Reelin transiently promotes N-cadherin–dependent neuronal adhesion during mouse cortical development

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Reelin is an essential glycoprotein for the establishment of the highly organized six-layered structure of neurons of the mammalian neocortex. Although the role of Reelin in the control of neuronal migration has been extensively studied at the molecular level, the mechanisms underlying Reelin-dependent neuronal layer organization are not yet fully understood. In this study, we directly showed that Reelin promotes adhesion among dissociated neocortical neurons in culture. The Reelin-mediated neuronal aggregation occurs in an N-cadherin–dependent manner, both in vivo and in vitro. Unexpectedly, however, in a rotation culture of dissociated neocortical cells that gradually reaggregated over time, we found that it was the neural progenitor cells [radial glial cells (RGCs)], rather than the neurons, that tended to form clusters in the presence of Reelin. Mathematical modeling suggested that this clustering of RGCs could be recapitulated if the Reelin-dependent promotion of neuronal adhesion were to occur only transiently. Thus, we directly measured the adhesive force between neurons and N-cadherin by atomic force microscopy, and found that Reelin indeed enhanced the adhesiveness of neurons to N-cadherin; this enhanced adhesiveness began to be observed at 30 min after Reelin stimulation, but declined by 3 h. These results suggest that Reelin transiently (and not persistently) promotes N-cadherin–mediated neuronal aggregation. When N-cadherin and stabilized β-catenin were overexpressed in the migrating neurons, the transfected neurons were abnormally distributed in the superficial region of the neocortex, suggesting that appropriate regulation of N-cadherin–mediated adhesion is important for correct positioning of the neurons during neocortical development.

Reelin | N-cadherin | aggregation | corticogenesis | neuronal migration

The mammalian neocortex is highly organized into six neuronal layers. This laminar structure is responsible for the complex motor, sensory, and cognitive functions of the mammalian brain (1). Neuronal migration plays an important role in the establishment of this layered structure. Cortical neurons are generated within the ventricular zone (VZ) or subventricular zone (SVZ), and migrate along radial fibers toward the pial surface. Newly born excitatory neurons migrate radially into the cortical plate (CP) past the neurons born earlier, resulting in a birth date-dependent "inside-out" alignment of the neurons in the CP (2–4).

Reelin is a glycoprotein that is secreted by the Cajal–Retzius cells in the marginal zone (MZ) of the cortex during neocortical development (5–7). This glycoprotein is essential for establishment of the aforementioned birth date-dependent layered structure of the neocortex, because the CP neurons show an almost inverted alignment in the neocortex of the Reelin-deficient, *reeler* mice. In addition, neurons born at the same time tend to be distributed broadly and not to form clear layers in the *reeler* cortex (8, 9). Although extensive studies, including at the molecular level, have been conducted to determine the role of Reelin in neuronal migration in the neocortex (10–14), the

cellular and molecular functions of Reelin in neuronal layer formation in the neocortex are not yet fully understood.

In recently carried out studies, we revealed that ectopic expression of Reelin by in utero electroporation caused neuronal aggregation in the developing mouse neocortex (15–17). In addition, we found that immature neurons were densely packed in the outermost region of the developing cortex (i.e., beneath the MZ), which is termed the primitive cortical zone (PCZ) (18), supporting the notion that Reelin may somehow be involved in the aggregation of migrating neurons beneath the MZ in vivo. However, it is not yet clear whether Reelin directly promotes adhesion among neurons or, instead, causes them to form aggregates as a result of being repelled by the surrounding cellular environment. Thus, in this study, we investigated the role of Reelin in neuronal aggregation/adhesion during neocortical development.

Results

To determine whether Reelin can cause aggregation of cortical cells in vitro, we dissociated cortical cells from embryonic day (E) 14.5 *reeler* mice and cultured them on polyethylenimine-coated dishes, which allowed the cells to move around at least to some extent. The cultured cells were then treated with mock or Reelin-containing medium for 24 h. The experiment revealed that more cell aggregates were formed after Reelin stimulation than after mock treatment (Fig. 1 A, A', B, B', and D and Fig. S1). Formation

