Brief Communications

Necdin Promotes Tangential Migration of Neocortical Interneurons from Basal Forebrain

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Necdin is a pleiotropic protein that promotes neuronal differentiation and survival. In mammals, the necdin gene on the maternal chromosome is silenced by genomic imprinting, and only the paternal necdin gene is expressed in virtually all postmitotic neurons. Necdin forms a complex with the homeodomain protein Dlx2 to enhance its transcriptional activity. Dlx2 plays a major role in controlling tangential migration of GABAergic interneurons from the basal forebrain to the neocortex. Here, we examined whether Dlx2-expressing interneurons migrate properly *in vivo* in mutant mice lacking the paternal necdin gene. In necdin-deficient mice at birth, the population of Dlx2-expressing cells significantly decreased in the neocortex but increased in the preoptic area. DiI-labeled cell migration assay using organotypic forebrain slice cultures revealed that the number of cells migrating from the medial ganglionic eminence into the neocortex was significantly reduced in necdin-deficient embryos. Furthermore, necdin-deficient mice had a decreased population of neocortical GABA-containing neurons and were highly susceptible to pentylenetetrazole-induced seizures. These results suggest that necdin promotes tangential migration of neocortical GABAergic interneurons during mammalian forebrain development.

Introduction

Necdin is a neural differentiation-specific protein expressed in murine embryonal carcinoma cells (Maruyama et al., 1991). The necdin gene (*Ndn*) is expressed in virtually all postmitotic neurons from early development to adult age (Uetsuki et al., 1996). Necdin is a member of melanoma antigen (MAGE) family proteins, all of which contain a conserved MAGE homology domain (MHD) (Barker and Salehi, 2002). Via the MHD, necdin interacts with many regulatory proteins involved in cell proliferation, apoptosis, and differentiation (Hasegawa and Yoshikawa, 2008). Through these interactions, necdin promotes terminal differentiation and survival of various neurons.

Necdin forms a complex, via MAGE-D1, with Msx/Dlx family proteins, which are homeodomain proteins that govern cell fates and differentiation (Kuwajima et al., 2004, 2006). Dlx family proteins play major roles in differentiation and migration of GABAergic interneurons originating in the ventral telencephalon (Marin and Rubenstein, 2003). Mutant mice defective in Dlx1/Dlx2 homeodomain proteins show abnormal migration of neocortical interneurons from the ventral telencephalon (Anderson et al., 1997b). Together, these findings raise the possibility that necdin promotes tangential migration of Dlx2-expressing cortical interneurons.

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Genomic imprinting is an epigenetic mechanism by which certain genes are silenced in a parent-of-origin-specific manner. Although genomic imprinting is thought to be involved in embryonic growth and development of placental mammals, it remains unclear whether this phenomenon is involved in mammalian brain development. Human necdin gene (*NDN*) is located on chromosome 15q11-q13, a region responsible for the genomic imprinting-associated neurodevelopmental disease Prader-Willi syndrome (PWS) (Jay et al., 1997; MacDonald and Wevrick, 1997; Nakada et al., 1998). Mutant mice defective in paternal *Ndn* (*pNdn*) show various types of neuronal abnormalities, including those seen in PWS (Gerard et al., 1999; Muscatelli et al., 2000; Kuwako et al., 2005). Although it is as yet unknown how much necdin deficiency contributes to the pathogenesis of PWS, it is of interest to analyze the phenotypes of necdin-deficient mice by comparison with those of PWS.

In the present study, we quantified Dlx2-expressing neurons in the neocortex and preoptic area of *pNdn*-deficient mice. We demonstrate that necdin deficiency reduces the migratory activity of fluorescent dye-labeled cells from the basal forebrain to the neocortex. We also show that the neocortical GABA system is impaired in *pNdn*-deficient mice, which show a high susceptibility to the $GABA_A$ receptor antagonist pentylenetetrazole (PTZ). Based on these findings, we propose that necdin evolved to promote the development of neocortical GABAergic neurons in mammals.

Materials and Methods

Animals. Generation, breeding, and genotyping of necdin-deficient mice (Ndn^{tm1Ky}) were described previously (Kuwako et al., 2005). Heterozygous males ($Ndn^{+/-}$) (>15 generations in ICR background) were crossed with wild-type ICR females (SLC) to obtain wild-type ($Ndn^{+/+}$) and *pNdn*-deficient ($Ndn^{+m/-p}$) littermates. Experiments using gene-targeted mice were approved by the Recombinant DNA and Animal

Experiment Committees of the Institute for Protein Research, Osaka University (Suita, Japan) and performed in accordance with institutional guidelines and regulations.

