-</sup> double-mutant mice in vivo predict disruptions in the number and positioning of interneurons in the  $\alpha$ 3<sup>lox/-</sup>Dlx5/6-CIE, netrin-1<sup>-/-</sup> postnatal cortex. Analysis of P0 cortices revealed that the number of EGFP<sup>+</sup> interneurons in the upper cortical plate region in double-mutant mice indeed was reduced by 64% compared with  $\alpha$ 3<sup>lox/+</sup>Dlx5/6-CIE, netrin-1<sup>+/+</sup> controls ([Fig. 4A, D, and E](#)). Surprisingly, the total number of EGFP<sup>+</sup> interneurons in  $\alpha$ 3<sup>lox/-</sup>Dlx5/6-CIE, netrin-1<sup>-/-</sup> double-mutant cortices also was reduced substantially, to 45% of

that observed in controls ([Fig. 4A, D, and E'](#)). This reduction in the number of interneurons in  $\alpha$ 3<sup>lox/-</sup>Dlx5/6-CIE, netrin-1<sup>-/-</sup> double-mutant cortices at P0 may in part be the result of increased apoptosis of misplaced interneurons, because we detected an increase in cleaved caspase-3<sup>+</sup> cells in double-mutant cortices compared with controls ([Fig. S6](#)). The decrease in cortical interneurons in double mutants is unlikely to be caused by reduced neurogenesis. We did not detect any significant changes in mitotically active progenitors, as assessed by phospho-histone H3 labeling, in the GE ventricular zone of double-mutant forebrains compared with controls ([Fig. S6](#)).

To evaluate this disruption in interneuronal organization further, we immunolabeled subpopulations of interneurons with calbindin, calretinin, GABA, somatostatin, neuropeptide Y (NPY), and distal-less homeobox 2 (Dlx2) antibodies. The cerebral wall was divided into 10 equal bins, and interneurons in each of these bins were quantified ([Figs. 4 and Fig. S7](#)). Selective changes in interneuronal positioning in the absence of netrin-1- $\alpha$ 3 integrin interactions are evident. Strikingly, interneuronal placement in the upper cortical plate is reduced consistently ([Fig. 4D, I, and N, arrowhead](#)). The number of calbindin<sup>+</sup> interneurons at the marginal zone (i.e., bin 10, [Fig. S7](#)) and throughout the cerebral wall were decreased by 58% and 66%, respectively, in  $\alpha$ 3<sup>lox/-</sup>Dlx5/6-CIE, netrin-1<sup>-/-</sup> brains, compared with controls ([Fig. 4F, I, J, and J', arrowhead and arrow](#)). Similarly, a substantial decrease in GABA<sup>+</sup> interneurons is evident in  $\alpha$ 3<sup>lox/-</sup>Dlx5/6-CIE, netrin-1<sup>-/-</sup> double mutants, particularly at the upper cortical plate region. The number of GABA<sup>+</sup> cells at the marginal zone and throughout the cerebral cortex was reduced by 57% and 58%, respectively, in  $\alpha$ 3<sup>lox/-</sup>Dlx5/6-CIE, netrin-1<sup>-/-</sup> brains, compared with controls ([Fig. 4K, N, O, and O', arrowhead and arrow](#)). Furthermore, similar reductions in Dlx2<sup>+</sup> interneurons of 40% and 35% were noticed at the marginal zone ([Fig. S7](#)) and throughout the cerebral cortex, respectively, in  $\alpha$ 3<sup>lox/-</sup>Dlx5/6-CIE, netrin-1<sup>-/-</sup> double mutants ([Fig. S8A and D–F](#)). Calretinin<sup>+</sup> interneurons in  $\alpha$ 3<sup>lox/-</sup>Dlx5/6-CIE, netrin-1<sup>-/-</sup> brains did not exhibit deficits at the upper cortical plate region but displayed 44% and 36% reductions in number at the ventricular