Significance

It has long been thought that Reelin is a stop signal for migrating neocortical neurons, because the neurons stop just beneath Reelin-rich regions. Our recent studies revealed that ectopically expressed Reelin can also cause neuronal aggregation with clear demarcation in vivo, suggesting that Reelin is not simply a stop signal. However, it remains unclear whether Reelin directly promotes adhesion among neurons or only causes them to be repelled by the surrounding cellular environment, facilitating their aggregation. We show here that Reelin directly promotes N-cadherin–dependent neuronal adhesion, causing neuronal aggregation. This Reelin-dependent neuronal adhesion occurs only transiently, and is not sustained. This dynamic change of N-cadherin–mediated cellular adhesiveness is important for establishment of the appropriate layering of neocortical neurons.
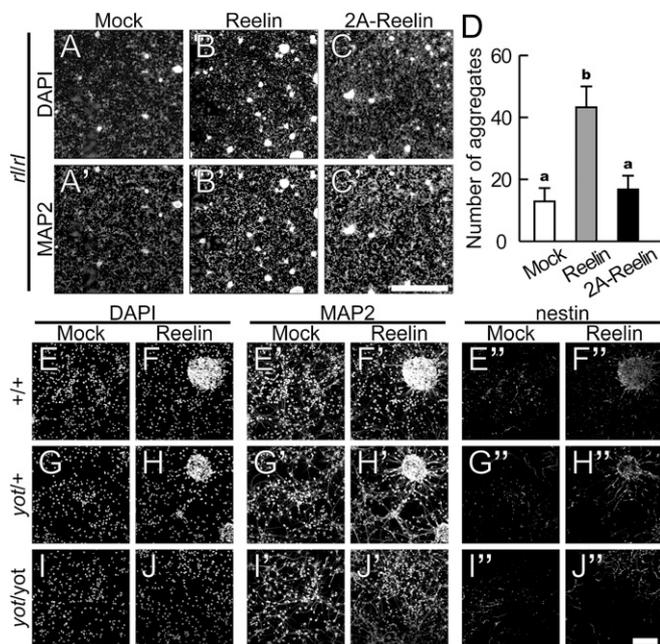
Fig. 1. Effect of Reelin treatment on a primary culture of cortical neurons. MAP2-positive neurons in a primary culture of dissociated E14.5 *reeler* mouse cortical cells after addition of control medium (Mock) (*A*), Reelin-containing medium (*B*), and 2A-Reelin–containing medium (*C*) for 24 h are shown. (*A*–*C*) Nuclei were visualized by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). (Scale bar, 5 mm.) (*D*) Number of aggregates in the chamber well. Results are expressed as mean ± SEM. Mock, $n = 8$; Reelin, $n = 8$; 2A-Reelin, $n = 6$. Means that are significantly different from each other are represented by different small letters ($P < 0.05$). Nuclei (*E*–*J*), MAP2-positive neurons (*E′*–*J′*), and nestin-positive RGCs (*E′′*–*J′′*) in a primary culture of dissociated E14.5 cortical cells from a wild-type mouse (*E*–*E′′* and *F*–*F′′*), heterozygous *yotari* mouse (*G*–*G′′* and *H*–*H′′*), and homozygous *yotari* mouse (*I*–*I′′* and *J*–*J′′*) are shown after the addition of mock medium (*E*–*E′′*, *G*–*G′′*, and *I*–*I′′*) or Reelin medium (*F*–*F′′*, *H*–*H′′*, and *J*–*J′′*) for 24 h. (Scale bar, 200 μm.)

of cell aggregates was observed at a level similar to the level observed in the mock control when 2A-Reelin was used; 2A-Reelin is the product of a point mutation of *Reelin* that contains two mutant lysine residues, which prevents binding of Reelin to its receptors (19) (Fig. 1 *C*, *C′*, and D and Fig. S1). These results suggest that Reelin can directly induce cortical cell aggregation via its receptors.

To examine further whether this cell aggregation is mediated by the well-established Reelin signaling pathway, we performed similar experiments using neurons obtained from the cortex of *yotari* mutant mice, which are deficient in Disabled 1 (Dab1), an intracellular adaptor protein that binds to the Reelin receptors and is essential for transduction of the Reelin signal (20–25). This experiment revealed that Reelin was unable to cause clear aggregation of the cells derived from the *yotari* homozygous mice (Fig. 1*J*), indicating that the Reelin-induced cortical cell aggregation is mediated by the well-known Reelin pathway involving Dab1. Immunohistochemical analyses showed that the Reelin-induced cell aggregates were mainly composed of neurons that were positive for microtubule-associated protein 2 (MAP2) (Fig. 1 *F*–*F′′* and *H*–*H′′*) and neuronal nuclei (NeuN) (Fig. S2 *A* and *B*), which is consistent with our previous finding that ectopically overexpressed Reelin caused aggregation of migrating neurons in vivo (15). It is worthy of note, however, that some nestin-positive and paired box protein 6 (Pax6)-positive neural progenitor cells [radial glial cells (RGCs)] were also incorporated into these aggregates. Because the RGCs tended to be located

near the surface of the aggregates, the RGCs may participate in the cell aggregation by adhering to the neurons, or may be excluded from the aggregated neurons. Reelin treatment did not significantly affect the number of NeuN-positive neurons or Pax6-positive RGCs in this culture (24 h) (Fig. S2 *C*–*E*).