Western blotting. Proteins (10 μg/lane) were separated by 10% SDS-PAGE, blotted, and detected with antibodies against necdin (NC243; 1:3000) (Niinobe et al., 2000) and β-tubulin (TUB2.1; Sigma-Aldrich) as described previously (Kuwajima et al., 2006).

Immunohistochemistry. Brain tissues were fixed with 4% paraformaldehyde solution in 0.1 M phosphate buffer, pH 7.4, overnight and cryoprotected by immersion in 30% sucrose overnight. Frozen, 12-μm-thick coronal brain sections at the rostral and caudal positions were prepared, and five sections with close morphological similarities between wild-type and *pNdn*-deficient mice were selected. The sections were incubated overnight or 36 h with primary antibodies at 4°C and fluorescence dye-conjugated secondary antibodies at room temperature for 90 min. Primary antibodies used are anti-Dlx2 (GDlx2; 1:3000) (Kuwajima et al., 2006), anti-GABA (1:3000; Sigma-Aldrich), and anti-necdin (NC243; 1:1000). The secondary antibodies fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:500; Cappel), cyanine 3-conjugated anti-guinea pig IgG (1:500), cyanine 2-conjugated anti-guinea pig IgG (1:500), cyanine 3-conjugated anti-mouse IgG (1:500), and cyanine 3-conjugated anti-rabbit IgG (1:500) are from Jackson ImmunoResearch Laboratories. Chromosomal DNA was detected with 3.3 μM Hoechst 33342 (Sigma-Aldrich). The images were observed with a fluorescence microscope (BX50–34-FLAD1; Olympus) and taken by CCD camera system (M-3204C; Olympus). For quantification of Dlx2- and GABA-containing cells, the immunopositive cells per 200-μm-wide radial column of the neocortical areas were counted.

Cell migration assay. Organotypic forebrain slice cultures were prepared from mouse embryos as described previously (Anderson et al., 1997a; Kuwajima et al., 2006). Selected 300-μm-thick sections containing lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), caudal ganglionic eminence (CGE), or preoptic area (POA) were placed on a collagen-coated culture membrane (diameter, 12 mm; pore size, 3.0 μm; Coaster, Corning), incubated with DMEM/F12 medium containing 10% fetal calf serum for 30 min, and cultured with Neurobasal medium supplemented with B27 supplement (Invitrogen) and 2 mM L-glutamine in a humidified 5% CO_2 incubator at 37°C for 48 h. To examine the migratory pathways of neurons generated in the ganglionic eminences (GEs) and POA, similar-sized crystals (~100 μm) of DiI (1,1′-dyoctadecyl-3,3,3′.3′-tetramethylindocarbocyanine; Invitrogen) were set on the slices using an insect pin and a dissection microscope. After 48 h incubation, the slices were fixed in 4% paraformaldehyde, stained with Hoechst 33342, and observed with the fluorescence microscope using rhodamine fluorescence filters.