**Fig. 5.** Disorganization and reduced numbers of hippocampal interneurons in  $\alpha 3^{\text{lox/-}}$ -Dlx5/6-CIE, *netrin-1*<sup>-/-</sup> mutants at P0. (A–H) A reduction in the number and organization of EGFP<sup>+</sup> interneurons throughout the hippocampal subfields of  $\alpha 3^{\text{lox/-}}$ -Dlx5/6-CIE, *netrin-1*<sup>-/-</sup> double mutants is evident (D and H, arrow) compared with controls (A and E). (Scale bar, 500  $\mu\text{m}$ .)

surface and throughout the cerebral wall, respectively, compared with controls (Fig. S7). No significant changes in the number or positioning of NPY<sup>+</sup> interneurons were observed in double mutants (Fig. 4 P–T'). Both the conditional  $\alpha 3$  integrin single mutants and *netrin-1* single mutants displayed deficits in the positioning of interneurons as well.  $\alpha 3$  integrin single-mutant mice displayed a 26% reduction in the number and ectopic positioning of calbindin<sup>+</sup> interneurons (Fig. 4 F, G, and J'). In *netrin-1*-deficient mice, a general dispersal of calbindin<sup>+</sup> interneurons in the cortical plate was noticed (Fig. 4H). Somatostatin<sup>+</sup> interneurons were detected only in the very lateral piriform cortex, where their numbers were decreased in  $\alpha 3^{\text{lox/-}}$ -Dlx5/6-CIE, *netrin-1*<sup>-/-</sup> double nulls (data not shown).

Because the hippocampus represents the end point of long-distance migration from the ventral telencephalon through the marginal zone region of the cerebral cortex (17, 18), analysis of interneuronal positioning in the hippocampal cortex provides another reliable measure of defects in long-distance interneuronal migration through the marginal zone region. We find a significant reduction in the number and organization of EGFP<sup>+</sup> interneurons throughout the hippocampus in  $\alpha 3^{\text{lox/-}}$ -Dlx5/6-CIE, *netrin-1*<sup>-/-</sup> double mutants compared with controls (Fig. 5 A, D, E, and H). In sum, these analyses of interneuronal organization indicate that *netrin-1*- $\alpha 3$  integrin interactions are necessary for the appropriate migration and positioning of distinct subsets of interneurons within the developing cerebral cortex.

## Discussion

In this study, we provide genetic evidence for the significance of *netrin-1*- $\alpha 3$  integrin interactions in distinct aspects of interneuronal migration in the developing cerebral cortex.  $\alpha 3\beta 1$  integrin is expressed by tangentially migrating interneurons, and *netrin-1* is present along their migratory route. In vitro, *netrin-1* activates  $\beta 1$  integrin and modulates the migration of interneurons in an  $\alpha 3\beta 1$  integrin signaling-dependent manner. In vivo, interneuron-specific  $\alpha 3\beta 1$  integrin, *netrin-1*-deficient mice display deficits in the migration of interneurons in the upper cortical plate region. Significant and selective disruptions in the number and positioning of subsets of interneurons, especially calbindin<sup>+</sup>, GABA<sup>+</sup>, Dlx2<sup>+</sup>, and calretinin<sup>+</sup> interneurons, but not NPY<sup>+</sup> interneurons, were observed in the absence of *netrin-1*- $\alpha 3\beta 1$  integrin interactions. Thus, *netrin-1*- $\alpha 3\beta 1$  integrin interactions guide the appropriate navigation of subsets of GABAergic interneuronal populations through the developing cerebral wall and are necessary for the proper placement of interneurons in the cerebral cortex.