Reelin is well known to control some cell adhesion molecules, such as N-cadherin (11, 14, 26) and integrin α5β1 (12). In regard to the adhesion molecule mediating the Reelin-dependent neuronal aggregation, we thought that N-cadherin might be a good candidate because N-cadherin is present in abundance in the cortical MZ (27). Therefore, we examined whether N-cadherin could also be detected when the migrating neurons aggregated in vivo in response to ectopically overexpressed Reelin in the developing neocortex (15). As expected, immunohistochemical examination revealed strong detection of N-cadherin in the central cell body-sparse, dendrite-rich, MZ-like region of the ectopic aggregates in vivo (12) (Fig. 2*A*). When expression vectors for the dominant-negative (DN) form of N-cadherin and green fluorescent protein (GFP) were cotransfected with the Reelin expression vector, the central MZ-like regions of the in vivo aggregates were invaded by migrating neurons and the margin of the aggregates became blurred, losing their spheroidal shape (Fig. 2*D*). The GFP-positive cells, which were aligned near the surface of the aggregates in a radial manner, with their processes directed toward the central MZ-like region when the control vector or N-cadherin expression vector was transfected (Fig. 2 *B*, *C*, and *E*), appeared to be randomly oriented within the aggregates when the DN form of N-cadherin was transfected (Fig. 2*D*). N-cadherin knockdown (KD) with Reelin overexpression also disrupted the centers of the neuronal aggregates and the radial orientation of the GFP-positive cells (Fig. 2*F*). These N-cadherin/KD phenotypes were rescued by cotransfection of the cells with a KD-resistant type of N-cadherin (Fig. 2*G*). These results imply that Reelin promotes neuronal aggregation via N-cadherin in vivo. In vitro also, inhibition of Reelin-dependent neuronal aggregation was observed following KD of N-cadherin in the cultured cortical cells (Fig. 2 *H*–*N*), further confirming the involvement of N-cadherin in the Reelin-induced neuronal aggregation.

Genotype-dependent differences in the cortical cell aggregation pattern between *reeler* and wild-type mice were previously analyzed using a rotation culture of dissociated cortical cells (6, 28). In this study, we took advantage of this rotation culture system, using the original system with some modifications, to investigate whether exogenous Reelin could modulate the aggregation pattern of *reeler* cortical cells. To this end, we first transfected an expression plasmid for Reelin (or control plasmid) with a GFP expression construct into the E12.0 *reeler* cortical cells by electroporation. The transfected cortices were then dissociated into single cells and cultured in a rotating tube for 5 d. As reported previously, the cortical cells showed reaggregation into spheres during the culture; this finding was confirmed by immunohistochemical analyses using markers for neurons (MAP2) and RGCs (nestin) (Fig. 3 *A* and *B* and Fig. S3 *A* and *B*). Interestingly, overexpression of Reelin in a fraction of the cultured cells caused clustering of the RGCs in the inner part of the reaggregated *reeler* cells (Fig. 3*B*, arrowhead, and Fig. S3*B*, dotted line). Although we initially assumed, based on the above-mentioned results (Figs. 1 and 2), that it is the neurons, rather than the RGCs, that form clusters in response to Reelin, neurons were unexpectedly distributed more diffusely than the RGCs and tended to be excluded from the nestin-positive RGC clusters.