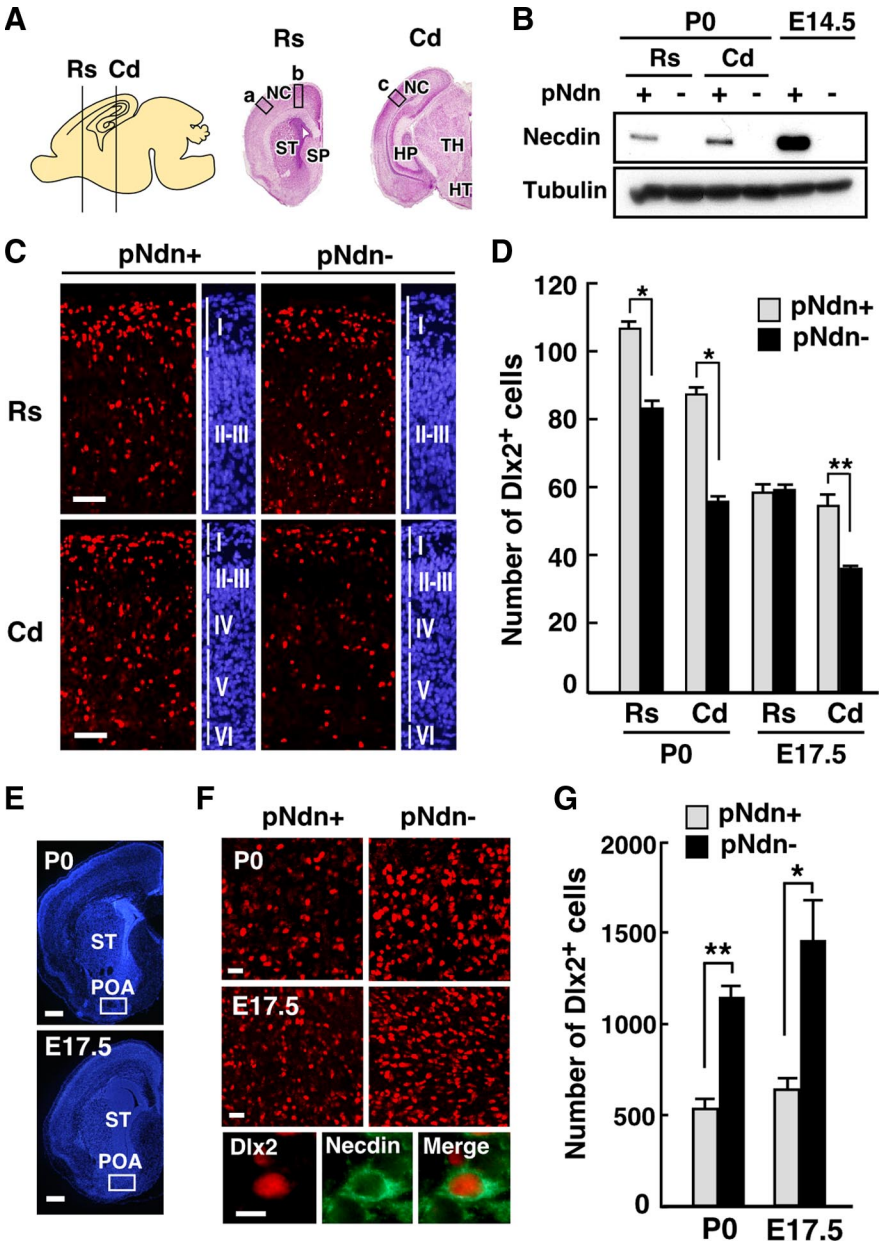

Figure 1. Immunohistochemical analysis of Dlx2-expressing cells in the neocortex and preoptic area. ***A***, Coronal sections of the cerebral cortex. Nissl-stained sections of P0 mouse forebrain at rostral and caudal positions are shown schematically. Boxes a–c are the areas analyzed. Rs, Rostral; Cd, caudal; NC, neocortex; SP, septum; ST, striatum; HP, hippocampus; TH, thalamus; HT; hypothalamus. ***B***, Western blot analysis. Expression levels of necdin in the rostral (Rs) and caudal (Cd) neocortices at P0 and forebrain at E14.5 of wild-type (pNdn+) and paternal *Ndn*-deficient (pNdn−) mice were analyzed. Necdin (43 kDa) and β-tubulin (52 kDa) bands are shown. ***C***, Dlx2 immunostaining patterns in the neocortex. Dlx2-immunopositive cells (red) in the neocortex at the rostral (Rs) and caudal (Cd) positions (boxes a and c, respectively, in ***A***) of pNdn+ and pNdn− mice at P0 are shown. The cortical layers were judged by staining DNA (blue). Scale bars, 50 μm. ***D***, Quantification of Dlx2-expressing cells. Dlx2-immunopositive ($Dlx2^+$) cells in cortical layers I–VI were counted and presented as the number per 200-μm-wide radial column (examined 5 sections per embryo, mean ± SEM, $n = 5$); $^*p < 0.05$, $^{**}p < 0.001$. ***E***, Coronal sections of P0 and E17.5 mouse rostral forebrains. Sections stained for DNA are shown schematically. Scale bars, 500 μm. ***F***, Dlx2 immunostaining patterns in POA. Dlx2-immunopositive cells (red) in a ventrolateral POA region (boxed in ***E***) of pNdn+ and pNdn− mice at P0 (top panels) and E17.5 (middle panels) are shown. A POA section of P0 mouse was double-immunostained for Dlx2 and necdin (bottom panels). Scale bars, 20 μm. ***G***, Quantification of Dlx2-expressing cells in POA. Dlx2-immunopositive ($Dlx2^+$) cells in POA (observed area, 200 × 200 μm) of pNdn+ and pNdn− mice at P0 and E17.5 were counted and presented as the number per square millimeter (examined 5 sections per animal, mean ± SEM, $n = 3$); $^*p < 0.05$, $^{**}p < 0.01$.