**Altered Migration and Positioning of Interneurons in the Absence of *Netrin-1*- $\alpha 3\beta 1$  Integrin Signaling.** The heterogeneity of cortical interneuron origins and the differential distribution of interneuron subtypes in the cerebral cortex suggest that cortical interneuron subtypes may take distinct migratory routes into the developing cerebral cortex (see *SI Text*). Our data show that *netrin-1*- $\alpha 3$  integrin interactions modulate interneuronal migration through the cortical marginal zone from the beginning at E13.5. Deficits in this migration become progressively more severe in double-mutant mice. By E16.5, the migration of GABAergic interneurons through the upper cortical plate is significantly retarded in  $\alpha 3^{\text{lox/-}}$ -Dlx5/6-

CIE, *netrin-1*<sup>-/-</sup> double mutants (Fig. S4 A and C). Correspondingly,  $\alpha 3^{\text{lox/-}}$ -Dlx5/6-CIE, *netrin-1*<sup>-/-</sup> double-mutant mice exhibited a significant increase in the number of interneurons aberrantly migrating through the ventricular zone, a region largely devoid of *netrin-1* expression (Fig. S4 B and D). Therefore, in the absence of *netrin-1*- $\alpha 3\beta 1$  integrin interactions, cortical interneurons may take alternate, aberrant routes into the developing cortex. Real-time imaging analysis of tangential migration in E15.5  $\alpha 3^{\text{lox/-}}$ -Dlx5/6-CIE, *netrin-1*<sup>-/-</sup> double-mutant mice reveals, at the marginal zone, an increase in tangential to radial transitions directed toward the ventricular zone (Fig. 3 B and F and Movie S4). This increase in ventricular surface-directed radial migration at the cortical marginal zone provides an explanation for the decrease in the number of interneurons at the marginal zone of E16.5  $\alpha 3^{\text{lox/-}}$ -Dlx5/6-CIE, *netrin-1*<sup>-/-</sup> double-mutant mice and underscores the relevance of *netrin-1*- $\alpha 3\beta 1$  integrin interactions for appropriate, dorsal cortex directed tangential migration in this region. Furthermore, the absence of *netrin-1*- $\alpha 3\beta 1$  integrin interactions also disrupts the local migration of interneurons within the cortical plate (Fig. 3 D and G and Movie S6). It remains to be determined if the aberrant tangential to radial transition of interneurons in double mutants affects the areal-specific distribution of interneurons in the cerebral cortex. By P0, a substantial change in the number and positioning of interneurons within the marginal zone and cerebral wall is observed. No significant changes were evident in interneuronal progenitor proliferation in the GE (Fig. S6). Aberrant neuronal migration patterns resulting in abnormal neuronal positioning may lead to apoptosis of misplaced neurons. Consistently, we observed increased apoptosis in the developing cerebral cortex (Fig. S6). In addition, the presence of a large ectopic aggregation of interneurons in the ventral telencephalon (data not shown) suggests that apoptosis and misrouting of cortical interneurons in the absence of *netrin-1*- $\alpha 3\beta 1$  integrin interactions may contribute to the deficiency in the number of cortical interneurons in double-mutant mice. Together, these data suggest that *netrin-1*- $\alpha 3\beta 1$  integrin interactions are necessary for interneurons to sustain directed tangential migration along the upper cortical plate and for the appropriate positioning of interneurons in the developing telencephalon.

Clearly, *netrin-1* and  $\alpha 3\beta 1$  integrin do not have an exclusive ligand-receptor relationship and the less severe deficits seen in single mutants are suggestive of redundancies in these interactions (see *SI Text*). Furthermore, how the loss of *netrin-1*- $\alpha 3\beta 1$  integrin interactions may lead to non-cell-autonomous changes in other signaling pathways (e.g., CXCL12/CXCR4; 19, 20) capable of influencing interneuronal also needs to be deciphered.

Deficits in the timing of cortical plate invasion by GABAergic interneurons results in aberrant laminar and regional distribution of cortical interneurons. Specifically, disrupted CXCL12/CXCR4 signaling results in premature migration of GABAergic interneurons into the cortical plate and, consequently, aberrant laminar and regional positioning of cortical interneurons (19, 20). Furthermore, GABAergic interneurons migrating through the marginal zone, including calbindin<sup>+</sup> interneurons, exhibit slow multidirectional movement, a local positioning phase, before descent and integration into the cortical plate (21, 22). Disruption of *netrin-1*- $\alpha 3\beta 1$