To determine the reason for this unexpected clustering of the RGCs in the inner part of the reaggregated cellular spheres, we used mathematical modeling of the reaggregation culture based on a recently described method (29), in an attempt to identify the factor(s) that could be important for recapitulating the cell reaggregation patterns in the presence or absence of Reelin.
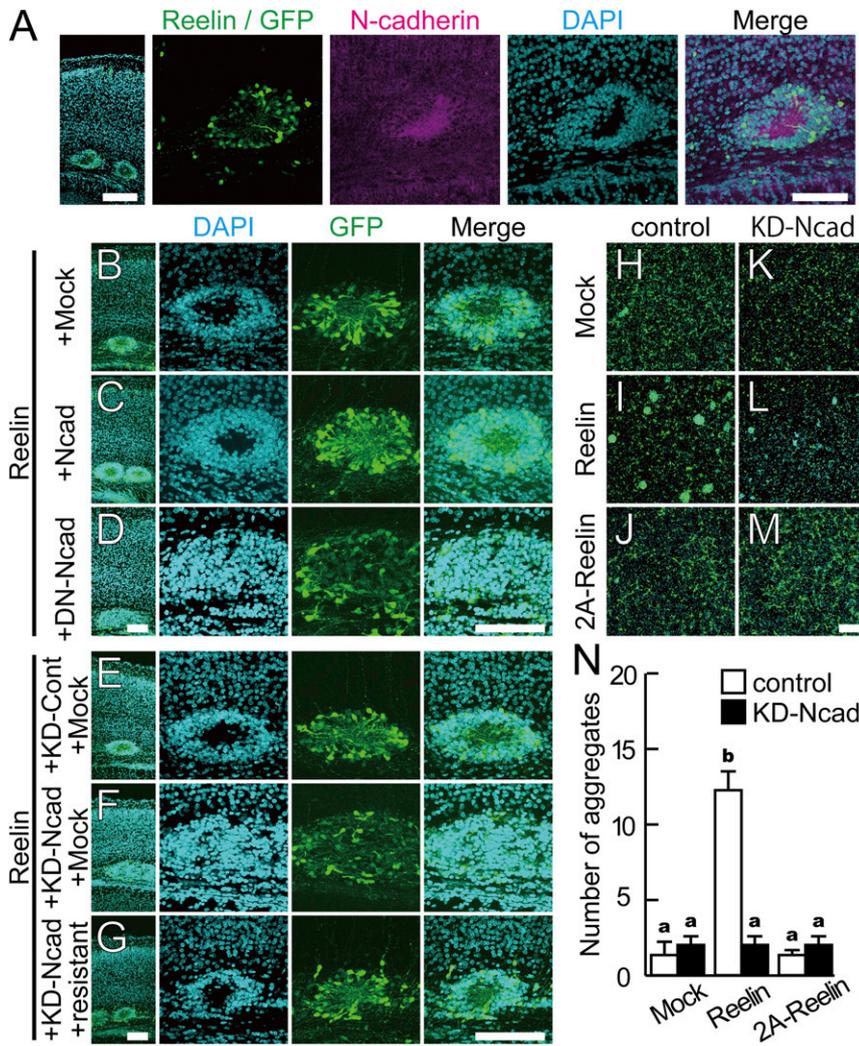
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Fig. 2. N-cadherin is necessary for neuronal aggregation both in vivo and in vitro. Representative immunostained images of cell aggregates formed by the mediation of Reelin in vivo. (*A*) Aggregate stained with an N-cadherin antibody (magenta) and DAPI (cyan). P1.5 brain transfected with expression vectors for Reelin and GFP plasmid at E14.5. (Scale bar, 100 μm.) Cotransfection with expression vectors for Reelin and Mock (*B*), with expression vectors for Reelin and N-cadherin (Ncad; *C*), and with expression vectors for Reelin and a DN form of N-cadherin (DN-Ncad; *D*). (Scale bars, 100 μm.) Cotransfection with the expression vectors for Reelin, control siRNA vector (KD-Cont), and Mock (*E*); with the expression vectors for Reelin and an siRNA expression vector to KD mouse N-cadherin (KD-Ncad) and Mock (*F*); and with expression vectors for Reelin, KD-Ncad, and an RNAi-resistant form of N-cadherin (resistant) (*G*). (Scale bars, 100 μm.) MAP2-positive neurons (green) in a primary culture of dissociated E14.5 *reeler* mouse cortical cells transfected with a control vector (*H–J*) or an N-cadherin KD vector (*K–M*) after addition of mock medium (*H* and *K*), Reelin medium (*I* and *L*), or 2A-Reelin medium (*J* and *M*) for 24 h. Nuclei were visualized by DAPI. (Scale bar, 100 μm.). (*N*) Number of aggregates transfected with the control vector (open columns) or N-cadherin KD vector (filled columns) after addition of mock medium, Reelin medium, or 2A-Reelin medium for 24 h. Results are expressed as mean ± SEM. Mock (control), $n = 3$; Mock (KD-Ncad), $n = 3$; Reelin (control), $n = 4$; Reelin (KD-Ncad), $n = 3$; 2A-Reelin (control), $n = 3$; 2A-Reelin (KD-Ncad), $n = 3$. Means that are significantly different from each other are represented by different small letters ($P < 0.05$).

In this mathematical model, we hypothesized that Reelin would enhance the cell–cell adhesion among neurons (Fig. 3*C*). In the absence of Reelin ($r = 0$, where r denotes the density of Reelin-secreting cells or the local concentration of Reelin itself), cell adhesion among neurons and RGCs was set to be identical (10 for any combination of cell types; Fig. 3*C*). In the presence of Reelin ($r > 0$), the cell adhesion among neurons was set to be increased ($10 + 20r$; Fig. 3*C*). The simulation assumed that the RGCs and neurons would be homogeneously distributed in the absence of Reelin (Fig. 3*E* and Movie S1). On the other hand, in the Reelin-present condition ($r > 0$), RGC clusters were seen in the aggregates, even though the RGC–RGC interaction was not set to be promoted (Fig. 3*F* and Movie S2). Nevertheless, the RGC clusters formed near the periphery of the aggregates tended to be localized along the edges of the aggregates (Fig. S3*D*), which appeared to be inconsistent with the aforementioned finding observed in living cells (Fig. 3*B* and Fig. S3*B*), wherein the RGC clusters were located in the inner parts of the reaggregated cells and the periphery was mainly occupied by neurons.