PTZ-induced seizures. PTZ-induced seizures were induced and scored according to the method described previously (Tornberg et al., 2005). Male wild-type ($n = 5$) and necdin-deficient ($n = 9$) mice (P120-150) were injected with PTZ (Sigma-Aldrich) intraperitoneally at a dose of 65 mg/kg. At this dose, mice showed 100% seizure frequency. Each mouse was observed for 20 min after PTZ injection. Lethality (percentage) was

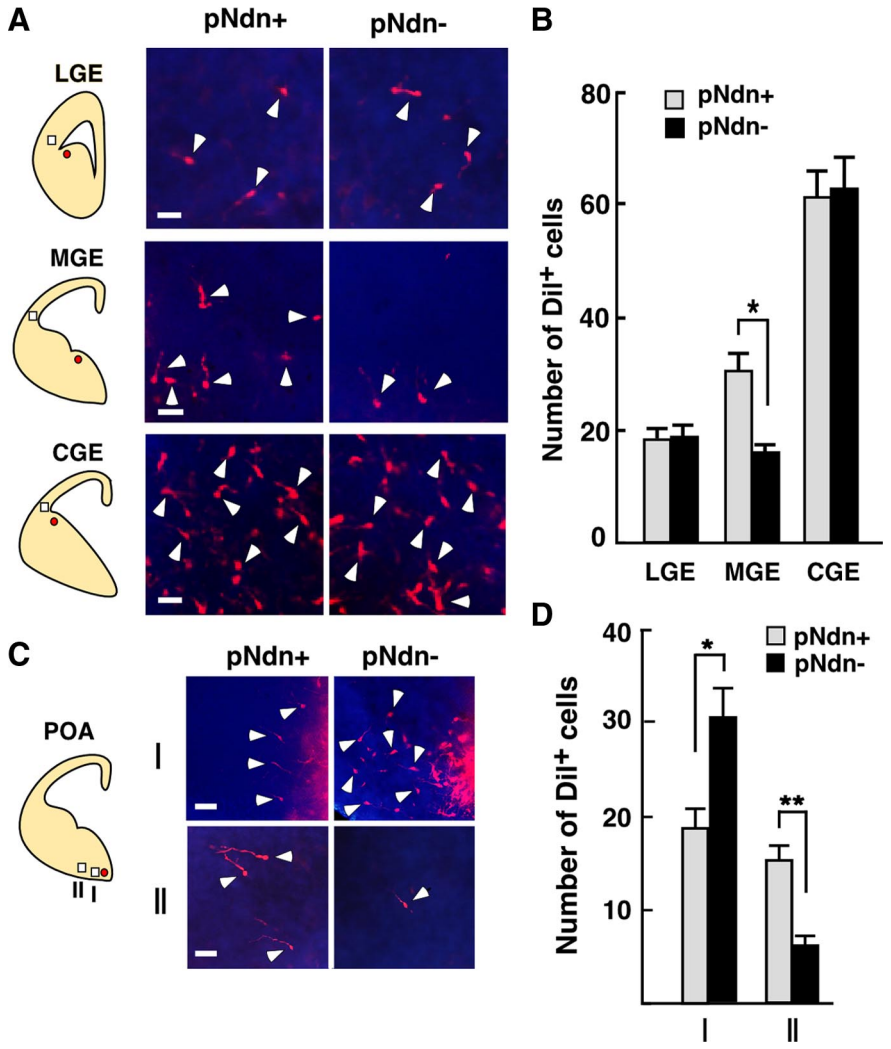

Figure 2. Tangential migration assay. ***A***, Tangential migration of DiI-labeled cells to the neocortex. Organotypic slice cultures containing lateral (LGE) (top), medial (MGE) (middle), and caudal (CGE) (bottom) ganglionic eminences were prepared from pNdn+ and pNdn− mice at E14.5. DiI crystals were set on the above areas, and DiI-positive cells were observed 48 h later. Schemes on the left show the positions of DiI crystals (red circles) and observed areas (white squares). Arrowheads indicate representative DiI-labeled cells. Scale bars, 20 μm. ***B***, Quantification of DiI-labeled cells in the cortex. DiI-positive (DiI $^+$) cells in the observed areas were counted and presented as the total number per 500-μm-wide radial column (mean ± SEM, $n = 10$); $^*p < 0.001$. ***C***, Tangential migration of DiI-labeled cells in the POA. DiI crystals were set on the POA in organotypic slices prepared from pNdn+ and pNdn− mice at E14.5 and cultured for 48 h. DiI-labeled cells in areas I and II (white squares) at ~200 and ~600 μm, respectively, distant from DiI crystals (red circle) were observed. Scale bars, 20 μm. Arrowheads point to representative DiI-labeled cells. ***D***, Quantification of DiI-labeled cells in POA. DiI-labeled (DiI $^+$) cells in observed areas I and II (100 × 100 μm each) were counted and presented as the number per observed area (examined 3 nonoverlapping areas per slice, mean ± SEM, $n = 16$); $^*p < 0.05$, $^{**}p < 0.01$.

calculated from the number of dead mice during the test period. The seizure frequency and lethality were calculated as the percentage of mice in each group with seizures scored 3. Latency to the onset of the first clonic seizure was recorded, and durations of clonic seizures during the test period in each mouse were averaged. Data were presented as mean ± SEM. Mice to which the first seizure was lethal were excluded from the data.

Statistical tests. Statistical significance was tested using an unpaired Student *t* test or one-way ANOVA followed by Tukey's *post hoc* test. A significance of $p < 0.05$ was required for rejection of the null hypothesis. For analysis of lethality after PTZ-induced convulsions, Fisher's exact probability test was used.

Results