integrin-mediated cell migration through the cortical marginal zone may lead to temporal and spatial alterations in the local positioning phase of interneurons and subsequent deficits in the integration of interneurons within the cortical plate. Indeed, the calbindin<sup>+</sup> cell distribution of  $\alpha 3^{\text{lox/-}}\text{Dlx5/6-CIE}$ , *netrin-1*<sup>-/-</sup> double-mutant mice is altered significantly compared with controls, indicating that netrin-1- $\alpha 3\beta 1$  integrin interactions at the cortical marginal zone may modulate the laminar positioning of calbindin<sup>+</sup> interneurons within the cortical plate. In contrast to the established “inside-out” gradient of cortical plate invasion, calretinin<sup>+</sup> interneurons populate the cortex in an “outside-in” gradient (30). Therefore, netrin-1- $\alpha 3\beta 1$  integrin interactions may differentially influence the guidance of calretinin<sup>+</sup> interneurons into the cortical plate compared with other interneuron subpopulations. The reduction of calretinin<sup>+</sup> interneurons at the ventricular zone instead of the marginal zone, in the absence of netrin-1- $\alpha 3\beta 1$  integrin interactions, supports this idea. Furthermore, the calbindin<sup>+</sup> and calretinin<sup>+</sup> cell distribution is affected more severely than the NPY<sup>+</sup> cell distribution of  $\alpha 3^{\text{lox/-}}\text{Dlx5/6-CIE}$ , *netrin-1*<sup>-/-</sup> double-mutant mice, suggesting that netrin-1- $\alpha 3\beta 1$  integrin interactions influence the migration and placement of specific interneuronal subpopulations. Whether the reduced expression of Dlx2<sup>+</sup> interneurons in the cerebral wall also is indicative of potential changes in oligodendrocyte cell fate in  $\alpha 3^{\text{lox/-}}\text{Dlx5/6-CIE}$ , *netrin-1*<sup>-/-</sup> double-mutant mice remains to be determined (31).

Disruptions in the placement and development of cortical interneuron subtypes may underlie the pathogenesis of neurodevelopmental disorders such as epilepsy, schizophrenia, and autism (see *SI Text*). This emerging evidence for interneuronal subtype-specific dysfunction in a variety of neurodevelopmental disorders highlights the importance of understanding the mechanisms underlying in-

terneuron subtype development. Our study demonstrates that the differential regulation of interneuronal subtype migration by directional guidance molecules, such as netrin-1, ultimately may modulate the placement and connectivity of interneuronal subpopulations in distinct regions of the cerebral cortex and contribute to normal cortical function.

## Materials and Methods

**Mutant Mouse Strains.** The generation and characterization of  $\alpha 3$  integrin floxed alleles is described in Liu et al. (35). See *SI Text* for details on the generation and characterization of  $\alpha 3^{\text{lox/-}}\text{Dlx5/6-CIE}$ , *netrin-1*<sup>-/-</sup> double mutants and related controls.

**Quantification of Interneuronal Cell Distribution in Cerebral Cortex.** Interneuronal cell counts in the E16.5 and P0 cortex were performed in sections corresponding to the somatosensory cortex as described previously (36). See *SI Text* for details.

**$\beta 1$  Activation Assay.** Netrin-1-mediated  $\beta 1$ -integrin activation in MGE cells was assayed using methods described in *SI Text*.

**Explant Co-cultures.** Explant co-cultures were prepared as described previously (14). For details, see *SI Text*.

**Cortical Slice-MGE Explant Co-cultures.** Slice co-cultures were prepared as described previously, with minor alterations (3). For details, see *SI Text*.

**Real-Time Imaging.** Coronal slices (200  $\mu\text{m}$ ) of E15.5  $\alpha 3^{\text{lox/+}}\text{Dlx5/6-CIE}$ , *netrin-1*<sup>+/-</sup> control and littermate  $\alpha 3^{\text{lox/-}}\text{Dlx5/6-CIE}$ , *netrin-1*<sup>-/-</sup> double-mutant brains were imaged repeatedly and analyzed as described earlier (4). For details, see *SI Text*. Immunohistochemical, immunoprecipitation, and immunoblotting methods are described in the *SI Text*.

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