In our previous study, we showed that when the neurons completed radial migration, they were transiently localized in the most superficial region of the CP, or the PCZ, where they accumulated at a high cell density (18). After the neurons were released from the PCZ and settled in the CP, the neuronal cell density became lower than in the PCZ. Thus, we hypothesized that Reelin might cause a transient, rather than persistent,

increase of cell–cell adhesion among neurons (Fig. 3*D*; transition from immature neurons with strong adhesiveness to mature neurons with weak adhesiveness was hypothesized). Simulation of this transition showed that in the Reelin-present and transition-present conditions, RGC clusters were formed only in the inner parts of the aggregates [Fig. 3*G*, Fig. S3*E* (a space between an RGC cluster and the edge of the aggregate is indicated by white asterisks), and Movie S3], resembling the pattern observed in the reaggregated living cells described above (Fig. 3*B* and Fig. S3*B*). Thus, our mathematical model predicted that the Reelin-induced increase in cell–cell adhesion among neurons would need to be only transient for the RGC clusters to form in the inner part of the reaggregated *reeler* cell spheres.

To investigate whether Reelin indeed promotes N-cadherin–mediated neuronal adhesion only transiently, we next applied atomic force microscopy (AFM)-based single-molecule force spectroscopy to quantify the adhesive interaction between the N-cadherin–coated cantilever tip and a cultured neuron. We stimulated primary cortical neurons with Reelin-containing medium or control medium (Mock) for 30 min or 3 h. Then, we measured the "work for detachment," which means the work required for detaching the N-cadherin–coated cantilever from the single neuron, and the "unbinding force," which reflects the maximal adhesive force between the N-cadherin–coated cantilever and a single neuron, from the obtained force-distance curves (Fig. S4*A*). This experiment revealed that Reelin
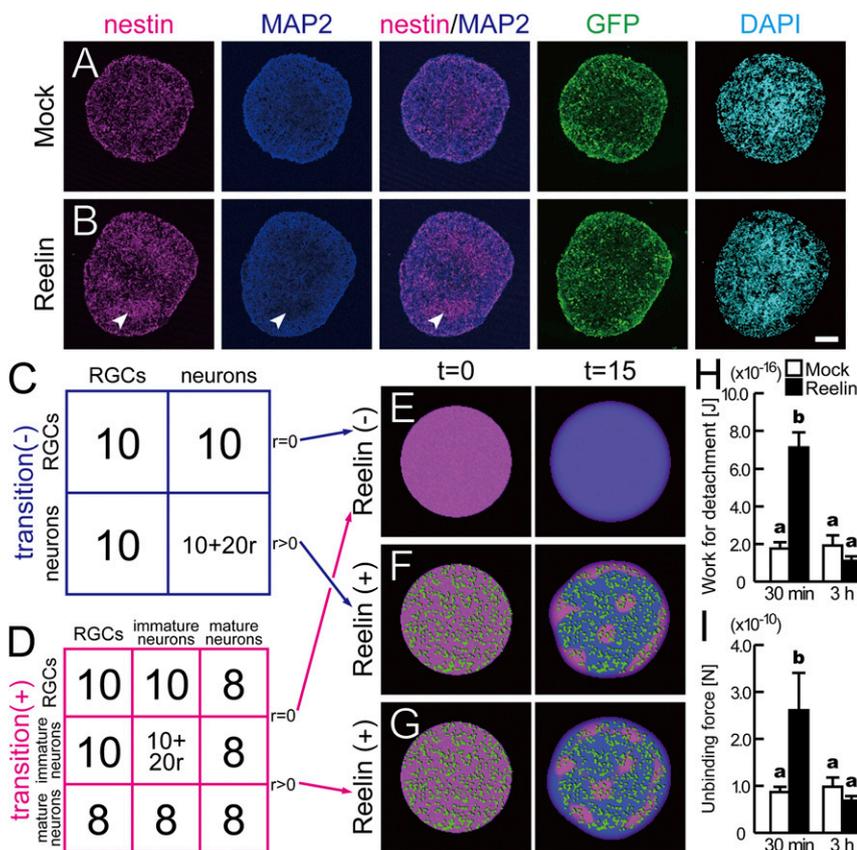
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Fig. 3. Comparison of the cell-sorting patterns between a rotation culture of living cells and a mathematical model. Representative immunostaining images of a rotation culture of cortical cells obtained from an E12.0 *reeler* mouse cortex transfected with an empty vector (Mock; *A*) or Reelin expression vector (*B*). A cell aggregate stained with an anti-nestin antibody (magenta), anti-MAP2 antibody (blue), and DAPI (cyan) is shown. Note the clustering of nestin-positive cells located in the inner part of the reaggregate (arrowheads). MAP2-positive cells were sorted out from the nestin-positive areas. (Scale bar, 100 μm.) (*C* and *D*) Adhesion strength parameters for simulation with the mathematical model. The transition from strong adhesion to weak adhesion among neurons was predicted to be absent (*C*) or present (*D*). (*E–G*) Time frames of numerical simulations of the aggregated patterns in the rotation culture. Each panel shows the distribution of the Reelin-secreting cells (green), neurons (blue), and RGCs (magenta) under the Reelin-negative, transition-negative condition (*E*); Reelin-positive, transition-negative condition (*F*); and Reelin-positive, transition-positive condition (*G*). (*H* and *I*) Single-cell adhesion measurement by AFM. Differences in the work for detachment (*H*) and the unbinding force (*I*) in primary culture of dissociated E14.5 ICR mouse cortical cells after addition of mock medium or Reelin medium for 30 min or 3 h. Results are expressed as mean ± SEM. Mock (30 min), $n = 10$; Mock (3 h), $n = 12$; Reelin (30 min), $n = 12$; Reelin (3 h), $n = 18$. Means that are significantly different from each other are represented by different small letters ($P < 0.05$). J, Joule; N, newton.