We first analyzed the expression levels of necdin in the rostral and caudal neocortices of wild-type and *pNdn*-deficient littermates at postnatal day 0 (P0) by Western blot analysis (Fig. 1*A*,*B*). Necdin was detectable in these areas of wild-type mice, although the levels were much lower in the neocortex than in the forebrain at embryonic day 14.5 (E14.5). Necdin was totally undetectable in *pNdn*-deficient mice. We next quantified the Dlx2-expressing cells in the neocortex by immunohistochemistry and found that the number of Dlx2-immunopositive cells was significantly reduced in *pNdn*-deficient mice (Fig. 1*C*,*D*). The number of Dlx2-expressing cells was more reduced (36% reduction) in the caudal cortex than in the rostral cortex (22% reduction). In particular, *pNdn*-deficient mice exhibited a marked reduction in the number of Dlx2-immunopositive cells in layers I–III of the caudal cortex (56% of the wild-type control) (wild type, 45.4 ± 1.8; *pNdn* deficient, 25.2 ± 0.4: $p < 0.001$). On the other hand, no appreciable change in the cortical population of glutamic acid-containing neurons was noted between wild-type and *pNdn*-deficient mice at P0 (data not shown), suggesting that cortical Dlx2-dependent neurons originating from the ventral telencephalon are specifically affected in *pNdn*-deficient mice.

We then examined when the reduction of neocortical interneurons expressing Dlx2 becomes evident in *pNdn*-deficient mice. At E17.5, there was a significant reduction in the number of Dlx2-expressing cells in the caudal neocortex in *pNdn*-deficient mice (34% reduction). In contrast, the number of Dlx2-expressing neurons was unchanged in the rostral neocortex of *pNdn*-deficient mice (Fig. 1*D*), suggesting that the abnormal distribution of late-born interneurons, which are still migrating at E17.5, becomes apparent in this region at P0. At E13.5, there was no difference in the number of Dlx2-positive cells in the marginal-to-intermediate zone between the wild-type and *pNdn*-deficient mice (wild type, 25.0 ± 1.4; *pNdn* deficient, 25.9 ± 0.7: $p = 0.58$), suggesting that the migration of Dlx2-expressing neurons that are born later than E13.5 is impaired in *pNdn*-deficient mice.

We have previously found that cells expressing high levels of Dlx2 are distributed in the ventral telencephalon, including the GEs and POA (Kuwajima et al., 2006). Thus, we quantified Dlx2-expressing cells in the POA as a control (Fig. 1*E*). We counted the Dlx2-immunopositive cells in a ventrolateral part of the POA in *pNdn*-deficient and wild-type littermates at P0 and E17.5 (Fig. 1*F*). Remarkably, the numbers of Dlx2-expressing cells in *pNdn*-deficient mice were 2.0 and 2.2 times those of wild-type controls in this region at P0 and E17.5, respectively (Fig. 1*G*). These results suggest that Dlx2-expressing neuron migration via the POA is impaired in necdin-deficient mice, leading to their accumulation in this area.