stimulation indeed caused a significant increase in both of these parameters within 30 min of its addition, indicating that the adhesive force between N-cadherin and neurons was strengthened by Reelin stimulation (Fig. 3 *H* and *I* and Fig. S4 *B* and *C*). As predicted from the mathematical modeling, these parameters decreased again to their initial levels by 3 h after the addition of Reelin (Fig. 3 *H* and *I* and Fig. S4 *D* and *E*). These results support our hypothesis that Reelin only transiently promotes N-cadherin–mediated neuronal adhesion.

To understand the biological role of the decrease in the adhesive forces between N-cadherin and neurons, we next examined the effect of persistent enhancement of N-cadherin–dependent adhesion. β-Catenin plays an important role in generating the cadherin-dependent adhesive force (30), and it is known to be destabilized by phosphorylation of the serine/threonine residues at positions 29, 33, 37, 41, and 45 (31, 32). Deletion of a segment that included these sites (amino acids 29–48 of β-catenin) results in the stabilization of β-catenin proteins (33). We thus transfected migrating neurons in E14.5 mice with expression vectors for N-cadherin and β-catenin (Δ29–48). This experiment revealed that neurons coexpressing N-cadherin and β-catenin (Δ29–48) exhibited abnormal positioning at postnatal day (P) 7.5 (Fig. 4 *A*–*E*). More neurons remained immediately beneath layer I compared with the control. It is noteworthy that overexpression of the expression vectors for N-cadherin and/or β-catenin (Δ29–48) at the same concentration (2.5 mg/mL) did not significantly affect neuronal migration when observed at P0.5 (Fig. S5 *A*–*E*), whereas overexpression of N-cadherin at a higher concentration (5.0 mg/mL) of the expression plasmid affected neuronal migration (Fig. S5 *F*–*J*), as reported previously (34). These results suggest that appropriate regulation, but not persistent increase, of the adhesive forces mediated by N-cadherin is necessary for the cortical neurons to settle in their proper positions eventually.

Discussion

Our recent investigation demonstrated that ectopically overexpressed Reelin in the developing neocortex caused aggregation of the migrating neurons in the CP in vivo (15). However, it remained unclear as to whether Reelin directly strengthened adhesion among neurons or indirectly caused aggregation of the neurons by, for example, causing the local cellular environment to repel the migrating neurons. In this study, we found evidence to suggest that Reelin directly causes neuronal cell aggregation (Fig. 1) through N-cadherin (Fig. 2).

Previous studies have shown that Reelin regulates the plasma membrane localization of N-cadherin through small GTPase Rap1 and serine/threonine kinase Akt in the migrating multipolar neurons (11), and that it regulates cadherin functions through Dab1 and Rap1 to control radial glia-independent somal translocation and lamination during neocortical development (14). Reelin signaling to Rap1 promotes N-cadherin functions via nectin3 and afadin toward mediating cell–cell interactions between the migrating neurons and Cajal–Retzius cells (26). However, these studies did not address the time-dependent dynamic changes of cell adhesion mediated by N-cadherin. Herein, we demonstrate that Reelin transiently, but not persistently, increases cell–cell adhesion via N-cadherin (Fig. 3). These transient, but not persistent, changes in N-cadherin–mediated adhesiveness of the neurons would appear to be important for transient formation of the cell-dense PCZ beneath the MZ and also for enabling the neurons born later to pass by this zone appropriately to settle in their correct positions in the CP (Fig. 4).