Most of the cortical interneurons are derived from the GEs during the embryonic period (Marin and Rubenstein, 2003). We then quantified DiI-labeled neurons migrating from the GEs to the cerebral cortex (Fig. 2*A*,*B*). DiI crystals were set on the specific points in E14.5 forebrain slices, and DiI-labeled cells that migrated into basal neocortical areas were quantified 48 h later (Fig. 2*A*). The number of DiI-labeled cells migrating from the MGE was significantly reduced in *pNdn*-deficient mice (48% reduction), whereas there was no appreciable difference in the number of DiI-labeled cells migrating from the LGE or the CGE between *pNdn*-deficient and wild-type mice (Fig. 2*B*), suggesting that necdin promotes the tangential migration of MGE-derived interneurons during this period. In this assay, we failed to evaluate the accumulation of DiI-labeled, MGE-derived interneurons within the MGE of *pNdn*-deficient mice.

We also examined the migratory activity of the cells located in the POA at this stage. We quantified DiI-labeled neurons present in the proximal and distal areas at ~200 μm (area I) and ~600 μm (area II), respectively, distant from the DiI spots in the POA (Fig. 2*C*). The number of DiI-labeled cells in area I of *pNdn*-deficient mice was 1.6 times that of the wild-type control, whereas the number of DiI-labeled cells in area II of *pNdn*-deficient mice was 38% of the control number (Fig. 2*D*). These results suggest that necdin deficiency impairs tangential migration of neurons, resulting in their accumulation in the POA on the route to their destinations.

Dlx2 is a master regulatory gene that induces differentiation of cortical GABAergic interneurons. We thus examined the population of GABA-containing cells in the neocortex (motor cortex) (Fig. 3*A*). We found that the number of intensely GABA-immunopositive neurons in this area of *pNdn*-deficient mice was significantly reduced to 59% of the wild-type control number (wild type, 11.9 ± 0.8; *pNdn* deficient, 7.0 ± 0.5; $p < 0.01$). Most of the intensely GABA-immunopositive neurons were positive for Dlx2 in the nucleus. The necdin immunoreactivity was distributed throughout the neocortical layers in wild-type mice. GABA-containing neurons overlapped with Dlx2- and necdin-expressing neurons (Fig. 3*B*). The Dlx2 immunoreactivity was localized in the nucleus, whereas the GABA and necdin immunoreactivities were detected predominantly in the cytoplasm.

Neocortical GABAergic interneurons form inhibitory circuits to suppress the excitation of glutamatergic neurons. Thus, we examined whether *pNdn*-deficient mice show a higher susceptibility to $GABA_A$ receptor antagonist PTZ than wild-type mice. There was no significant difference in the lethality between wild-type controls and *pNdn*-deficient mice (wild type, 4, $n = 6$; *pNdn* deficient, 7, $n = 9$: $p = 0.45$ on Fisher's exact probability test; expected frequency in χ^2 test, <5). However, the seizure latency in *pNdn*-deficient mice was markedly reduced to 33% of the wild-type control (wild type, 490.6 ± 43.0 s; *pNdn* deficient, 162.6 ± 25.4 s: $p < 0.001$). In addition, the seizure duration of *pNdn*-deficient mice was 2.9 times that of the wild-type control (wild type, 15.5 ± 1.5 s; *pNdn* deficient, 45.0 ± 7.9 s: $p < 0.02$). These data indicate that the inhibitory function of cortical GABAergic interneurons is impaired in *pNdn*-deficient mice, which are hypersensitive to PTZ-induced seizures. To confirm that the neocortical interneuron population remains decreased in