The adhesive force among cells imparted by N-cadherin is known to be controlled by several mechanisms. The cytoplasmic domain of N-cadherin is known to play an important role in generating the adhesive force through interaction with β-catenin and p120 catenin (30). β-Catenin has a key role in the formation of adherens junctions through its interactions with cadherin and α-catenin (35). β-Catenin contains several serine and threonine
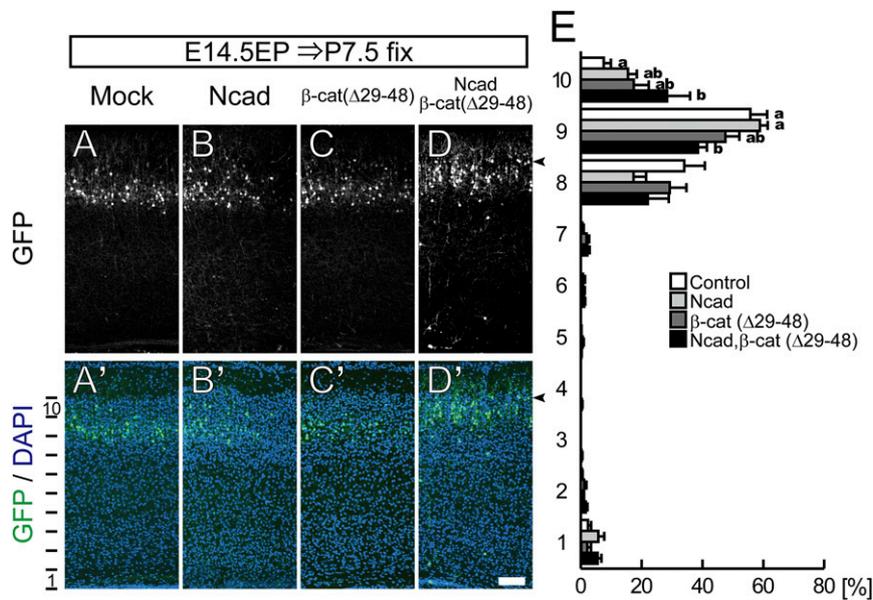
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Fig. 4. Decrease of the adhesive forces is necessary for appropriate neuronal positioning in vivo. Cerebral cortices at P7.5 electroporated with control (*A*), N-cadherin (*B*; 2.5 mg/mL), β-catenin (β-cat; Δ29–48) expression plasmids (*C*; 2.5 mg/mL), or N-cadherin/β-catenin (Δ29–48) expression plasmids (*D*; 2.5 mg/mL + 2.5 mg/mL) at E14.5. A mock vector (an empty pCAGGS vector) was cotransfected to make the total DNA concentration constant. Details of the plasmid DNA concentrations are described in *Materials and Methods* (Table S1). (*A′–D′*) GFP expression plasmid was also cotransfected to allow visualization of the transfected cells. Nuclei were labeled with DAPI. (Scale bar, 100 μm.) (*E*) Bin analysis. The distance between the layer I/layer II boundary (arrowhead) and the ventricular surface was divided into 10 bins. Results are expressed as mean ± SEM. Control, $n = 7$ brains; N-cadherin, $n = 6$ brains; β-catenin (Δ29–48), $n = 5$ brains; N-cadherin/β-catenin (Δ29–48), $n = 7$ brains. Means that are significantly different from each other in each bin are represented by different small letters ($P < 0.05$).

residues in the consensus positions for phosphorylation by glycogen synthase kinase 3β (GSK3β). This phosphorylation site is known to be a prerequisite for ubiquitination of β-catenin and regulation of β-catenin stability (31, 32). Because Reelin-Dab1 signaling triggers the phosphorylation of Akt (protein kinase B) and inactivation of GSK3β in cultured cortical neurons (36, 37), β-catenin is one of the strong candidates with respect to molecules mediating the Reelin-dependent control of N-cadherin–mediated cell adhesion.

Similarly, p120 catenin also controls the cadherin-mediated adhesion among cells by stabilizing cadherin at the plasma membrane; in its absence, the cadherins are internalized through endocytosis (38, 39). The p120 catenin–cadherin binding is strengthened by the interaction of p120 catenin with nectin-associated afadin in a Rap1-dependent manner (40). Reelin recruits p120 catenin and N-cadherin to the nectin and afadin complexes, enhancing the N-cadherin–mediated cell adhesion (26). Thus, p120 catenin is also a candidate molecule connecting the Reelin–Dab1 pathway and N-cadherin. Future studies are required to elucidate the detailed mechanism underlying the time-dependent changes of N-cadherin–mediated cell adhesion.