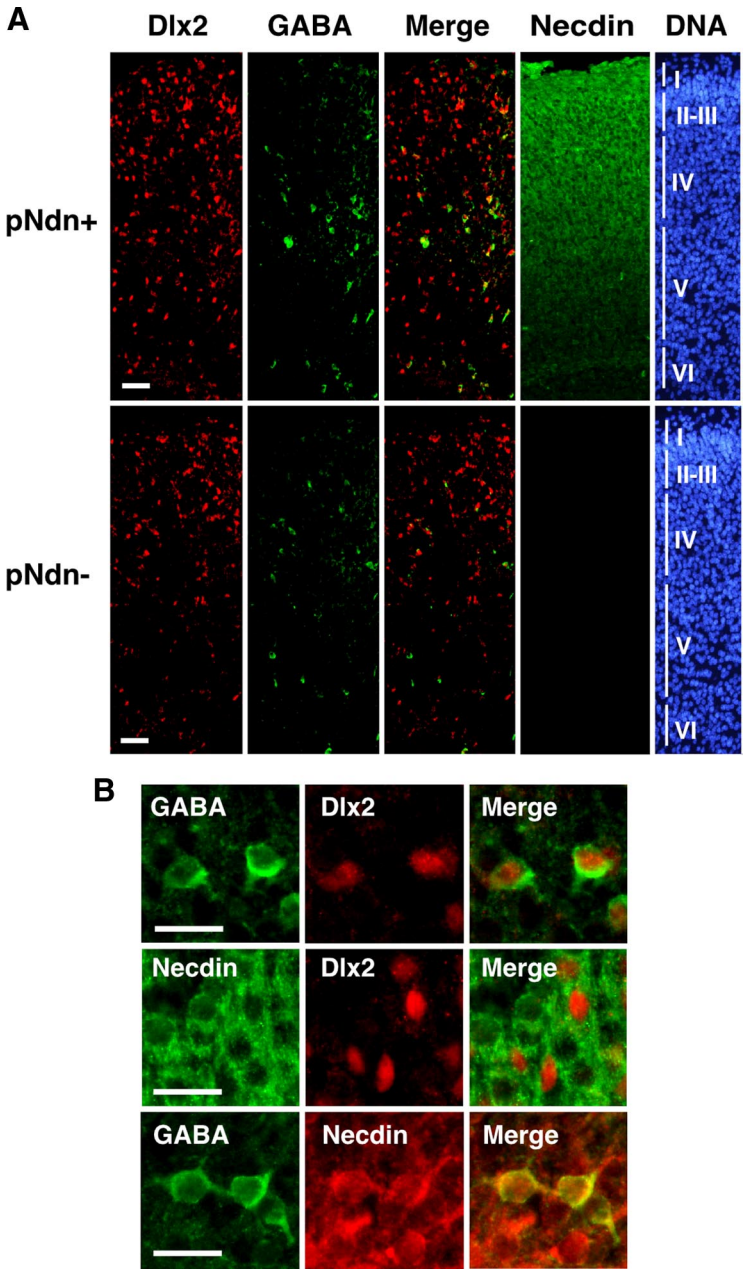

Figure 3. Immunohistochemical analysis of neocortical GABAergic neurons. ***A***, Double immunostaining for Dlx2 and GABA. Cells in the rostral neocortex at P0 (shown as box b in Fig. 1*A*) of pNdn+ and pNdn− mice were immunostained for Dlx2 (red) and GABA (green), and two images were merged. Necdin was also immunostained for reference. The cortical layers were judged by staining DNA. Scale bars, 50 μm. Quantitative data are given in Results. ***B***, Colocalization of GABA, Dlx2, and necdin. Coronal sections of pNdn+ mice at P0 were double immunostained for GABA and Dlx2 (top panels), necdin and Dlx2 (middle panels), or GABA and necdin (bottom panels). Images in neocortical layer V are shown. Two images are merged for colocalization. Scale bars, 20 μm.

adulthood, we quantified cortical Dlx2-expressing cells in necdin-deficient mice at P120. The number of Dlx2-immunopositive cells in layers I–IV of the *pNdn*-deficient mouse cortex was 69% that of the wild-type control (wild type, 21.2 ± 1.5; *pNdn* deficient, 14.6 ± 1.4: $p < 0.05$).

Discussion

The present study has shown that necdin-deficient mice display impaired migration of Dlx2-containing interneurons from the subpallium to the neocortex. Consistent with the fact that cells from the MGE give rise to a majority of cortical GABAergic interneurons (Marin and Rubenstein, 2003), the migratory activities of the MGE cells were specifically reduced in *pNdn*-deficient embryos (Fig. 2*A*). These observations suggest that endogenous necdin promotes tangential migration of Dlx2-dependent neurons from the MGE into the neocortex at the embryonic stage. Similarly, tangential migration of gonadotropin-releasing hormone (GnRH) neurons is attenuated from the olfactory epithelium to the anterior hypothalamus in necdin-deficient mouse embryos (Miller et al., 2009). GnRH neuron development is also dependent on Msx/Dlx family proteins (Givens et al., 2005). These findings support the notion that necdin collaborates with Msx/Dlx homeodomain transcription factors to modulate the migratory activities of both neocortical interneurons and GnRH neurons.