When we inhibited the N-cadherin functions in the Reelin-induced cell aggregates in vivo, the cell body-sparse (MZ-like) center of the neuronal aggregates and the radial orientation of the GFP-positive cells were disrupted, but blurred cell aggregates were still formed. This result could have occurred because we transfected the DN form of N-cadherin (Fig. 2*D*) or N-cadherin KD vector (Fig. 2*F*), which could have led to only incomplete inhibition of the functions of N-cadherin, and the remaining exogenous N-cadherin might have mediated the residual weak adhesion. Another possibility is that residual adhesion, especially adhesion among the cell bodies, mediated by adhesion molecules other than N-cadherin contributed to the formation of the blurred aggregates without the central MZ-like regions. In the mathematical model that we used, we rather simplified the conditions

for simulating the essential features of the cell aggregates. For example, in this model, neurons and RGCs were assumed to be distributed evenly within the aggregates in the absence of Reelin (Fig. 3*E*), which might be somewhat different from the actual pattern produced by the living cells (Fig. 3*A*). However, the degree of cell sorting between living neurons and RGCs was obviously increased in the presence of Reelin (Fig. 3*B*), which was successfully recapitulated with this model (Fig. 3 *F* and *G* and Fig. S3 *D* and *E*). The living neurons and RGCs might, to some extent, interact in a homophilic manner even in the absence of Reelin. In that case, it may be considered that the homophilic adhesion among neurons is transiently enhanced by Reelin stimulation.

In this study, we provide evidence indicating that Reelin causes a transient, but not persistent, increase in cell–cell adhesion among neurons through N-cadherin. In vivo, when migrating neurons reach the outermost zone, the PCZ, of the developing CP and receive the Reelin signal, the immature neurons show strong cell adhesiveness and become packed into the PCZ at a high cell density. This "packing" may be beneficial for appropriate sorting of the neurons in a birth date-dependent manner (41), although further investigation is required to confirm this contention. Because the increase in the cell adhesiveness is presumably only transient, it is thought that the cell–cell adhesion among these neurons in the PCZ becomes weak subsequently, resulting in the release of these neurons from the PCZ to a deeper position in the CP (Fig. S6). Considering what the effect of a persistent increase in the adhesiveness of the migrating neurons might be on their final disposition (Fig. 4), this transient increase, followed by a subsequent decrease in the cell adhesiveness, appears to be necessary for establishment of the highly organized layered structure of the neurons in the mammalian neocortex. In the future, it will be important to clarify the molecular mechanisms of the dynamic changes of the cellular adhesiveness induced by Reelin and to investigate the effect of inhibition of the "decrease mechanism" on neuronal positioning in vivo.

Materials and Methods

Animals and Animal Treatment. Pregnant ICR mice were purchased from Japan SLC. The colony of *reeler* mice (B6CFe *a/a-Reln*^{rl}/J) obtained from The Jackson Laboratory was maintained by allowing heterozygous or homozygous females to mate with homozygous males. The day of vaginal plug detection was considered as E0. For example, "E14" was used to denote experiments performed on the morning of E14 and "E14.5" was used for experiments performed in the afternoon of E14. All animal experiments were performed using protocols approved by the Keio University Institutional Animal Care and Use Committee.

Details of construction of plasmids, in utero electroporation, dissociated cell culture, rotation culture, preparation of frozen sections, immunohistochemistry and immunocytochemistry, high-content analysis, counting of the primary culture cells, AFM, cell culture and transfection to prepare Reelin medium, and mathematical models are provided in *SI Materials and Methods*.

Statistical Analyses. All numerical data are expressed as mean ± SEM.

For comparing two groups, Student's *t* test was used when neither normality nor homogeneity of variances of the dataset was rejected by the Shapiro–Wilk normality test and Levene's test, respectively. In cases where normality was proven but homogeneity was rejected, Welch's *t* test was used. In cases where normality of the dataset alone was rejected, the Mann–Whitney *U* test was conducted as a nonparametric test.

For comparing multiple groups, the normality of the data was tested by the Shapiro–Wilk test, and the variance homogeneity among all groups was tested by Levene's test. When neither normality nor homogeneity of variance of the dataset to be analyzed was rejected by the above tests, ANOVA, followed by the Tukey–Kramer test, was used. In cases where either normality or variance homogeneity was rejected, the Kruskal–Wallis test was used, followed by the Games–Howell test. For all of the statistical tests mentioned above, the α-value was set at 0.05.

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