Neuronal apoptosis is significantly increased in the dorsal root ganglia and the superior cervical ganglia of *pNdn*-deficient mice (Kuwako et al., 2005; Tennese et al., 2008). In contrast, necdin deficiency exerts no appreciable effects on the number of Dlx2-expressing neurons differentiating both *in vivo* and *in vitro* (Kuwajima et al., 2006). Thus, the reduction of neocortical Dlx2-expressing interneurons may not be attributable to the increased apoptosis but mainly to the attenuated migration in *pNdn*-deficient mice. Consistent with this idea, the number of Dlx2 neurons was markedly increased in the ventrolateral part of the POA (Fig. 1*F*,*G*) in which migrating DiI-labeled cells are prone to accumulate (Fig. 2*C*,*D*). Because a substantial number of cortical GABAergic interneurons originate in the POA (Gelman et al., 2009), the POA-derived cells may accumulate within the POA of *pNdn*-deficient mice. However, it is unlikely that the reduction of neocortical interneurons in necdin-deficient mice is attributable to the abnormal migration of POA-derived cells, which contribute to a relatively small population in the neocortex.

Mice lacking both *Dlx1* and *Dlx2* show no detectable cell migration from the subcortical telencephalon to the neocortex, where few GABA-expressing cells are present (Anderson et al., 1997b). In contrast, the phenotype of *pNdn*-deficient mice is milder than that of the *Dlx1*/*Dlx2* double-mutant mice. This may be because necdin potentiates the effects of Dlx2 only on the differentiation and migration of GABAergic interneurons. Necdin interacts with Dlx2 via MAGE-D1 (also known as NRAGE or Dlxin1), regulates Dlx2-dependent transcriptional activities, and promotes GABAergic neuron differentiation (Kuwajima et al., 2006). Thus, necdin and Dlx2 may cooperate to transactivate certain genes required for the tangential migration of GABAergic interneurons. For example, Dlx2 upregulates TrkB expression in retinal ganglion cells (de Melo et al., 2008). The TrkB signaling via PI3-kinase (phosphoinositide 3-kinase) activation plays an important role in controlling interneuron migration during development of the cerebral cortex (Polleux et al., 2002). Dlx2 also transcriptionally activates the expression of Arx, another homeodomain transcription factor contributing to the migration of GABAergic interneurons (Colasante et al., 2008). We found that a cortical population of Arx-expressing cells, like that of Dlx2-expressing cells, is significantly reduced in necdin-deficient mice (T. Kuwajima and K. Yoshikawa, unpublished observations). Thus, necdin may enhance Dlx2-dependent expression of regulatory genes that are involved in the tangential migration of GABAergic interneurons. We are currently investigating such specific genes that are upregulated through the cooperation between Dlx2 and necdin.

The present study has shown that the neocortical GABAergic system is abnormal in necdin-deficient mice. The GABAergic system is required for preventing hyperexcitation of cortical pyramidal neurons. Although there is little information about neocortical GABAergic system in PWS, it has been reported that the frequency of generalized seizure disorder in PWS patients is significantly higher than in the general pediatric population (Fan et al., 2009). Furthermore, cortical GABAergic interneurons are thought to be involved in the pathogenesis of autism (Rubenstein and Merzenich, 2003). The majority of PWS individuals show a striking autistic-like behavioral phenotype (Descheemaeker et al., 2006). Thus, the present results may account, at least in part, for the mechanisms of such neurobehavioral abnormalities seen in PWS.

More than 25 members of MAGE family genes have been identified in human and mouse genomes (Barker and Salehi, 2002). In view of the evolution of necdin and other MAGE family genes, it is noteworthy that necdin is absent from primitive mammals such as the monotreme platypus and the marsupials tammar wallaby and opossum (Rapkins et al., 2006) and that chicken has only a single MAGE, which is functionally similar to necdin (Lopez-Sanchez et al., 2007). These findings indicate that MAGE family genes were rapidly diversified during the course of mammalian evolution. It is tempting to speculate that necdin coevolved with neocortical interneuron diversification in eutherian brains. The present findings may provide valuable insights into the molecular mechanisms underlying forebrain development in placental mammals.

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